UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Identificação e caracterização de peptídeos da peçonha de serpentes botrópicas que interferem na agregação plaquetária

Aluna: Bruna Barbosa de Sousa Simamoto

Orientador: Prof. Dr. Fábio de Oliveira

UBERLÂNDIA - MG
2017

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Identificação e caracterização de peptídeos da peçonha de serpentes botrópicas que interferem na agregação plaquetária

Aluna: Bruna Barbosa de Sousa Simamoto

Orientador: Prof. Dr. Fábio de Oliveira

Tese apresentada à Universidade Federal de Uberlândia como parte dos requisitos para obtenção do Título de Doutor em Genética e Bioquímica (Área Bioquímica).

UBERLÂNDIA - MG
2017

Dados Internacionais de Catalogação na Publicação (CIP)
Sistema de Bibliotecas da UFU, MG, Brasil.

S588i 2017

Simamoto, Bruna Barbosa de Sousa, 1989
Identificação e caracterização de peptídeos da peçonha de serpentes botrópicas que interferem na agregação plaquetária / Bruna Barbosa de Sousa Simamoto. - 2017.
111 f. : il.

Orientador: Fábio de Oliveira.
Tese (doutorado) - Universidade Federal de Uberlândia, Programa de Pós-Graduação em Genética e Bioquímica.
Disponível em: http://dx.doi.org/10.14393/ufu.te.2018.40
Inclui bibliografia.

1. Bioquímica - Teses. 2. Serpente peçonhenta - Peçonha - Teses. 3. Peptídeos - Teses. 4. Bothrops - Teses. I. Oliveira, Fábio de. II. Universidade Federal de Uberlândia. Programa de Pós-Graduação em Genética e Bioquímica. III. Título.

CDU: 577.1

Angela Aparecida Vicentini Tzi Tziboy – CRB-6/947

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Identificação e caracterização de peptídeos da peçonha de serpentes botrópicas que interferem na agregação plaquetária

Aluna: Bruna Barbosa de Sousa Simamoto

COMISSÃO EXAMINADORA

Presidente: Prof. Dr. Fábio de Oliveira

Examinadores:
Profa. Dra. Carla Cristine Neves Mamede
Profa. Dra. Fabiana de Almeida Araújo Santos
Profa. Dra. Kelly Aparecida Geraldo Yoneyama Tudini
Prof. Dr. Norival Alves Santos Filho

Data da defesa: 15 / 12 / 2017

As sugestões da comissão examinadora e as normas da PGGB para o formato da tese foram contempladas

_____

Prof. Dr. Fábio de Oliveira

Dedico este trabalho aos meus familiares e amigos, em especial aos meus pais, minha irmã e meu marido por todo amor, apoio e dedicação concedidos.

AGRADECIMENTOS

Agradeço a Deus pela proteção e sabedoria concedidas, me conduzindo sempre para o melhor caminho.

À minha família e amigos, em especial meus pais Ana Paula e Luis Henrique, minha irmã Carolina e minha avó Verônica. Obrigada pelo amor, educação, conselhos, confiança e todos os esforços dedicados à minha formação. O apoio de vocês foi essencial para a conclusão desse trabalho.

Ao meu marido Mário, por todo amor, carinho, compreensão, companheirismo e paciência. Obrigada pelo incentivo, por acreditar em mim e estar ao meu lado durante todo esse tempo. Com certeza esse caminho teria sido mais difícil sem você ao meu lado.

Agradeço ao professor Dr. Fábio de Oliveira pela orientação, incentivo, dedicação, confiança e amizade ao longo de todos esses anos. Muito obrigada por todas as oportunidades, conhecimento e por proporcionar todas as ferramentas necessárias para a realização desse trabalho. Você é um exemplo de profissional e ser humano.

Aos meus amigos do Laboratório de Biologia Molecular e Celular: Carla, Débora, Edigar, Júnia, Kellen, Mari, Mayara e Nádia. Agradeço por toda ajuda, experiência e aprendizado compartilhados. Mas antes de tudo, agradeço imensamente pelas boas risadas, companheirismo e amizade. Tudo isso foi fundamental para tornar a realização desse trabalho mais agradável e alegre. Espero levar a amizade de vocês para sempre.

Agradeço a todos os funcionários e professores do Instituto de Genética e Bioquímica pela colaboração e ensinamentos.

Aos membros da comissão examinadora, Dra. Carla, Dra. Fabiana, Dra. Kelly e Dr. Norival pela disponibilidade e atenção.

À Coordenação de Aperfeiçoamento de Pessoal do Ensino Superior (CAPES), ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), ao INCT Nano Biofar, à Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) e à Universidade Federal de Uberlândia (UFU) pelo apoio financeiro para o desenvolvimento desse trabalho.

Essa minha vitória merece ser compartilhada com todos vocês que contribuíram para a consolidação desse sonho. Muito obrigada!

LISTA DE FIGURAS

CAPÍTULO I

CAPÍTULO II

CAPÍTULO III

LISTA DE TABELAS

CAPÍTULO I

LISTA DE ABREVIATURAS

5-HT - 5-hidroxitriptamina

AA - Ácido araquidônico

ADP - Adenosina difosfato

ATP - Adenosina trifosfato

***Cys-rich* -** domínio rico em cisteína

COX-1 - cicloxigenase-1

CRD - Domínio de reconhecimento ao carboidrato

DFP - Diisopropilfluorofosfato

Dis - domínio desintegrina

***Dis-like* -** domínio semelhante à desintegrina

EDTA - Ácido etilenodiaminotetracético

FAD - Flavina adenina dinucleotídeo

FMN - Flavina mononucleotídeo

FvW - Fator de von Willebrand

GP - Glicoproteína

H_2O_2 - Peróxido de hidrogênio

LAAO - L-aminoácido-oxidase

mRNA - RNA mensageiro

NGF - Fator de crescimento neuronal

OMS - Organização Mundial da Saúde

PARs - Receptores ativados por proteases

PDGF - Fator de crescimento derivado de plaquetas

PMSF - Fluoreto de fenilmetilsulfonilo

PLA_2 - Fosfolipase A_2

PARs - receptores ativados por proteases

SAB - Soro antibotrópico

SABC - Soro antibotrópico-crotálico

SABL - Soro antibotrópico-laqético

Sinan - Sistema de Informação de Agravos e Notificação

Snaclecs - do inglês, *snake C-type lectins*

SV-LAAO - L-aminoácido-oxidases de peçonha de serpentes

SVMPs - Metaloproteases de peçonha de serpentes

SVSPs - Serinoproteases de peçonha de serpentes

TXA_2 - Tromboxano A_2

VEGF - Fator de crescimento endotelial vascular

Resumo

As serpentes botrópicas representam o grupo mais importante das serpentes peçonhentas brasileiras pois são responsáveis pela maioria dos acidentes ofídicos notificados no país. Em geral, o envenenamento botrópico é caracterizado por dor e edema no local da picada, hemorragia local e bolhas, que podem vir acompanhadas de necrose tecidual e muscular, principal complicação local do envenenamento. Hemorragia sistêmica e distúrbios de coagulação também podem ocorrer. Nos casos mais graves, observa-se também choque e insuficiência renal aguda, decorrentes, principalmente, da hemorragia ocasionada. Cerca de 90% do peso seco da peçonha das serpentes corresponde a proteínas e peptídeos, como L-aminoácido-oxidases, fosfolipases A_2, metaloproteases, serinoproteases, 5'-nucleotidases, lectinas e desintegrinas, as quais são responsáveis pela maioria dos efeitos deletérios observados durante o envenenamento. Esses componentes apresentam diferentes atividades farmacológicas, principalmente sobre a hemostasia. Nos últimos anos, uma grande quantidade de toxinas de peçonha de serpentes foi identificada por interferirem na função plaquetária, promovendo ou inibindo a agregação das plaquetas, devido à sua seletividade aos receptores plaquetários, como GPIb-V-IX, GPVI, $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$ ou aos seus respectivos ligantes, como FvW e/ou fibrinogênio. Uma investigação detalhada dessas moléculas fornece informações importantes para o desenvolvimento de testes de diagnósticos e novos agentes terapêuticos para distúrbios hemostáticos. O presente trabalho teve como objetivo identificar e caracterizar novas toxinas da peçonha de serpentes botrópicas capazes de interferirem na função plaquetária. Como resultado foram identificados dois peptídeos denominados de BaltPAi e BmooPAF isolados da peçonha de *Bothrops alternatus* e *Bothrops moojeni*, respectivamente. BaltPAi é um peptídeo capaz de inibibir a agregação plaquetária induzida por colágeno. Além disso, BaltPAi também apresentou efeito citotóxico sobre células de adenocarcinoma cervical humano (HeLa) e células de adenocarcinoma de próstata humano (PC-3). Por sua vez, BmooPAF é um peptídeo capaz de induzir a agregação plaquetária via GPIbα. Esses pequenos peptídeos têm atraído a atenção e interesse de empresas farmacêuticas que procuram por novas drogas

em virtude de sua simplicidade estrutural e excelente potencial terapêutico, podendo ser sintetizados facilmente e a um custo mais baixo, comparado com moléculas maiores. Essas propriedades tornam-os particularmente adequados para serem utilizados no desenvolvimento de novos agentes farmacológicos.

Palavras-chave: Peçonha de serpentes; Bothrops; Agregação plaquetária; Peptídeos.

Abstract

Botrophs snakes represent the most important group of Brazilian venomous snakes because they are responsible for the majority of snake accidents reported in the country. In general, Bothrops poisoning is characterized by pain and edema at the site of the bite, local hemorrhage which may be accompanied by tissue and muscular necrosis, the main local complication of poisoning. Systemic bleeding and clotting disorders may also occur. In the most severe cases, shock and acute renal failure are also observed, mainly due to the hemorrhage caused. About 90% of the dry weight of snake venom corresponds to proteins and peptides, such as L-amino acid oxidases, phospholipases A2, metalloproteases, serine proteases, 5'-nucleotidases, lectins and disintegrins, which are responsible for most deleterious effects observed during poisoning. These components present different pharmacological activities, mainly on hemostasis. In recent years, a large number of snake venom toxins have been identified as interfering in platelet function, promoting or inhibiting platelet aggregation, due to their selectivity to platelet receptors, such as GPIb-V-IX, GPVI, $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$ or to their respective linkers, such as vWF and/or fibrinogen. Detailed investigation of these molecules provides important information for the development of diagnostic tests and novel therapeutic agents for hemostatic disorders. The present work aimed to identify and characterize new venom toxins from Bothrops snake venom capable of interfering in platelet function. As a result, two peptides named BaltPAi and BmooPAF isolated from *Bothrops alternatus* and *Bothrops moojeni* snake venom, respectively, were identified. BaltPAi is a peptide capable of inhibiting collagen-induced platelet aggregation. In addition, BaltPAi also showed cytotoxic effect on human cervical adenocarcinoma cells (HeLa) and human prostate adenocarcinoma cells (PC-3). In turn, BmooPAF is a peptide capable of inducing platelet aggregation via GPIbα. These small peptides have attracted the attention and interest of pharmaceutical companies that search for new drugs because of their simpler structures and therapeutic potential which can be easily synthesized and at a low cost. These properties make them particularly suitable probes for the development of novel pharmacological drugs.

Keywords: Snake venom; Bothrops; Platelet aggregation; Peptides.

SUMÁRIO

APRESENTAÇÃO

As peçonhas de serpentes contêm grande quantidade de peptídeos e proteínas farmacologicamente ativos capazes de interagir com componentes do sistema hemostático humano, os quais podem provocar coagulopatias, hemorragia, choque hipovolêmico, trombose, entre outros. Alguns desses constituintes interferem na função plaquetária, podendo induzir e/ou inibir a agregação das plaquetas. Devido ao seu potencial terapêutico, esses componentes hemostaticamente ativos têm sido isolados e utilizados como potentes drogas antiplaquetárias para o tratamento de doenças trombóticas e/ou estão em fase de estudos pré-clínicos. Diante desse contexto, o trabalho desenvolvido durante o doutorado teve como objetivo a descoberta e a caracterização de novas moléculas da peçonha de serpentes botrópicas capazes de interferir na função plaquetária com potencial uso terapêutico.

A presente tese foi desenvolvida de acordo com as normas do Programa de Pós-Graduação em Genética e Bioquímica da Universidade Federal de Uberlândia para obtenção do título de Doutor, e foi subdividida em capítulos.

O capítulo I (Fundamentação teórica) é constituído por uma revisão bibliográfica, demonstrando a importância da serpente do gênero *Bothrops* no Brasil e os principais componentes da peçonha responsáveis pelos efeitos deletérios observados durante o acidente ofídico. Além disso, demonstra a importância do papel das plaquetas na hemostasia e estabelece uma relação entre os componentes da peçonha de serpentes e sua capacidade de interferir com o sistema hemostático humano.

Os capítulos II e III apresentam os artigos referentes ao trabalho experimental realizado durante o doutorado, os quais foram intitulados de "BaltPAi: A peptide platelet aggregation inhibitor from *Bothrops alternatus* snake venom" e "BmooPAF: a new platelet-activating factor from *Bothrops moojeni* snake venom". Ambos os artigos se referem a pequenos peptídeos isolados da peçonha de serpentes botrópicas com potenciais efeitos terapêuticos para doenças do sistema hemostático. O primeiro, trata-se de um peptídeo capaz de inibir a agregação plaquetária induzida por colágeno, com potencial uso para doenças trombóticas, enquanto que o segundo trata de um ativador da agregação

plaquetária via glicoproteína Ib, com potencial uso para a Doença de von willebrand. Ambos os trabalhos foram redigidos em formato e linguagem de artigo científico, de acordo com os padrões textuais e científicos exigidos pelo periódico a ser submetido.

Ao final da tese, encontra-se em anexo o artigo intitulado "A new platelet-aggregation-inhibiting factor isolated from *Bothrops moojeni* snake venom" publicado no periódico BioMed Research International (fator de impacto = 2,476). Este trabalho foi anexado à tese devido à similaridade de assunto. Esses resultados foram obtidos durante o mestrado e finalizados durante o doutorado e trata-se da purificação e caracterização de uma molécula isolada da peçonha de *Bothrops moojeni* com efeito inibitório sobre a agregação plaquetária.

CAPÍTULO I:
FUNDAMENTAÇÃO TEÓRICA

Toxinas ofídicas e sua interferência na função plaquetária

1 Serpentes brasileiras

As serpentes são animais muito bem adaptados e com uma grande diversidade fisiológica e morfológica, podendo ser encontradas em diferentes tipos de ambientes terrestres ou aquáticos (VITT; CALDWELL, 2013). Elas pertencem ao reino Animalia, filo Chordata, subfilo Vertebrata, classe Reptilia, ordem Squamata e subordem Serpentes. Existem, aproximadamente, 3 mil espécies de serpentes no mundo, sendo que cerca de 10 a 14% são consideradas peçonhentas e despertam interesse para a saúde pública (BARRAVIERA, 1993; HICKMAN; ROBERTS; LARSON, 2004; MELGAREJO, 2009).

As serpentes peçonhentas são aquelas capazes de inocular, através de dentes modificados, substâncias tóxicas, farmacologicamente ativas, produzidas por glândulas bucais modificadas (WARREL, 2010). Elas são responsáveis por cerca de 5,5 milhões de acidentes por ano, em todo o mundo, com cerca de 20.000 a 120.000 óbitos e 400.000 amputações. As áreas mais afetadas são os países tropicais e subtropicais da África, Sudeste Asiático e América Latina (BOCHNER, 2013; KASTURIRATNE et al., 2008).

O Brasil apresenta uma das mais ricas faunas de serpentes do mundo (UETZ; FREED; HOSEK, 2015). Segundo a Sociedade Brasileira de Herpetologia, o território brasileiro possui, de forma catalogada, 392 espécies de serpentes, distribuídas em 10 famílias: Anomalepididae (7 espécies), Typhlopidae (6 espécies), Leptotyphlopidae (17 espécies), Aniliidae (1 espécie), Tropidophiidae (3 espécies), Boidae (12 espécies), Colubridae (35 espécies), Dipsadidae (248 espécies), Elapidae (33 espécies) e Viperidae (30 espécies) (COSTA; BÉRNILS, 2015). Apenas duas famílias (Viperidae e Elapidae) reúnem as serpentes peçonhentas, as quais estão distribuídas em quatro gêneros: *Bothrops*, *Crotalus* e *Lachesis*, pertencentes à família Viperidae; e *Micrurus*, pertencente à família Elapidae (MELGAREJO, 2009).

O principal representante de importância médica da família Elapidae é o gênero *Micrurus*. As serpentes desse gênero são popularmente conhecidas por coral, coral verdadeira ou boicorá e apresentam um padrão de cor característico em anéis vermelhos, brancos e pretos em diferentes combinações. São animais

de pequeno e médio porte, e diferentemente das demais serpentes peçonhentas, não apresentam fosseta loreal, órgão termorreceptor situado entre o olho e a narina (BRASIL, 2001). Essas serpentes apresentam aparelho inoculador do tipo proteróglifo, com dentes inoculadores de peçonha pouco desenvolvidos, não retráteis e posicionados na região anterior da maxila (figura 01) (BRASIL, 2001; JACKSON, 2003). Essas características, associadas ao seu hábito fossorial (vivem enterradas, habitando, preferencialmente, buracos) explicam o reduzido número de acidentes registrados por esse gênero (BRASIL, 2009).

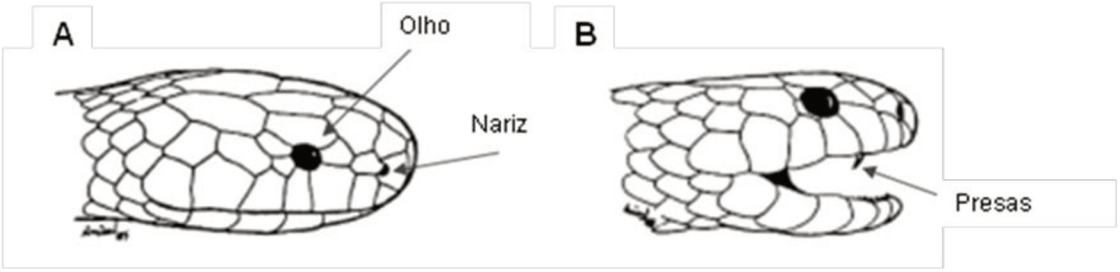

Figura 01: Esquema representativo de uma serpente do gênero *Micrurus*. **(A)** Ausência de fosseta loreal. **(B)** Dentição proteróglifa: presas pouco desenvolvidas, não retráteis e posicionadas na região anterior da maxila. Fonte: BRASIL, 2001.

A família Viperidae representa o grupo de serpentes mais importante para a saúde pública brasileira, pois são responsáveis pela maioria dos acidentes ofídicos registrados no país (BRASIL, 2014). Ela é constituída por três subfamílias: Azemiopinae, Viperinae e Crotalinae. Todas as espécies de viperídeos presentes no Brasil pertencem à subfamília Crotalinae (COSTA; BÉRNILS, 2015; VITT; CALDWELL, 2013). Uma das principais características dessa subfamília é a presença de fosseta loreal e aparelho inoculador do tipo solenóglifo (dentes inoculadores muito desenvolvidos, localizados na região anterior da maxila, e retráteis, que se projetam para frente no momento do ataque). Esses dentes são canaliculados e ligados à glândula produtora de peçonha através de ductos, o que garante maior eficiência durante a inoculação (figura 02) (BRASIL, 2001; JACKSON, 2003; WARREL, 2010). No Brasil, a subfamília Crotalinae é representada pelos gêneros *Bothrocophias* (2 espécies), *Bothrops* (26 espécies), *Crotalus* (1 espécie) e *Lachesis* (1 espécie) (COSTA; BÉRNILS, 2015).

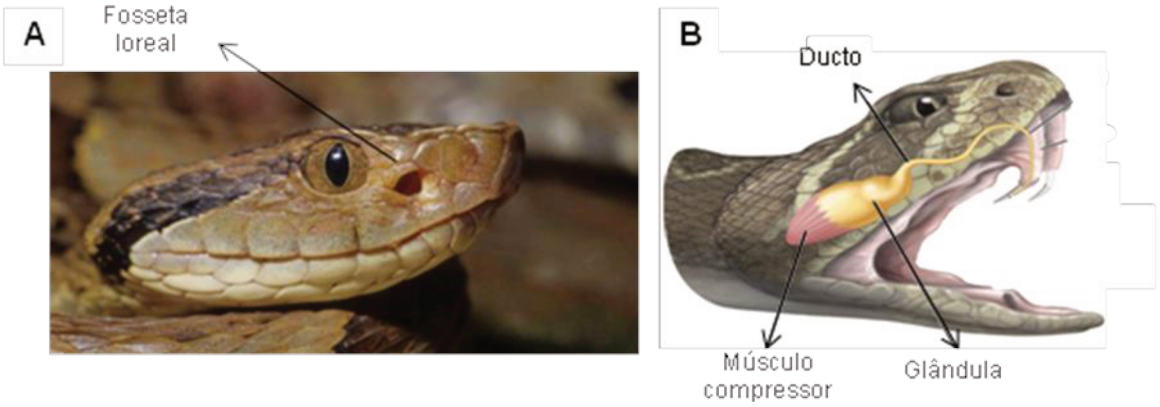

Figura 02: Representação de uma serpente do gênero *Bothrops*. **(A)** Presença de fosseta loreal. Fonte: <http://cobrasvenenosas.com/sistema-de-espionagem-defesa-e-ataque-das-serpentes-peconhentas/>. Acesso em 20 nov. 2017. **(B)** Dentição solenóglifa: presas muito desenvolvidas, retráteis e posicionadas na região anterior da maxila. Fonte: <http://keywordsuggest.org/gallery/1050462.html>. Acesso em 20 nov. 2017.

As serpentes do gênero *Crotalus* são representadas no país por uma única espécie (*C. durissus*) (COSTA; BÉRNILS, 2015). Elas são popularmente conhecidas por cascavel, boicininga, maracambóia e maracá e sua principal característica é a presença de guizo ou chocalho na extremidade caudal (BRASIL, 2001; BRASIL, 2009). Para o gênero *Lachesis*, a espécie que se encontra no território brasileiro é a *L. muta* (COSTA; BÉRNILS, 2015). São popularmente conhecidas por surucucu, surucucu-pico-de-jaca, surucutinga e malha-de-fogo e são as maiores serpentes peçonhentas das Américas, podendo atingir até 4,0 m de comprimento (BRASIL, 2001; BRASIL, 2009).

O gênero *Bothrops* representa o grupo mais importante de serpentes peçonhentas brasileiras, pois são responsáveis pela maioria dos acidentes ofídicos notificados no país (BRASIL, 2014). Possui espécies distribuídas por todo território nacional, sendo que as mais conhecidas são: *B. atrox*, encontrada, principalmente, no Norte do Brasil; *B. erythromelas*, encontrada na região Nordeste; *B. neuwiedi*, distribuída em todo território nacional, exceto região Norte do país; *B. jararaca*, distribuída nas regiões Sul e Sudeste; *B. jararacussu*, espécie que pode alcançar maior comprimento (até 1,8 m), predominante no Sul e Sudeste; *B. alternatus*, encontrada desde a região Centro-Oeste até a Sul e *B. moojeni*, principal espécie do cerrado brasileiro (BRASIL, 2009; PINHO;

PEREIRA, 2001). As serpentes deste gênero são popularmente conhecidas por jararaca, jararacuçu, urutu-cruzeira, combóia e caiçaca. Habitam, principalmente, zonas rurais e periferias de grandes cidades. Têm preferência por ambientes úmidos e locais com facilidade para proliferação de roedores e são serpentes com hábitos noturnos e crepusculares (BRASIL, 2001).

1.1 Serpente *Bothrops moojeni*

A serpente *B. moojeni*, conhecida popularmente por jararaca, jararacão ou caiçaca, foi descrita por Hoge em 1966. É a principal espécie botrópica encontrada no cerrado brasileiro, distribuindo-se desde o Paraná até o Maranhão (figura 03) (FRANÇA; MÁLAQUE, 2009). São serpentes de médio porte (tamanho em torno de 1,5 m de comprimento), comportamento agressivo e capazes de adaptar-se bem a ambientes modificados (MELGAREJO, 2009). São encontradas predominantemente em áreas ripárias do cerrado, como matas de galerias e pastagens úmidas adjacentes, mas, ocasionalmente, encontram-se em áreas interfluviais mais secas. Apresentam hábitos noturnos, são mais ativas durante os meses mais quentes e úmidos (outubro a abril) e possuem dieta generalista, alimentando-se de pequenos mamíferos, anfíbios, lagartos, serpentes e aves (NOGUEIRA; SAWAYA; MARTINS, 2003).

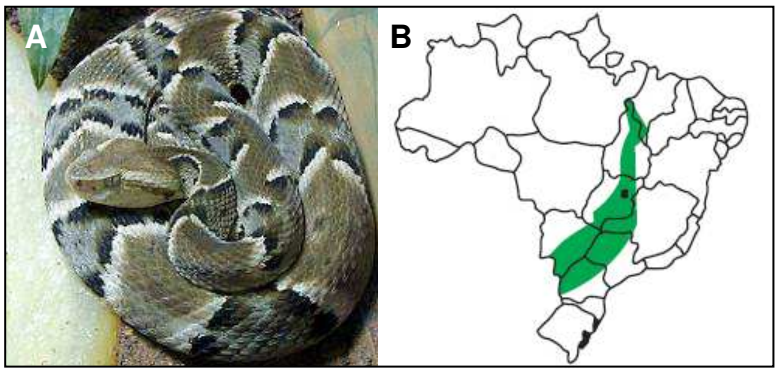

Figura 03: (A) Exemplar da espécie *B. moojeni*. Fonte: <https://www.flickr.com/photos/momcilo56/4034963478/in/photostream/>. Acesso em: 1 mar. 2017. **(B)** Distribuição geográfica da espécie *B. moojeni* no Brasil. Fonte: BRASIL, 2001.

1.2 Serpente *B. alternatus*

A serpente *B. alternatus*, conhecida popularmente por urutu, foi descrita por Duméril, Bibron e Duméril em 1854. É uma serpente com ampla distribuição geográfica, podendo ser encontrada no Centro-Oeste, Sudeste e Sul do Brasil. Apresenta porte grande (podendo atingir até 1,7 m de comprimento) e é encontrada, predominantemente, em áreas abertas e úmidas, como brejos e pântanos, e, ocasionalmente, em áreas de campos mais secas (figura 04) (ROCHA; FURTADO, 2005; WARREL, 2004). Ao contrário da maioria das espécies do gênero (generalistas), a serpente *B. alternatus* é uma espécie especialista em pequenos mamíferos em todas as fases da vida (MARTINS; MARQUES; SAZIMA, 2002).

Popularmente, a peçonha da serpente *B. alternatus* é conhecida por causar graves acidentes, por isso foi criado o ditado "quando não mata, aleija". Contudo, contrariando a crença popular, estudos têm demonstrado que acidentes causados por essa serpente não são tão graves, se comparado com acidentes causados por outras serpentes botrópicas (BAUAB et al., 1994; QUEIROZ; PETTA, 1984). A peçonha dessa espécie possui uma menor atividade enzimática, causando menores complicações (CAMPOS et al., 2013; MAMEDE et al., 2016; ROCHA; FURTADO, 2005; SOUZA et al., 2015).

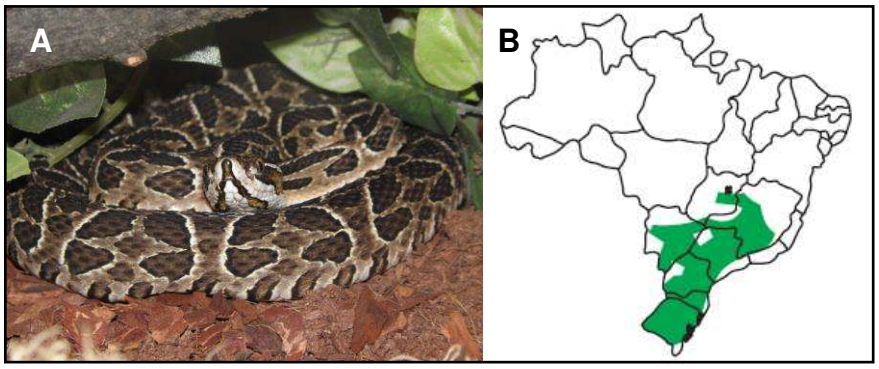

Figura 04: (A) Exemplar da espécie *B. alternatus*. Fonte: <http://carnivoraforum.com/topic/10307745/1/>. Acesso em: 1 mar. 2017. **(B)** Distribuição geográfica da espécie *B. alternatus* no Brasil. Fonte: BRASIL, 2001.

2 Acidentes ofídicos

Os acidentes ofídicos constituem um grave problema de saúde pública em muitas partes do mundo, especialmente nos países tropicais e subtropicais, devido à frequência com que ocorrem e gravidade dos casos (BOCHNER, 2013; CHIPPAUX, 2015). Apesar disso, eles recebem pouca atenção do governo e das agências de saúde global. Somente em abril de 2009 essa doença foi incluída na lista de "doenças tropicais negligenciadas" da Organização Mundial da Saúde (OMS) (HARRISON et al., 2009; WHO, 2017).

Diferentes estudos têm demonstrado que os acidentes ofídicos estão relacionados à fatores climáticos e ao aumento da atividade dos trabalhadores em áreas rurais. Nas épocas de calor e chuva (início e final do ano), que coincide com o período de maior atividade do homem no campo, ocorre um aumento do número de casos. As vítimas, geralmente, são homens em idade profissional ativa (20 a 39 anos) e as regiões mais acometidas são os membros inferiores (pés e pernas) (BRASIL, 2001; BRASIL, 2009; BRASIL, 2017; CHIPPAUX, 2015; SOTELO-CRUZ; GÓMEZ-RIVERA, 2017; WHO, 2017).

O Brasil é o país da América do Sul que apresenta maior número de acidentes por ano. As regiões Centro-Oeste e Norte apresentam maior incidência. No entanto, é possível que haja uma subnotificação nas regiões mais remotas do país, em especial a região Norte, devido à dificuldade de acesso aos serviços de saúde (BRASIL, 2001; WARREL, 2004).

Dados do Sistema de Informação de Agravos de Notificação (Sinan) mostraram que no ano de 2015 foram notificados 18.741 acidentes ofídicos no Brasil. Destes acidentes, 13.490 foram provocados por serpentes do gênero *Bothrops* (71,98%), 1.438 por serpentes do gênero *Crotalus* (7,67%), 529 por serpentes do gênero *Lachesis* (2,82%), 139 por serpentes do gênero *Micrurus* (0,74%) e 908 por serpentes não peçonhentas (4,85%). Em 2.237 casos (11,94%), o gênero da serpente não foi identificado (BRASIL, 2017). Vários estudos apontam para uma média de 20.000 acidentes por ano, sendo o gênero *Bothrops* o principal agente causador. Isso demonstra a importância epidemiológica do acidente botrópico para o Brasil (BOCHNER; STRUCHINER, 2003; CHIPPAUX, 2015; RIBEIRO; JORGE, 1997).

A maioria dos acidentes ofídicos é clinicamente classificada como leve, entretanto alguns fatores podem aumentar consideravelmente sua gravidade, como a quantidade de peçonha inoculada, profundidade e número de picadas, presença de patógenos na boca da serpente, idade e tamanho da vítima, região acometida, tratamento recebido e tempo decorrido entre o acidente e o atendimento. Além disso, tamanho e espécie da serpente envolvida são fatores determinantes (BRASIL, 2009; FRANÇA; MÁLAQUE, 2009; RUSSEL, 1973). Dentre os acidentes ofídicos registrados pelo Sinan em 2015, 85 casos (0,45%) evoluíram para óbito (BRASIL, 2017). Em geral, o maior índice de letalidade corresponde ao gênero *Crotalus* (1,87%), seguido do gênero *Lachesis* (0,95%), *Micrurus* (0,36%) e *Bothrops* (0,31%) (BOCHNER; STRUCHINER; 2003; BRASIL, 2001).

O elevado índice de letalidade atribuído ao acidente crotálico deve-se às suas frações neurotóxica, miotóxica e hemolítica, que podem provocar paralisia motora, lesões nas fibras musculares e incoagulabilidade sanguínea, os quais podem evoluir para insuficiência renal aguda, principal causa de óbito. O acidente crotálico, geralmente, é grave e quase sempre fatal na ausência de tratamento específico e adequado (AZEVEDO-MARQUES et al., 1985; AZEVEDO-MARQUES; HERING; CUPO, 1987; BANCHER; ROSA; FURLANETTO, 1973; BRASIL, 2001; TOKARNIA; PEIXOTO, 2006). O acidente laquético é caracterizado, principalmente, por destruição tecidual na região da picada, devido à sua fração proteolítica, acompanhado de incoagulabilidade sanguínea, hemorragia e síndrome vagal (BRASIL, 2001; PINHO; PEREIRA, 2001; TOKARNIA; PEIXOTO, 2006). Por sua vez, o acidente elapídico, provocado, principalmente, por serpentes do gênero *Micrurus*, apresenta um quadro clínico semelhante ao acidente crotálico, com fraqueza muscular progressiva devido à ação de neurotoxinas que bloqueiam e/ou competem com acetilcolina. Essa fraqueza muscular pode evoluir para insuficiência respiratória aguda, principal causa de óbito (BRASIL, 2001; TOKARNIA; PEIXOTO, 2006).

O acidente botrópico, apesar de apresentar baixo índice de letalidade, corresponde ao acidente ofídico de maior importância epidemiológica do país, devido à frequência com que ocorre. Isso explica a necessidade de conhecer sua fisiopatologia para padronização de um tratamento adequado (BRASIL, 2009). Em

geral, o envenenamento por serpentes botrópicas manifesta-se por dor e edema no local da picada, de intensidade variável, e geralmente, de instalação precoce e caráter progressivo. Podem surgir também reações inflamatórias graves, equimose, hemorragia local e bolhas, que podem vir acompanhadas ou não de necrose tecidual e muscular, principal complicação local do envenenamento. A necrose pode resultar em perda irreversível da função ou do tecido, havendo possibilidade de amputação do membro afetado (BRASIL, 2014; GUTIERREZ et al., 2009; GUTIERREZ; LOMONTE; 1989; PINHO; PEREIRA, 2001; STÁBELLI et al., 2006; TOKARNIA; PEIXOTO, 2006; WARREL, 2004).

O envenenamento botrópico pode desencadear também alterações sistêmicas, como nâuseas, vômitos, sudorese, hipotensão arterial, alterações cardiovasculares e distúrbios de coagulação. Hemorragias sistêmicas também podem ocorrer, como gengivorragias, epistaxes, hematêmese e hematúria. A hemorragia pode afetar também pulmões e rins, e, às vezes, o sistema nervoso central, geralmente letal. Nos casos mais graves, observa-se também choque e insuficiência renal aguda, decorrentes, principalmente, da hemorragia ocasionada (ALBUQUERQUE et al., 2013; BRASIL, 2014; GUTIÉRREZ; ESCALANTE; RUCAVADO, 2009; HERRERA et al., 2012; MACHADO et al., 2010; PINHO; PEREIRA, 2001; TOKARNIA; PEIXOTO, 2006; WARREL, 2004).

O procedimento terapêutico mais utilizado para o tratamento de acidentes ofídicos é a soroterapia, que consiste na administração endovenosa, o mais precoce possível, do soro (antiveneno) específico para cada tipo de envenenamento, de acordo com a sua gravidade (BRASIL, 2014). O antiveneno consiste em imunoglobulinas ou fragmentos de imunoglobulinas gerados a partir do fracionamento do plasma de animais de grande porte (equinos e ovinos) previamente sensibilizados com aplicações sucessivas de pequenas quantidades de peçonha de uma (soro monoespecífico) ou mais espécies de serpentes (soro poliespecífico) (SLAGBOOM et al., 2017; WHO, 2010). Dessa forma, a presença de títulos elevados de anticorpos capazes de neutralizar as toxinas presentes na peçonha impede o agravamento do envenenamento (BRASIL, 2001). Soros poliespecíficos possuem uma eficiência inferior quando comparado aos monoespecíficos (CARDOSO; YAMAGUCHI; SILVA, 2009).

No caso do acidente botrópico deve-se utilizar prioritariamente o soro antibotrópico (SAB) mas, na ausência deste ou impossibilidade de diferenciação do tipo de serpente, utiliza-se a associação dos soros antibotrópico-crotálico (SABC) ou antibotrópico-laquético (SABL) (BRASIL, 2014). No Brasil, o SAB é fabricado no Instituto Butantan, em São Paulo, e, geralmente, é preparado a partir da imunização com a peçonha de *B. alternatus*, *B. jararaca*, *B. jararacussu*, *B moojeni* e *B. neuwiedi*, espécies responsáveis pela maioria dos acidentes ofídicos desse gênero no Brasil (CAMEY; VELARDE; SANCHEZ, 2002; FRANÇA; MÁLAQUE, 2009; FURTADO et al., 2010).

A soroterapia reverte grande parte dos efeitos provocados pelo envenenamento, desempenhando um papel crucial para reduzir a mortalidade e morbidade do acidente ofídico, entretanto, apresenta algumas deficiências (WHO, 2010). A administração do antiveneno pode causar reações de hipersensibilidade tardia ou precoce, como urticárias, náuseas, broncoespasmo e hipotensão. Por esse motivo, é necessário um monitoramento rigoroso do paciente durante a administração do soro para detectar precocemente a ocorrência dessas reações (BRASIL, 2014; CARON et al., 2009; WARREL, 2010). Variações inter e intra-específicas na composição da peçonha interferem nos efeitos patológicos e na eficiente neutralização dos efeitos da peçonha pelo soro antiofídico. Assim, para que haja uma neutralização eficiente, deve-se incluir toxinas de serpentes de diferentes idades, coletadas em diferentes épocas do ano e em diferentes regiões (CALVETE et al., 2009; CARDOSO; YAMAGUCHI; SILVA, 2009; CHIPPAUX; WILLIAMS; WHITE,1991; SILVA et al., 2013; TAN et al., 2016). Além disso, embora a soroterapia seja eficaz contra a toxicidade sistêmica, ela é ineficaz contra a toxicidade local induzida pelo envenenamento (GIRISH; KEMPARAJU, 2011; GUTIÉRREZ et al., 2009; WEN, 2009).

Em virtude dessas limitações e complicações, o tratamento dos acidentes ofídicos ainda é um problema de saúde pública. Há uma necessidade urgente de estudos e incentivos políticos a fim de melhorar a produção e distribuição desses antivenenos, bem como o treinamento da equipe médica para utilização do soro e outros medicamentos envolvidos durante o tratamento (GUTIÉRREZ et al., 2010; WARREL, 2010; WHO, 2010).

3 Componentes da peçonha ofídica

A peçonha ofídica consiste em um complexo de substâncias biologicamente ativas produzidas por glândulas bucais diretamente ligadas às presas através de ductos (KERKKAMP; CASEWELL; VONK, 2015). Ela tem como função a imobilização, morte e digestão da presa, além de contribuir para a defesa contra predadores (HARDY; COCHRANE; ALLAVENA, 2014). A composição da peçonha pode diferenciar-se de acordo com a família, gênero, espécie e subespécie. Variações intraespecíficas também podem ocorrer, uma vez que sua composição é influenciada pela origem geográfica, dieta, idade, dimorfismo sexual e habitat (CHIPPAUX; WILLIAMS; WHITE, 1991; MENEZES et al., 2006).

Cerca de 90% do seu peso seco corresponde a proteínas e peptídeos, enzimáticos e não enzimáticos. Aminotransferases, acetilcolinesterases, ADPases, ATPases, hialuronidases, L-aminoácido-oxidases, fosfolipases A_2, metaloproteases, serinoproteases e 5'-nucleotidases são exemplos de enzimas encontradas na peçonha, as quais são responsáveis pela maioria dos efeitos deletérios observados durante o envenenamento. Os constituintes não-enzimáticos incluem as lectinas, os ativadores de proteína C, proteínas ligantes ao fator de Von Willebrand (FvW), fatores de crescimento do endotélio vascular (VEGF) e neuronal (NGF), precursores de peptídeos bioativos e desintegrinas (ANGULO; LOMONTE, 2009; BRAUD; BON; WISNER, 2000; FRANÇA; MÁLAQUE, 2009; KANG et al., 2011; KINI, 2006; MARLAND, 1998; RAMOS; SELISTRE-DE-ARAÚJO, 2006).

Uma fração não proteica também faz parte da constituição da peçonha ofídica, sendo representada por compostos orgânicos de baixo peso molecular, como carboidratos, lipídios, aminoácidos livres, nucleosídeos, aminas biogênicas e citrato; e compostos inorgânicos, como cálcio, ferro, fósforo, potássio, magnésio, manganês, cobalto, sódio e zinco (BJARNASON; FOX, 1994; FRANÇA; MÁLAQUE, 2009; KOH; ARMUGAM; JEYASEELAN, 2006; MARLAND, 1998; MATSUI; FUJIMURA; TITANI, 2000; RAMOS; SELISTRE-DE-ARAÚJO, 2006). O cálcio, manganês e magnésio são importantes para estabilização estrutural de algumas proteínas. O zinco, cobre, ferro e cobalto atuam nos

mecanismos catalíticos de alguns componentes enzimáticos, como as metaloproteases (BJARNASON; FOX, 1994).

Os componentes da peçonha ofídica apresentam diferentes atividades farmacológicas e o estudo dessas toxinas isoladas tem contribuído para a elucidação de mecanismos moleculares envolvidos na fisiologia do envenenamento, desenvolvimento de poderosas ferramentas de pesquisa, técnicas de diagnóstico e de novos agentes terapêuticos, como agentes antiplaquetários, anticoagulantes, antitumorais, antivirais, antimicrobianos, analgésicos, entre outros (CHAN et al., 2016; CUNHA; MARTINS, 2012; MCCLEARY; KINI, 2012).

3.1 Metaloproteases

As metaloproteases da peçonha de serpentes (do inglês: Snake Venom Metalloproteinases - SVMPs) são enzimas que dependem da ligação de um metal (Zn^{2+}, Ca^{2+} ou Mg^{2+}) em seu sítio catalítico para a manifestação de suas atividades biológicas (FOX; SERRANO, 2009). Em geral, as SVMPs são zinco dependentes, pertencentes à família metzincina e possuem uma sequência peptídica conservada de ligação de zinco, HEBXHXBGBXHZ, constituindo o domínio catalítico metaloprotease; onde H representa a histidina, E ácido glutâmico, G glicina, B um resíduo hidrofóbico, X um aminoácido qualquer e Z um aminoácido diferente entre as subfamílias, mas conservado dentro das mesmas (BODE; GOMIS-RUTH; STOCKLER, 1993). Agentes quelantes de Zn^{2+}, tais como ácido etilenodiaminotetracético (EDTA) e 1,10-fenantrolina inibem completamente a atividade das SVMPs (MATSUI; FUJIMURA; TITANI, 2000).

As SVMPs são classificadas em três classes (P-I, P-II e P-III), conforme suas massas moleculares e organização de seus domínios estruturais. As toxinas da classe P-I apresentam massas moleculares entre 20 - 30 KDa e são constituídas apenas pelo domínio metaloprotease. A classe P-II apresenta toxinas com massa molecular entre 30 - 60 KDa e possuem, além do domínio metaloprotease, um domínio desintegrina, os quais são frequentemente separados por um processo proteolítico pós-traducional. Ambos os produtos são estáveis após o processamento. As toxinas da classe P-III possuem massas

moleculares entre 60 - 100 KDa e apresentam um domínio semelhante ao desintegrina (*Dis-like*) e um domínio rico em cisteína (*Cys-rich*) adicionais ao domínio catalítico (FOX; SERRANO, 2008). A principal diferença entre os domínios desintegrina e *dis-like* é a ausência do motivo RGD, característico de desintegrinas de classe P-II (CALVETE et al., 2005; GUTIÉRREZ; RUCAVADO, 2000).

As classes P-II (P-IIa, P-IIb, P-IIc, P-IId e P-IIe) e P-III (P-IIIa, P-IIIb, P-IIIc e P-IIId) foram subdivididas de acordo com o processamento proteolítico pós-traducional para liberação dos domínios adicionais e formação de estruturas diméricas. Em especial, a subclasse P-IIId possui um domínio semelhante à lectina (*Lectin-like*) ligada por pontes dissulfeto aos demais domínios característicos de SVMPs P-III (FOX; SERRANO, 2008). A figura 05 mostra um esquema representativo da classificação das SVMPs.

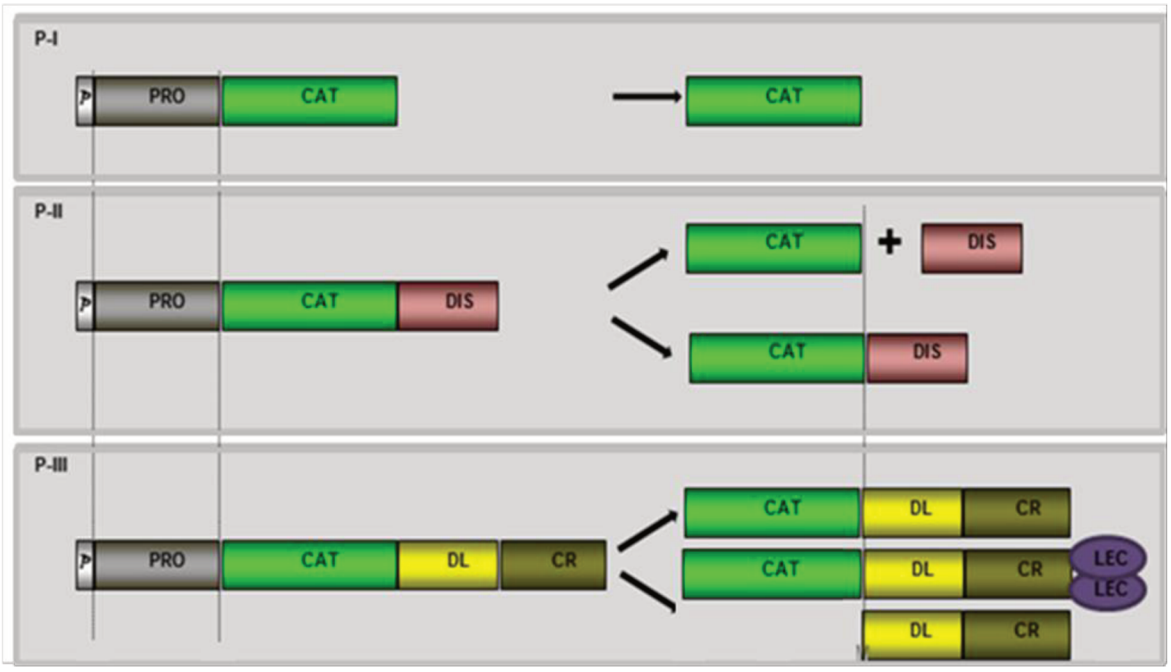

Figura 05: Esquema representativo da classificação das SVMPs, antes e após o processamento proteolítico pós-traducional. P: peptídeo sinal; PRO: pró-domínio, removido durante sua ativação; CAT: domínio catalítico ou metaloprotease; DIS: domínio desintegrina; DL: domínio semelhante à desintegrina (*Dis-like*); CR: domínio rico em cisteína (*Cys-rich*); LEC: domínio semelhante à lectina (*Lectin-like*). Fonte: adaptado de MOURA-DA-SILVA et al., 2016.

Estima-se que cerca de 32% da peçonha de viperídeos seja constituída por SVMPs, o que sugere seu papel significativo nas patologias associadas ao envenenamento por essas serpentes (FOX; SERRANO, 2009).

As SVMPs são uma das principais enzimas responsáveis pelo aparecimento de hemorragia, uma vez que apresentam afinidade específica sobre a membrana basal de capilares, provocando ruptura da integridade vascular, principal mecanismo indutor de hemorragia (BJARNASON; TU, 1978; GUTIÉRREZ; RUCAVADO, 2000; MOREIRA et al, 1994). A habilidade de causar hemorragia pode ser atribuída a todas classes de SVMPs. Entretanto, as enzimas da classe P-III são as mais hemorrágicas (FOX; SERRANO, 2005). A presença dos domínios não catalíticos favorece a fixação e direcionamento aos componentes específicos da microvasculatura (ESCALANTE et al., 2011). Além da hemorragia, as SVMPs induzem mionecrose, que parece ser secundária à isquemia que ocorre no tecido muscular em decorrência da hemorragia (GUTIÉRREZ et al., 1995; GUTIÉRREZ; RUCAVADO, 2000).

Outras atividades comumente associadas às SVMPs são: atividades fibrin(ogen)olítica e apoptótica, ativação de protrombina e do fator X da cascata de coagulação e inibição da agregação plaquetária; a maioria delas relacionadas a distúrbios hemostáticos. As SVMPs também causam reações inflamatórias e edemas (FOX; SERRANO, 2005, 2009). A presença dos domínios não catalíticos facilita, também, a interação das SVMPs com os receptores plaquetários (MATSUI; FUJIMURA; TITANI, 2000).

3.2 Serinoproteases

As serinoproteases da peçonha de serpentes (do inglês: Snake Venom Serine Proteinase - SVSPs) são enzimas que contêm em seu sítio ativo um resíduo de serina altamente reativo, estabilizado pela presença de um resíduo de histidina e aspartato. A organização desses resíduos gera um sítio catalítico muito bem caracterizado, que define esse grupo de proteases (BARRETT; RAWLINGS, 1995). Essas enzimas são inibidas por compostos que reagem com o resíduo de serina, como fluoreto de fenilmetilsulfonilo (PMSF) e diisopropilfluorofosfato

(DFP), e também são inibidas competitivamente por benzamidina e por p-aminobenzamidina (SERRANO; MAROUN, 2005).

Apesar de possuírem uma característica estrutural homogênea, as SVSPs apresentam diversos efeitos farmacológicos. Elas são caracterizadas como enzimas que têm atividade semelhante à trombina (*thrombin-like*). Podem afetar a cascata de coagulação pela ativação de componentes sanguíneos envolvidos na coagulação (fator V, proteína C e plasminogênio) e ação sobre o sistema fibrinolítico, calicreína-cinina (*kallikrein-like*) e sobre a agregação plaquetária, causando um desequilíbrio no sistema hemostático. As SVSPs calicreína-símile (*Kallikrein-like*) liberam bradicinina a partir da proteólise do cininogênio plasmático provocando hipotensão, quadro comum do acidente ofídico (COSTA et al., 2010; MATSUI; FUJIMURA; TITANI, 2000; OLIVEIRA et al., 2016; SERRANO, 2013; SERRANO; MAROUN, 2005).

As SVSPs com atividade trombina-símile (*thrombin-like*) clivam o fibrinogênio plasmático, semelhantemente à trombina, liberando os fibrinopeptídeos A e B. Todavia, essas enzimas não ativam outros fatores da coagulação, como o fator XIII. Por esse motivo, elas formam apenas um coágulo de fibrina frouxo, o qual é rapidamente degradado pelo sistema fibrinolítico (CASTRO et al., 2004; KINI, 2006; MATSUI; FUJIMURA; TITANI, 2000). Por esse motivo, essas enzimas têm sido amplamente utilizadas como agentes desfibrinogenantes em várias condições trombóticas e em coagulopatias. Um exemplo é a Batroxobin, uma SVSP *thrombin-like* isolada de peçonha da serpente *B. atrox*, que atua como um desfibrinogenante devido à sua específica ação sobre o fibrinogênio (SERRANO, 2013).

3.3 Fosfolipases A_2

As fosfolipases A_2 (PLA_2s) são enzimas dependentes de Ca^{2+} que catalisam a hidrólise específica da ligação éster do carbono 2 de fosfolipídios, liberando ácidos graxos, como o ácido araquidônico (AA), e lisofosfolipídios. O AA liberado é precursor de prostaglandinas, tromboxanos e leucotrienos, mediadores químicos que iniciam uma série de reações inflamatórias (ARNI; WARD, 1996; KINI, 2003).

Essas enzimas podem ser classificadas em PLA_2s secretadas ($sPLA_2$s), citosólicas ($cPLA_2$s), independentes de Ca^{2+} ($iPLA_2$s), acetil-hidrolases do fator ativador de plaquetas (PAH-AH), PLA_2s lisossomais e PLA_2s tecido adiposo-específica. As PLA_2s encontradas em peçonha de serpentes são do tipo $sPLA_2$s. As PLA_2s também foram divididas em 15 grupos (I à XV) de acordo com localização celular, peso, dependência de Ca^{2+}, entre outros. As PLA_2s de peçonha de serpente pertencem aos grupos I e II e apresentam baixa massa molecular (cerca de 14 - 18 KDa), cinco a oito pontes dissulfeto, possuem em seu sítio ativo um resíduo de histidina e aspartato e necessitam de Ca^{2+} para suas atividades enzimáticas (DENNIS et al., 2011; VALENTIN; LAMBEAU, 2000). Aquelas do grupo I correspondem às PLA_2s da família Elapidae, enquanto que as do grupo II correspondem às PLA_2s da família Viperidae (BURKE; DENNIS, 2009; DENNIS et al., 2011).

As PLA_2s do grupo II são subdivididas em dois subgrupos principais: as PLA_2s Asp49, assim denominadas por possuírem um resíduo de aspartato na posição 49, enzimaticamente ativas; e as PLA_2s Lys49, nas quais o resíduo de aspartato é substituído por um resíduo de lisina na mesma posição, com nenhuma atividade catalítica sobre os fosfolipídios. A substituição do resíduo Asp49 por Lys49 altera a ligação do Ca^{2+} às PLA_2s, comprometendo sua atividade enzimática (ARNI; WARD, 1996; OWNBY et al., 1999; SOARES; FONTES; GIGLIO, 2004).

Apesar de apresentarem significante similaridade estrutural, as PLA_2s compreendem um dos componentes mais relevantes da peçonha, sendo responsáveis por diversos efeitos provocados pelo envenenamento, como: neurotoxicidade, miotoxicidade, cardiotoxicidade, hipotensão, hemorragia, indução de edema, reação inflamatória, hemólise, ação anticoagulante e sobre a atividade plaquetária (DUTTA; GOGOI; MUKHERJEE, 2015; FAURE; GOWDA; MAROUN, 2007; KINI, 2003; SANTOS-FILHO et al., 2008; SILVEIRA et al., 2013; TEIXEIRA et al., 2011). Sugere-se que a grande diversidade de efeitos farmacológicos induzidos pelas PLA_2s esteja relacionada com sua alta afinidade a receptores endógenos específicos, as quais podem atuar enzimaticamente ou por mecanismos não enzimáticos (KINI, 2003; SAJEVIC; LEONARDI; KRIZAJ, 2011).

3.4 L-aminoácido-oxidases

As L-aminoácido-oxidases de peçonha de serpentes (do inglês: Snake Venom L-amino acid oxidases - SV-LAAOs) são enzimas amplamente distribuídas em peçonhas ofídicas, que catalisam a desaminação oxidativa estereoespecífica de L-aminoácidos, produzindo o alfa-ceto ácido correspondente, peróxido de hidrogênio (H_2O_2) e amônia (IZIDORO et al., 2014; KOMMOJU; MACHEROUX; GHISLA, 2007; MOUSTAFA et al., 2006; RODRIGUES et al., 2009; SUN et al., 2010). Em geral, as SV-LAAOs são estruturas homodiméricas, unidas por ligações não covalente e com massa molecular de aproximadamente 110 - 150 KDa em condições não desnaturantes, e 50 - 70 KDa em sua forma monomérica (DU; CLEMETSON, 2002; IZIDORO et al., 2014; SOUZA et al., 1999). Elas são consideradas flavoenzimas, uma vez que cada subunidade apresenta um cofator flavina mononucleotídeo (FMN) ou flavina adenina dinucleotídeo (FAD) ligado covalentemente à sua estrutura química. A cor amarela da peçonha está relacionada à presença do pigmento riboflavina presente nesses cofatores (COSTA et al., 2014; GUO et al., 2012; JOHNSON; KARDONG; OWNBY, 1987).

Embora o papel fisiológico das SV-LAAOs ainda seja pouco conhecido, acredita-se que sua presença esteja relacionada com a proteção contra agentes naturais, parasitas e bactérias (FOX, 2013; VARGAS et al., 2013). Estudos têm mostrado que elas apresentam uma variedade de efeitos farmacológicos, como: indução ou inibição da agregação plaquetária; atividades antimicrobiana, antibacteriana e antitumoral; citotoxicidade; indução de edema e atividade apoptótica, inclusive de células endoteliais vasculares, contribuindo para o sangramento prolongado após o acidente (ANDE et al., 2008; CISCOTTO et al., 2009; IZIDORO et al., 2014; NAUMANN et al., 2011; SAMEL et al., 2006; TONISMAGI et al., 2006; TORRES et al., 2010). Vários estudos têm mostrado que o principal metabólito responsável por essas atividades é o H_2O_2 liberado durante a reação química catalisada pelas SV-LAAOs, o qual contribui para a ação citotóxica da enzima (DU; CLEMETSON, 2002; FOX, 2013).

3.5 Desintegrinas

As desintegrinas presentes na peçonha de serpentes compreendem uma família de pequenos polipeptídeos (40 - 100 aminoácidos) não enzimáticos ricos em cisteína, liberados pelo processamento proteolítico de SVMPs classe P-II e P-III (KINI; EVANS, 1992) ou sintetizados diretamente a partir de mRNAs (OKUDA; KOIKE; MORITA, 2002).

As desintegrinas são divididas em cinco grupos de acordo com seu tamanho e número de pontes dissulfeto (CALVETE et al., 2003). O primeiro grupo inclui desintegrinas curtas, com 41 a 51 resíduos de aminoácidos e quatro pontes dissulfeto. O segundo grupo é formado por desintegrinas de tamanho médio com, aproximadamente, 70 aminoácidos e seis pontes dissulfeto. O terceiro grupo inclui desintegrinas longas, com 84 resíduos e sete pontes dissulfeto. No quarto grupo estão as *disintegrins-like* derivadas do processamento proteolítico das SVMPs da classe P-III. Essas moléculas contêm, aproximadamente, 100 aminoácidos e oito pontes dissulfeto (CALVETE et al., 2000a; CALVETE et al., 2003). Diferentemente das desintegrinas já citadas, que são moléculas de cadeia simples, o quinto grupo é formado por desintegrinas diméricas. Cada subunidade contém cerca de 67 resíduos, dentre os quais 10 são de cisteína, envolvidos na formação de quatro pontes dissulfeto intracadeia e duas intercadeia (BILGRAMI et al., 2004; CALVETE et al., 2000b; CALVETE et al., 2003).

Apesar de serem considerados pequenos polipeptídeos, as desintegrinas apresentam grande versatilidade funcional. Elas são capazes de se ligar especificamente à integrinas presentes na superfície celular, especialmente integrinas β_1 e β_3, bloqueando sua atividade (CALVETE et al., 2005; CALVETE, 2013; SANZ et al., 2006). Por esse motivo, elas podem ser utilizadas na terapia de diversas patologias nas quais bloqueadores de integrinas desempenham papel relevante, incluindo isquemia coronariana aguda e trombose ($\alpha_{IIb}\beta_3$), metástase tumoral, osteoporose, reestenose e artrite reumatóide ($\alpha_v\beta_3$), infecção bacteriana e doenças vasculares ($\alpha_5\beta_1$), inflamação e doenças autoimunes ($\alpha_4\beta_1$, $\alpha_7\beta_1$, $\alpha_9\beta_1$) e angiogênese tumoral ($\alpha_1\beta_1$, $\alpha_v\beta_3$) (CALVETE et al., 2005; CALVETE, 2013; MARCINKIEWICZ, 2007).

A inibição das integrinas pelas desintegrinas depende do pareamento adequado dos resíduos de cisteína que determinam a conformação da alça inibitória, cujo ápice contém uma sequência tripeptídica específica (CALVETE et al., 2003; CALVETE et al., 2005). Em geral, nas desintegrinas derivadas das SVMPs da classe P-II, a sequência ativa é o tripeptídeo RGD (arginina-glicina-aspartato) (MCLANE et al., 1998). Entretanto, estudos recentes mostraram a presença de outros motivos, como KGD (lisina-glicina-aspartato), MVD (metionina-valina-aspartato), MLD (metionina-leucina-aspartato), VGD (valina-glicina-aspartato) e KTS (lisina-treonina-serina) (CALVETE et al., 2005; HITE et al., 1992; KOH; KINI, 2012; OSHIKAWA; TERADA, 1999; SCARBOROUGH et al., 1991). Nas *disintegrins-like*, o motivo RGD é substituído pela sequência XXCD (x-x-cisteína-aspartato) (CALVETE et al., 2005). Em geral, motivos RGD bloqueiam integrinas $\alpha_8\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$ e $\alpha_{IIb}\beta_3$; KGD e MVD bloqueiam a integrina $\alpha_{IIb}\beta_3$; MLD visa principalmente as integrinas $\alpha_4\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_9\beta_1$; VGD prejudica a função da integrina $\alpha_5\beta_1$ e KTS é um inibidor de $\alpha_1\beta_1$ (CALVETE, 2013).

A sequência C-terminal da desintegrina, junto à sequência de aminoácidos dentro da alça inibitória, determina a especificidade de ligação à integrina (EBLE, 2010). Isso explica o fato de que diferentes desintegrinas contendo um mesmo motivo de ligação apresentam afinidade e seletividade de ligação diferentes para as integrinas (CALVETE et al., 2005).

3.6 Lectinas tipo C

As lectinas tipo C são proteínas diméricas não enzimáticas, capazes de se ligar a mono e oligossacarídeos de forma reversível, não-covalente e Ca^{2+} dependentes. Essa capacidade de se ligar a carboidratos se deve à presença de uma região designada de domínio de reconhecimento ao carboidrato (CRD) presente na região C-terminal de cada subunidade da proteína, o qual é formado por uma sequência de três aminoácidos (glutamina-prolina-aspartato) (DODD; DRICKAMER, 2001; DRICKAMER, 1988). Esses domínios interagem com os açúcares presentes nas células por meio de pontes de hidrogênio e interações de van der Waals, estabelecidas entre o sítio ativo da proteína e os carboidratos de superfície celular (WEIS; DRICKAMER, 1996). Esses domínios são responsáveis

pela principal função dessa classe de proteínas, a aglutinação de eritrócitos *in vitro* (GARTNER; STOCKER; WILLIAMS, 1980; LAM; NG, 2011).

A principal forma de lectina encontrada em peçonha de serpentes são as lectinas tipo C-*like*, também denominadas de *Snaclecs*, as quais são Ca^{2+} independentes e possuem um domínio CRD incompleto ou ausente, e consequentemente não se ligam a açúcares (CLEMETSON, 2010; MORITA, 2005; ZELENSKY; GREADY, 2005).

As lectinas tipo C-*like* estão envolvidas, principalmente, com as alterações hemostáticas decorrentes do envenenamento ofídico, interagindo com receptores plaquetários ou fatores da cascata de coagulação sanguínea. Essas moléculas podem apresentar ação anticoagulante devido à sua ligação aos fatores IX e X, inibindo a conversão de protrombina em trombina ou ligando-se diretamente à trombina (AROCAS et al., 1996; ATODA; HYUGA; MORITA, 1991). Podem, ainda, apresentar ação coagulante devido à ativação do fator X e protrombina (TAKEYA et al., 1992; YAMADA; MORITA, 1997) e podem atuar como agonistas ou antagonistas plaquetários devido à sua afinidade por receptores plaquetários, como GPIb, FvW e receptores de colágeno (GPVI e $\alpha_2\beta_1$) (BERGMEIER et al., 2001; HAMAKO et al., 1996; KANAJI et al., 2003; LU et al., 2005; MARCINKIEWICZ et al., 2000; POLGAR et al., 1997; USAMI et al., 1993). Outros efeitos, também, atribuídos às lectinas de peçonha de serpentes são: indução de edema, hipotensão, aumento de atividade de neutrófilos e atividades anti-tumoral, anti-bacteriana e anti-microbiana (BARBOSA et al., 2010; LOMONTE et al., 1990; NUNES et al., 2011; PANUNTO et al., 2006; PEREIRA-BITTENCOURT; GAGLIARDI; COLLINS, 1999).

4 Hemostasia

A hemostasia é definida como uma série complexa de processos fisiológicos que tem a função de formar um tampão hemostático sobre uma superfície danificada do endotélio vascular, enquanto mantém o fluxo sanguíneo normal em outra parte da circulação. Dessa forma, a hemostasia minimiza a perda sanguínea e promove a restauração da arquitetura vascular (GALE, 2011; STASSEN; ARNOUT; DECKMYN; 2004).

O sistema hemostático é constituído principalmente pelas células endoteliais, plaquetas, fatores de coagulação e fatores fibrinolíticos. As células endoteliais são as células que revestem os vasos sanguíneos. No estado normal contribuem para a manutenção do fluxo sanguíneo em uma superfície não-trombogênica, enquanto que mediante uma injúria vascular estimulam a síntese de vários fatores pró-trombóticos. Esses fatores pró-trombóticos, juntamente com a exposição das moléculas adesivas do subendotélio, permitem que as plaquetas interajam com as paredes do vaso, favorecendo a formação do tampão plaquetário. Simultaneamente, fatores de coagulação (pró-enzimas) produzidos pelo fígado são ativados através de uma cascata de coagulação que irão resultar na formação de fibrina, a qual será depositada sobre o tampão plaquetário, transformando-o em um tampão hemostático sólido. Após a cobertura da área lesada pelo tampão hemostático, o endotélio libera um ativador de plasminogênio tecidual que converte plasminogênio em plasmina. A plasmina formada promove proteólise das fibras de fibrina e outras proteínas coagulantes, dissolvendo o coágulo a fim de controlar sua extensão e restabelecendo o fluxo sanguíneo no interior do vaso restaurado (CASTRO et al., 2006; COLLER et al., 2010; ISRAELS et al., 2011; QUEIROZ et al., 2017; STASSEN; ARNOUT; DECKMYN, 2004).

Os processos de formação do tampão plaquetário, ativação dos fatores de coagulação e sistema fibrinolítico também são conhecidos por hemostasia primária, secundária e terciária, respectivamente (STASSEN; ARNOUT; DECKMYN, 2004).

4.1 Hemostasia primária

A hemostasia primária compreende mecanismos dependentes de plaquetas. As plaquetas são pequenos fragmentos citoplasmáticos anucleados produzidos na medula óssea a partir dos megacariócitos. Elas são capazes de aderirem a vasos sanguíneos danificados e a outras plaquetas, a fim de formarem o tampão plaquetário (COLLER et al., 2010; SCHULE; SHIVDASANI, 2005).

Receptores de superfície presentes na membrana plaquetária regulam as interações plaqueta-plaqueta ou plaqueta-matriz sendo essenciais para a formação do tampão plaquetário. As glicoproteínas (GP) Ib-V-IX, GPVI, $5HT_{2A}$,

TP, receptores ativados por proteases (PARs), $P2Y_1$, $P2Y_{12}$ e as integrinas plaquetárias são alguns exemplos desses receptores envolvidos na hemostasia (COLLER et al., 2010). As integrinas são complexos heterodiméricos compostos por duas subunidades de glicoproteínas transmembrana (α e β) associadas por ligações não-covalentes. Elas estão amplamente distribuídas em diferentes tipos de células, e cada integrina demonstra propriedades únicas de ligação ao ligando. Cinco tipos de subunidades α (α_2, α_{IIb}, α_V, α_5, α_6) e dois tipos de subunidades β (β_1 e β_3) formam as integrinas plaquetárias: $\alpha_{IIb}\beta_3$ (receptor de fibrinogênio), $\alpha_V\beta_3$ (receptor de vitronectina), $\alpha_2\beta_1$ (receptor de colágeno), $\alpha_5\beta_1$ (receptor de fibronectina) e $\alpha_6\beta_1$ (receptor de laminina) (BENNETT; BERGER; BILLINGS, 2009; CALVETE et al., 2005; KAMIGUTI, 2005).

Sob condições fisiológicas, as plaquetas fluem pelos vasos sanguíneos sem interagir com o endotélio vascular. Quando a integridade do sistema vascular é rompida, as plaquetas são recrutadas da circulação para a matriz subendotelial e submetidas a uma resposta em série, altamente regulada que inclui: adesão das plaquetas às moléculas adesivas do subendotélio (adesão plaquetária), ativação das plaquetas e amplificação da resposta plaquetária (ativação plaquetária) e interação plaqueta-plaqueta (agregação plaquetária). Estas respostas são mediadas pelos receptores presentes na superfície das plaquetas e resultam na formação do tampão plaquetário (CASTRO et al., 2006; JURK; KEHREL, 2005).

4.1.1 Adesão plaquetária

A adesão plaquetária à matriz subendotelial é a primeira etapa da hemostasia que envolve as plaquetas (ANDREWS; BERND, 2004; STASSEN; ARNOUT; DECKMYN, 2004). Após o rompimento da integridade do sistema vascular as plaquetas são recrutadas da circulação para a matriz subendotelial, as quais interagem com os componentes da matriz expostos na parede do vaso (JURK; KEHREL, 2005). Este é um processo complexo que depende da tensão de cisalhamento. Sob altas forças de cisalhamento, como ocorre em pequenas artérias e arteríolas, as plaquetas interagem com o FvW através do complexo plaquetário GPIb-V-IX. Em seguida, as plaquetas podem interagir diretamente com o colágeno através de seus principais receptores de colágeno, GPVI e $\alpha_2\beta_1$.

Por sua vez, sob baixas forças de cisalhamento, as plaquetas aderem principalmente ao colágeno, fibronectina e laminina, através de integrinas β_1 (KAMIGUTI, 2005).

O FvW é uma glicoproteína plasmática multimérica de alto peso molecular sintetizado pelas células endoteliais. Os múltiplos sítios de ligação do FvW permitem a ligação à integrina plaquetária $\alpha_{IIb}\beta_3$ (também conhecida por GPIIb-IIIa), assim como à subunidade GPIbα do complexo GPIb-V-IX (JURK; KEHREL, 2005; MATSUI; HMAMAKO, 2005). O FvW desempenha um papel crítico na adesão plaquetária uma vez que ele permite o primeiro contato das plaquetas com os componentes expostos no subendotélio (JURK; KEHREL, 2005). Ele atua como uma ponte molecular entre os receptores plaquetários, principalmente a subunidade GPIbα, e o colágeno do subendotélio. A afinidade do FvW circulante com a GPIbα plaquetária é baixa, mas é aumentada em condições de alto cisalhamento, devido às alterações conformacionais ocasionadas no FvW (OBERT et al., 2006; RUGGERI; MENDOLICCHIO, 2015). Por isso, sob condições estáticas, a ligação FvW-GPIbα não ocorre. Indutores não fisiológicos, tais como o antibiótico ristocetina são utilizados para reproduzir esse evento *in vitro* (MATSUI et al., 2002; NAVDAEV et al., 2014).

A ligação FvW-GPIbα, apesar de desempenhar um papel crítico na adesão plaquetária, apresenta uma taxa de dissociação rápida, não sendo capaz de induzir uma adesão plaquetária estável (RUGGERI, 2002). Entretanto, essa interação facilita a ligação direta das plaquetas com o colágeno subendotelial através da GPVI, que, por sua vez, gera sinais intracelulares que medeiam a ativação de outros receptores necessários para uma adesão plaquetária estável e irreversível na superfície do subendotélio, como $\alpha_2\beta_1$ e $\alpha_{IIb}\beta_3$ (NIESWANDT; WATSON, 2003). Enquanto a integrina $\alpha_2\beta_1$ liga-se diretamente ao colágeno, $\alpha_{IIb}\beta_3$ medeia uma adesão irreversível por se ligar à sequência RGD do FvW (GAWAZ, 2004).

Além disso, o contato inicial da GPIbα e da GPVI com componentes do subendotélio inicia uma transdução de sinais que resultam na ativação das plaquetas aderentes induzindo o aumento da concentração de Ca^{2+} citosólico, que, por sua vez, promove a secreção de agonistas plaquetários, aumentando a afinidade da integrina $\alpha_{IIb}\beta_3$ pelo FvW. Esses eventos intensificam a adesão

plaquetária e contribuem para a posterior formação de um agregado plaquetário (CASTRO et al., 2006; KAMIGUTI, 2005; ISRAELS et al., 2011).

4.1.2 Ativação plaquetária

A adesão plaquetária, seja induzida pelo colágeno ou por outros agonistas, como ADP ou trombina, induz uma rápida transdução de sinais, desencadeando uma série de eventos que sustentarão a adesão e a subsequente agregação plaquetária (CASTRO et al., 2006).

As plaquetas aderentes sofrem uma reorganização das proteínas do citoesqueleto que resulta em mudança conformacional, passando da forma discóide para o formato de esferas irregulares, com projeção de pseudópodes provenientes da membrana plasmática. Os pseudópodes formados facilitam a adesão das plaquetas à parede do vaso lesado, permitindo uma vedação efetiva (GAWAZ, 2004; ISRAELS et al., 2011; JURK; KEHREL, 2005). Além disso, os grânulos internos homogeneamente distribuídos nas plaquetas em repouso sofrem centralização e fusão com a membrana plasmática, resultando em secreção de seu conteúdo via exocitose (JURK; KEHREL, 2005).

O aumento dos níveis de Ca^{2+} citosólico, tanto a partir dos estoques de armazenamento de Ca^{2+} dentro das plaquetas, conhecido como sistema tubular denso, como a partir do influxo de Ca^{2+} no citoplasma, permite uma série de processos, como: secreção do conteúdo dos grânulos, ativação das PLA_2s plaquetárias, reorganização da bicamada de fosfolípidos e ativação de integrinas plaquetárias (STASSEN; ARNOUT; DECKMYN, 2004).

As plaquetas possuem dois tipos de grânulos de armazenamento, os grânulos α e os grânulos densos. Os grânulos α contêm várias proteínas adesivas que atuam na interação das plaquetas com a parede do vaso lesado, como FvW, vitronectina e fibronectina, além de fatores de coagulação (fator V, VII, XI e XIII), fatores de crescimento derivados de plaquetas (PDGF) e fibrinogênio. Os grânulos densos contêm ADP, ATP, serotonina e Ca^{2+} (JURK; KEHREL, 2005; STASSEN; ARNOUT; DECKMYN, 2004). Esses componentes quando secretados após a ativação plaquetária modulam as funções das plaquetas aderentes e

estimulam o recrutamento de plaquetas adicionais para formação de agregados na parede do vaso lesado (JURK; KEHREL, 2005).

Acredita-se que o ADP secretado pelos grânulos densos seja o principal amplificador da ativação plaquetária inicial. Ele atua via dois receptores plaquetários, $P2Y_1$ e $P2Y_{12}$. O receptor $P2Y_1$ medeia a alteração conformacional das plaquetas e o aumento dos níveis de Ca^{2+} citosólico, o qual desempenha um papel central na ativação e atração plaquetária. Por sua vez, o receptor $P2Y_{12}$ potencializa a secreção plaquetária sustentando a ativação das plaquetas, e ativa a integrina $\alpha_{IIb}\beta_3$. A relação entre esses dois receptores plaquetários é que $P2Y_1$ inicia a resposta plaquetária, enquanto que $P2Y_{12}$ atua reforçando-a (ANDREWS; BERND, 2004; DORSAM; KUNAPULI, 2004; GACHET, 2001; JURK; KEHREL, 2005).

A serotonina (5-hidroxitriptamina ou 5-HT), um potente vasoconstritor, atua sobre o receptor $5HT_{2A}$ e amplifica, juntamente com o ADP, a resposta plaquetária. Além disso, a serotonina pode desempenhar um papel pró-coagulante devido à retenção de proteínas pró-coagulantes na superfície das plaquetas, como fibrinogênio (DALE et al., 2002; JURK; KEHREL, 2005).

O aumento dos níveis de Ca^{2+} citosólico resultam na ativação das PLA_2s plaquetárias. Essas enzimas clivam os fosfolipídeos, resultando na liberação de AA que por sua vez é convertido em tromboxano A_2 (TXA_2). Ao ligar ao seu receptor (TP), o TXA_2 libera mais Ca^{2+} para o citosol, amplificando a resposta plaquetária. Dessa forma, assim como o ADP, o TXA_2 atua como um mediador de *feedback* positivo na resposta plaquetária (STASSEN; ARNOUT; DECKMYN, 2004). Além disso, o TXA_2 tem uma atividade vasoconstritora e, portanto, favorece a formação do trombo pela redução do fluxo sanguíneo (GAWAZ, 2004).

Quando as plaquetas são ativadas, ocorre uma remodelação na distribuição dos fosfolipídeos da membrana, de forma que aqueles carregados negativamente ficam expostos na superfície externa das plaquetas. Estes se complexam com Ca^{2+}, que então formam uma ponte com alguns fatores de coagulação. A colocalização desses fatores de coagulação na superfície das plaquetas ativadas facilita a propagação da coagulação e formação de trombina a partir de protrombina, culminando com intensa deposição de fibrina que reforçará o tampão plaquetário. A trombina, além de seu papel na coagulação, é um dos

mais poderosos agonistas plaquetários. Ela amplifica a ativação plaquetária ao interagir com seus receptores plaquetários GPIbα e clivgem de receptores ativados por proteases (PARs), PAR1 e PAR4 (COLLER et al., 2010; STASSEN; ARNOUT; DECKMYN, 2004).

4.1.3 Agregação plaquetária

A adesão das plaquetas, reorganização de seu citoesqueleto e secreção do conteúdo de seus grânulos são passos fundamentais para a formação do tampão plaquetário, porém o passo final é a interação plaqueta-plaqueta (STASSEN; ARNOUT; DECKMYN, 2004). O receptor central desse processo é a integrina $\alpha_{IIb}\beta_3$ (também conhecida por GPIIb-IIIa) (JURK; KEHREL, 2005).

Cerca de 40.000 - 80.000 receptores $\alpha_{IIb}\beta_3$ estão presentes por plaqueta. Nas plaquetas em repouso, esses receptores apresentam-se em um estado de baixa afinidade, os quais precisam ser ativados para ligarem-se aos seus ligantes (STASSEN; ARNOUT; DECKMYN, 2004). Todos os agonistas plaquetários, incluindo ADP, TXA_2 e trombina, ao interagirem com seus receptores de membrana específicos, iniciam uma via de sinalização desencadeando mudança na conformação da integrina $\alpha_{IIb}\beta_3$, convertendo-a de seu estado de baixa afinidade para um estado ativado de alta afinidade a seus ligantes, devido à exposição de seus sítios de ligação. Assim, a integrina $\alpha_{IIb}\beta_3$ ativada liga-se ao fibrinogênio e/ou FvW, através de suas sequências RGD (arginina-glicina-aspartato), os quais funcionam como uma ponte entre integrinas $\alpha_{IIb}\beta_3$ de plaquetas adjacentes ativadas, resultando na interação plaqueta-plaqueta, e por fim, na formação do tampão plaquetário sobre a superfície do vaso lesado (GAWAZ, 2004; ISRAELS et al., 2011).

Após a formação do tampão plaquetário, o processo de coagulação é acelerado na superfície das plaquetas ativadas, levando à estabilização do tampão devido à deposição de fibrina, formando um tampão hemostático sólido sobre a superfície danificada do endotélio vascular (YIP et al., 2005). A figura 06 mostra um esquema simplificado dos processos de adesão, ativação e agregação plaquetária.

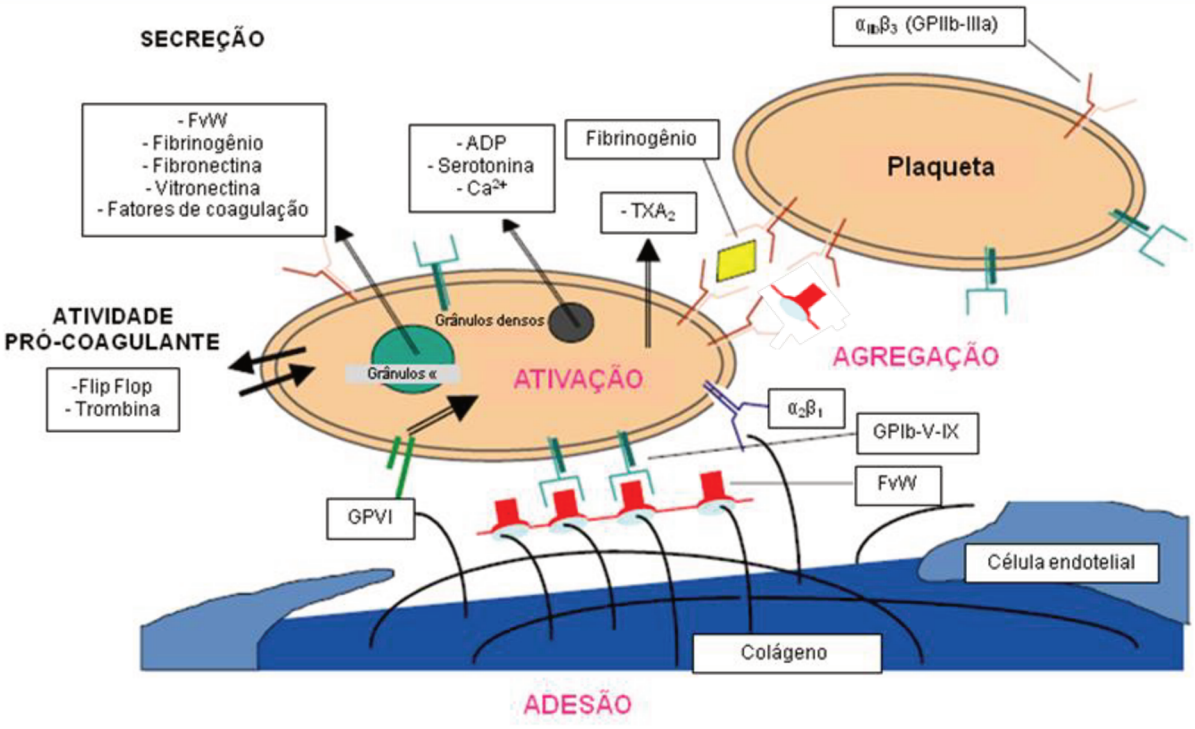

Figura 06: Esquema representatvo do processo de hemostasia primária. A lesão vascular resulta na exposição de colágeno e outras proteínas subendoteliais. A adesão plaquetária inicia-se com a ligação do complexo GPIb-V-IX ao FvW e ligação direta do colágeno à GPVI e $\alpha_2\beta_1$. A adesão das plaquetas induz sinalização intracelular e ativação das plaquetas, resultando em degranulação com liberação de ADP, serotonina e Ca^{2+}, síntese de TXA_2, exposição de fosfolipídeos ácidos (fosfatidilserina), gerando uma superfície pró-coagulante e ativação da integrina $\alpha_{IIb}\beta_3$ (GPIIb-IIIa). Esses eventos facilitam o recrutamento de outras plaquetas resultando na agregação plaquetária mediada pela ligação de fibrinogênio e FvW com $\alpha_{IIb}\beta_3$ ativada de plaquetas adjacentes. A geração de uma superfície pró-coagulante acelera a geração de trombina e deposição de fibrina, resultando em estabilização do tampão plaquetário. Fonte: adaptado de HARRISON, 2005.

A mesma sequência de eventos que resulta na formação de trombos durante a hemostasia normal, resulta em doenças trombóticas, como o infarto do miocárdio ou acidente vascular cerebral, duas das principais causas de mortalidade no mundo ocidental (ANDREWS; BERND, 2004; HARRISON, 2005). A ruptura de uma placa aterosclerótica expõe a matriz trombogênica subjacente às plaquetas circulantes e inicia o recrutamento de outras plaquetas em um processo semelhante à hemostasia primária (GAWAZ, 2004). Além disso, a alta tensão de cisalhamento em uma artéria ocluída pode induzir a formação de trombos mediada pelas interações FvW-GPIbα (ANDREWS; BERND, 2004). O

trombo resultante pode resultar em bloqueio do suprimento de sangue ao coração ou ao cérebro (YIP et al., 2005).

A terapia antiplaquetária pode, portanto, ser benéfica no tratamento e profilaxia de doenças trombóticas, devendo ser cuidadosamente administrada sem aumentar o risco de hemorragia (HARRISON, 2005). O agente antiplaquetário mais utilizado é o ácido acetilsalicílico (aspirina), embora existam também outros agentes, como clopidogrel, abciximab, eptifibatide e/ou tirofibana. Cada agente afeta as plaquetas de um modo diferente, podendo apresentar diferentes efeitos colaterais. Entretanto, todos interferem nos processos de adesão, ativação e/ou agregação plaquetária (CASTRO et al., 2006; GEIGER, 2001).

O clopidogrel atua como um antiplaquetário por se ligar de maneira irreversível aos receptores $P2Y_{12}$. Apesar de demonstrar eficácia, não pode ser considerado o antiagregante ideal devido à inibição irreversível dos receptores, aumentando o risco de sangramentos, à sua capacidade de apresentar resistência e a sua latência para atingir o pico de ação, o que reduz o seu benefício nos pacientes com síndrome coronariana aguda (FALCAO et al., 2013). Abciximab, eptifibatide e tirofibana inibem a agregação plaquetária por serem antagonistas da integrina $\alpha_{IIb}\beta_3$ (GPIIb-IIIa). Apesar de demonstrarem eficácia na redução do risco de complicações em pacientes com síndrome coronariana aguda, ainda apresentam possíveis efeitos adversos como hemorragia e trombocitopenia (GIORDANO et al., 2016; IBBOTSON; MCGAVIN; GOA, 2003; SHAH et al., 2009; WEI et al., 2016).

A aspirina inibe a agregação plaquetária por inibir o metabolismo do AA gerado a partir dos fosfolipídeos, através da inativação da cicloxigenase-1 (COX-1) (CLELAND, 2002). Esse medicamento pode ser utilizado de forma preventiva nos eventos trombóticos cardiovasculares, entretanto seu uso para prevenção em pacientes de baixo risco é controverso devido ao risco de episódios hemorrágicos, toxicidade gástrica e síndrome de "resistência à aspirina" (ALTMAN et al., 2004; BATES; LAU, 2005; GRAEME; HANKEY; EIKELBOOM, 2006).

Diante desse contexto, novos agentes antiplaquetários com maior eficiência e menores efeitos colaterais estão sendo investigados para serem utilizados no desenho de novos medicamentos na prevenção e tratamento de

doenças trombóticas. Sabendo-se que componentes presentes na peçonha de serpentes são capazes de interferir na hemostasia, inclusive na função plaquetária, uma investigação detalhada dessas moléculas pode fornecer informações para a descoberta de novas drogas que possam ser utilizadas como alvos anti-trombóticos.

4.2 Ação de diferentes toxinas ofídicas sobre a função plaquetária

Nos últimos anos, uma grande quantidade de toxinas de peçonha de serpentes foram identificadas por interferirem na função plaquetária, promovendo ou inibindo a agregação das plaquetas, devido à sua seletividade aos receptores plaquetários, como GPIb-V-IX, GPVI, $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$ ou aos seus respectivos ligantes, como FvW e/ou fibrinogênio. Geralmente, essas moléculas pertencem à família das SVMPs, SVSPs, PLA_2s, SV-LAAOs, desintegrinas e lectinas tipo C-*like* (ANDREWS; BERND, 2000; BRAUD; BON; WISNER, 2000; CLEMETSON; LU; CLEMETSON, 2007; KINI, 2004; KOH; KINI, 2012; MARSH; WILLIAMS, 2005). A tabela 01 mostra como, geralmente, os principais compostos, enzimáticos ou não, isolados de peçonha de serpentes interferem na função plaquetária.

Tabela 01: Efeito de diferentes classes de toxinas ofídicas sobre a função plaquetária.

Classes	**Efeito sobre a agregação plaqueária**
SVMPs	Inibe
SVSPs	Ativa
PLA_2s	Inibe e ativa
SV-LAAOs	Inibe e ativa
Desintegrinas	Inibe
Lectinas tipo C-*like*	Inibe e ativa

Fonte: adaptado de SAJEVIC; LEONARDI; KRIZAJ, 2011.

Vários estudos mostram que as SVMPs, geralmente, estão relacionadas à inibição da agregação plaquetária (KAMIGUTI, 2005; MOURA-DA-SILVA; BUTERA; TANJONI, 2007; SAJEVIC; LEONARDI; KRIZAJ, 2011). Essas enzimas podem interagir com proteínas adesivas enolvidas na hemostasia, como o

colágeno e FvW e também com receptores plaquetários, como $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$ e GPIb-V-IX (KAMIGUTI, 2005; MOURA-DA-SILVA; BUTERA; TANJONI, 2007).

A ação das SVMPs sobre a integrina $\alpha_{IIb}\beta_3$ geralmente está relacionada a SVMPs classe P-II, devido à presença do domínio desintegrina. A presença do motivo RGD no domínio desintegrina facilita a interação dessas enzimas com a integrina $\alpha_{IIb}\beta_3$ (MATSUI; FUJIMURA; TITANI, 2000). Elas ligam-se com alta afinidade à essa integrina e inibem a agregação por bloquear a ligação do fibrinogênio com seu receptor. Como esta é uma etapa final e essencial para a agregação plaquetária, essas enzimas inibem a resposta plaquetária por todos os agonistas e são considerados potentes inibidores enzimáticos (MATSUI; FUJIMURA; TITANI, 2000; NIEWIAROWSKI et al., 1994).

Por sua vez, o efeito das SVMPs sobre a agregação plaquetária induzida por colágeno geralmente está relacionada a SVMPs classe P-III, devido à presença dos domínios *dis-like* e *cys-rich*. Essas enzimas, por possuírem um motivo alternativo no domínio *dis-cys*, não bloqueiam a interação entre a integrina $\alpha_{IIb}\beta_3$ e o fibrinogênio. Sua ação sobre a função plaquetária está concentrada no bloqueio da interação do colágeno com seu receptor (CALVETE et al., 2005; MOURA-DA-SILVA; BUTERA; TANJONI, 2007). A maioria das SVMPs classe P-III parecem interferir com a integrina $\alpha_2\beta_1$ (KAMIGUTI, 2005). A Jararhagin (SVMP classe P-III isolada de *B. jararaca*) inibe a agregação plaquetária induzida por colágeno devido a clivagem da subunidade β_1 da integrina $\alpha_2\beta_1$, resultando em plaquetas incapazes de responder ao estímulo de colágeno (DE LUCA et al., 1995; KAMIGUTI; HAY; ZUZEL, 1996). Outro ponto importante envolvendo a ação das SVMPs na inibição da agregação plaquetária induzida por colágeno é a habilidade dessas enzimas de ligarem-se ao colágeno, como foi observado pela Jararhagin, Catrocollastatin (SVMP classe P-III isolada de *C. atrox*) e Crovidisin (SVMP classe P-III isolada de *C. viridis*). Essas enzimas se ligam às fibras de colágeno bloqueando sua ação com as plaquetas (LIU; HUANG, 1997; ZHOU; DANGELMAIER; SMITH, 1996).

A ação das SVMPs sobre o FvW é observada em ensaios de agregação plaquetária induzidos por ristocetina. Em geral, nesses estudos, a inibição da agregação plaquetária é atribuída a um efeito direto da enzima sobre o FvW. Alguns exemplos são: Jararhagin, Kaouthiagin (SVMP classe P-III isolada de *Naja*

Kaouthia), Acurhagin (SVMP classe P-III isolada de *Agkistrodon acutus*) e Crotalin (SVMP classe P-I isolada de *C. atrox*). Essas enzimas são capazes de hidrolisar o FvW tornando-o incapaz de desencadear as respostas necessárias para a agregação das plaquetas (HAMAKO et al., 1998; LAING; MOURA-DA-SILVA, 2005; WANG; HUANG, 2002; WU; PENG; HUANG, 2001). Efeitos sobre o receptor de FvW, a GPIb-V-IX, também já foram relatados por algumas SVMPs, como Kistomin (SVMP classe P-III isolada de *Calloselasma rhodostoma*), Mocarhagin (SVMP classe P-III isolada de *Naja mocambique mocambique*) e Crotalin (HUANG; CHANG; TENG, 1993; WARD et al., 1996; WU; PENG; HUANG, 2001).

A atividade catalítica das SVMPs parece ser responsável pela maioria de suas atividades biológicas, entretanto algumas SVMPs enzimaticamente inativas ou domínios *dis-like*/*cys-rich* isolados, como encontrado na Jararhagin-C e na Catrocollastatina-C, também interferem na função da integrina $\alpha_2\beta_1$, sugerindo que a atividade catalítica não desempenha um papel crucial nesse efeito (SHIMOKAWA et al., 1997; USAMI et al., 1994). Os domínios adicionais ao domínio cataítico direcionam a enzima para seus alvos (KAMIGUTI, 2005).

A figura 07 mostra os possíveis alvos plaquetários das SVMPs de acordo com cada classe.

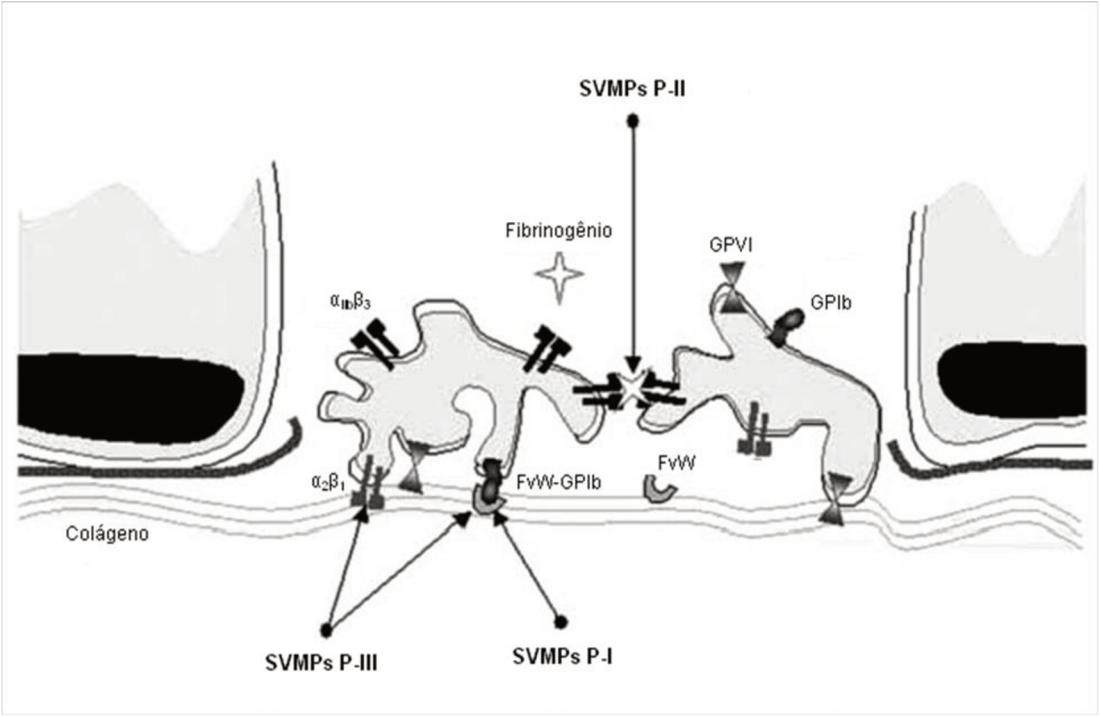

Figura 07: Ação das SVMPs sobre os receptores plaquetários: SVMPs classe P-II são capazes de inibir a ligação do fibrinogênio com a integrina $\alpha_{IIb}\beta_3$. SVMPs classe P-I podem hidrolizar o FvW e seu receptor GPIb-V-IX, enquanto que SVMPs classe P-III podem interferir com a agregação paquetária dependente de colágeno e FvW. Fonte: adaptado de MOURA-DA-SILVA; BUTERA; TANJONI, 2007.

Em geral, as SVSPs atuam promovendo a agregação das paquetas e secreção plaquetária (SAJEVIC; LEONARDI; KRIZAJ, 2011; SERRANO; MAROUN, 2005; SERRANO, 2013). Essas enzimas atuam de forma semelhante à trombina ativando as plaquetas através da clivagem dos PARs (PAR1 e PAR4) ou pela ligação à GPIb, os mesmos receptores responsáveis pelos efeitos da trombina nas plaquetas (CLEMETSON; LU; CLEMETSON, 2007; SANTOS et al., 2000). Essas enzimas induzem mobilização de Ca^{2+} nas plaquetas (SERRANO, 2013). Alguns exemplos são a PA-BJ e Bothrombin, ambas isoladas de *B. jararaca*. PA-BJ induz a agregação das plaquetas devido à clivagem proteolítica dos PARs, enquanto que Bothrombin se liga ao receptor GPIb (NISHIDA et al., 1994; SANTOS et al., 2000).

As PLA_2s afetam a função das paquetas por três mecanismos diferentes (SAJEVIC; LEONARDI; KRIZAJ, 2011). Essas enzimas podem induzir a agregação das plaquetas devido á hidrólise dos fosfolipídeos de membrana e liberação de AA e TXA_2, que é um agonista plaquetário. Podem inibir a agregação devido à hidrólise dos subprodutos do AA. E algumas fosfolipases apresentam um

efeito bifásico sobre as plaquetas. Sob baixas concentrações ou após um período curto de incubação elas induzem a agregação, entretanto em altas concentrações ou após um longo período de incubação inibem a agregação (CLEMETSON; LU; CLEMETSON, 2007; KINI; EVANS, 1990; MOUNIER; BON; KINI, 2001; SAJEVIC; LEONARDI; KRIZAJ, 2011; TENG; CHEN; OUYANG, 1984). Alguns exemplos de PLA_2s que interferem na função plaquetária são: Bthtx-II isolada de *B. jararacussu* induz a agregação paquetária (FULY et al., 2004), BmooPLA_2 isolada de *B. moojeni* inibe a agregação induzida por colágeno e ADP (SILVEIRA et al., 2013) e uma PLA_2 isolada de *Vipera russelii* apresenta um efeito bifásico (TENG; CHEN; OUYANG, 1984).

Algumas PLA_2s inibem a agregação plaquetária por um mecanismo independente de sua atividade enzimática, como é o caso das PLA_2s isoladas de *Ophiophagus hannah* e *Pseudechis papuanus*. A atividade anti-plaquetária dessas enzimas parece estar relacionada a uma alteração na morfologia das plaquetas devido à ruptura de seu citoesqueleto (HUANG; GOPALAKRISHNAKONE; KINI, 1997; LAING et al., 1995).

O papel das SV-LAAOs sobre as plaquetas ainda é controverso. Elas podem ativar ou inibir a agregação plaquetária. Os dois efeitos estão associados com a habilidade dessas enzimas produzirem H_2O_2, uma vez que a catalase atenua seu efeito. Entretanto, seu exato mecanismo de ação ainda não está esclarecido (CLEMETSON; LU; CLEMETSON, 2007; SAJEVIC; LEONARDI; KRIZAJ, 2011). O motivo da inibição da agregação plaquetária pode estar relacionado com a liberação ezimática de H_2O_2, o qual pode interferir com a interação entre receptores plaquetários (GPIIb-IIIa) e o fibrinogênio, prejudicando assim o mecanismo de agregação (CLEMETSON; LU; CLEMETSON, 2007; DU; CLEMETSON, 2002; SAKURAI et al., 2001). A indução da agregação plaquetária também está intimamente relacionada com a formação de H_2O_2 e subsequente síntese de TXA_2, um agonista plaquetário (IZIDORO et al., 2014; LI; YU; LIAN, 1994). Entretanto, o papel do H_2O_2 no processo de indução da agregação permanece incerto, uma vez que é improvável que o H_2O_2 sozinho seja responsável pelas atividades biológicas das SV-LAAOs. Provavelmente outros mecanismo desencadeiam essa potente atividade biológica (ZHONG et al., 2009). No caso da enzima NA-LAAO, isolada da peçonha de *Naja atra,* a sua ligação à

membrana plaquetária aumenta a sensibilidade das plaquetas ao H_2O_2 e, ao mesmo tempo, o H_2O_2 liberado pela enzima ativa as plaquetas por um mecanismo ainda desconhecido (LI et al., 2008).

As desintegrinas são consideradas potentes inibidores da agregação plaquetária (MARKLAND; SWENSON, 2013). Em geral, desintegrinas contendo o motivo RGD interferem na função plaquetária por bloquearem reversivelmente a integrina $\alpha_{IIb}\beta_3$ (GPIIb-IIIa), prevenindo a ligação do fibrinogênio ao seu receptor (KOH; KINI, 2012; MARSH; WILLIAMS, 2005). Antagonistas da integrina $\alpha_{IIb}\beta_3$ inibem a agregação induzida por diversos agonistas, como ADP, trombina, colágeno e AA (CLEMETSON; LU; CLEMETSON, 2007). As *disintegrins-like* também são capazes de inibir a agregação. Entretanto, seu mecanismo de ação não está completamente esclarecido. Dados na literatura indicam que as atividades biológicas dessas moléculas estão atribuídas à sua habilidade de bloquear a interação das plaquetas ao colágeno (CALVETE et al., 2005; JIA et al., 1997; ZHOU; SMITH; GROSSMAN, 1995). Duas drogas, Tirofibana (Aggrastat®) e Eptifibatide (Integrillin®), foram desenvolvidas com base em desintegrinas isoladas de peçonha de serpentes e estão disponíveis no mercado como agentes antiplaquetários (KOH; KINI, 2012).

As lectinas tipo C-*like* podem atuar como agonistas ou antagonistas plaquetários (SAJEVIC; LEONARDI; KRIZAJ, 2011). Em geral, promovem ou inibem a agregação por se ligar ao FvW, GPIb e/ou receptores de colágeno (ANDREWS; BERND, 2000). Botrocetin (isolada de *B. jararaca*) e Bitiscetin (isolada de *Bitis arietans*) induzem a agregação plaquetária por se ligarem ao FvW e à GPIb simultaneamente (FUKUDA et al., 2005; MAITA et al., 2003). As lectinas tipo C-*like,* que atuam via GPIb, podem inibir a agregação por bloquear a ligação ao FvW ou à trombina, ou então podem mimetizar o FvW, ativando a agregação das plaquetas (CLEMETSON; LU; CLEMETSON, 2007). Echicetin (isolada de *Echis carinatus*) se liga especificamente à GPIb e bloqueia a interação ao FvW e à trombina (NAVDAEV et al., 2001). Convulxin (isolada de *C. durissus terrificus*) pode se ligar à GPVI e/ou GPIb, desempenhando papel na ativação plaquetária (HORII; BROOKS; HERR, 2009; KANAJI et al., 2003). Rhodocytin (isolada de *Calloselasma rhodostoma*) induz a agregação via $\alpha_2\beta_1$ e GPIb (BERGMEIER et al., 2001). EMS16 (isolada de *E. multisquamatus*) inibe a

agregação plaquetária induzida por colágeno por se ligar à integrina $\alpha_2\beta_1$ (MARCINKIEWICZ et al., 2000).

Em suma, sabendo-se que as moléculas presentes na peçonha de serpentes têm capacidade de interagir com componentes do sistema hemostático humano interferindo na função plaquetária, uma investigação detalhada dessas moléculas pode fornecer ferramentas importantes no desenvolvimento de testes de diagnósticos e novos agentes terapêuticos para distúrbios hemostáticos. Além disso, a grande diversidade estrutural, de especificidade e mecanismos de ação desses componentes ampliam ainda mais o seu potencial uso terapêutico.

REFERÊNCIAS

ALBUQUERQUE, P. L. et al. Acute kidney injury caused by *Crotalus* and *Bothrops* snake venom: A review of epidemiology, clinical manifestations and treatment. *Revista do Instituto de Medicina Tropical*, v. 55, p. 295-301, 2013. https://doi.org/10.1590/S0036-46652013000500001

ALTMAN, R. et al. The antithrombotic profile of aspirin. Aspirin resistance, or simply failure? *Thrombosis Journal*, v. 2, p. 1-8, 2004. https://doi.org/10.1186/1477-9560-2-1

ANDE, S. R. et al. Induction of apoptosis in yeast by L-amino acid oxidase from the Malayan pit viper *Calloselasma rhodostoma*. *Yeast*, v. 25, p. 349-357, 2008. https://doi.org/10.1002/yea.1592

ANDREWS, R. K.; BERNDT, M. C. Snake venom modulators of platelet adhesion receptors and their ligands. *Toxicon*, v. 38, p. 775-791, 2000. https://doi.org/10.1016/S0041-0101(99)00187-7

ANDREWS, R. K.; BERNDT, M. C. Platelet physiology and thrombosis. *Thrombosis Research*, v. 114, p. 447-53, 2004. https://doi.org/10.1016/j.thromres.2004.07.020

ANGULO, Y.; LOMONTE, B. Biochemistry and toxicology of toxins purified from the venom of the snake *Bothrops asper*. *Toxicon*, v. 54, p. 949-957, 2009. https://doi.org/10.1016/j.toxicon.2008.12.014

ARNI, R. K.; WARD, R. I. Phospolipase A_2 - A structural review. *Toxicon*, v. 34, p. 827-841, 1996. https://doi.org/10.1016/0041-0101(96)00036-0

AROCAS, V. et al. Bothrojaracin: a potent two-site-directed thrombin inhibitor. *Biochemistry*, v. 35, p. 9083-9089, 1996. https://doi.org/10.1021/bi960043l

ATODA, H.; HYUGA, M.; MORITA, T. The primary structure of coagulation factor IX/factor X-binding protein isolated from the venom of *Trimeresurus flavoviridis*. Homology with asialoglycoprotein receptors, proteoglycan core protein, tetranectin, and lymphocyte Fc3 receptor for immunoglobulin E. *The Journal of Biological Chemistry*, v. 266, p. 14903-14911, 1991.

AZEVEDO-MARQUES, M. M. et al. Myonecrosis, myoglobinuria and acute renal failure induced by South American Rattlesnake (*Crotalus durissus terrificus*) envenomation in Brazil. *Toxicon*, v. 23, p. 631-636, 1985. https://doi.org/10.1016/0041-0101(85)90367-8

AZEVEDO-MARQUES, M. M.; HERING, S. E.; CUPO, P. Evidence that *Crotalus durissus terrificus* (South American Rattlesnake) envenomation in humans causes myolysis rather than hemolysis. *Toxicon*, v. 25, p. 1163-1168, 1987.

https://doi.org/10.1016/0041-0101(87)90134-6

BANCHER, W.; ROSA, R. R.; FURLANETTO, R. S. Estudos sobre a fixação eletiva e quantitativa do veneno de *Crotalus durissus terrificus* nos tecidos nervoso, renal, hepático e muscular de Mus-musculus Linnaeus. *Memórias do Instituto Butantã*, v. 37, p. 139-148, 1973.

BARBOSA, P. S. F. et al. Purification and biological effects of a C-type lectin isolates from *Bothrops moojeni*. *The Journal of Venomous Animals and Toxins including Tropical Diseases*, v. 16, p. 493-504, 2010. https://doi.org/10.1590/S1678-91992010000300016

BARRAVIERA, B. Estudo clínico dos acidentes ofídicos: revisão. *Jornal Brasileiro de Medicina*, v. 65, p. 209-250, 1993.

BARRETT, A. J.; RAWLINGS, N. D. Families and clans of serine peptidases. *Archives Biochemistry Biophysics*, v. 318, p. 247-250, 1995. https://doi.org/10.1006/abbi.1995.1227

BATES, E. R.; LAU, W. C. Controversies in antiplatelet therapy for patients with cardiovascular disease. *Circulation*, v. 111, p. 267-71, 2005. https://doi.org/10.1161/01.CIR.0000157158.63751.B2

BAUAB, F. A. et al. Clinical and epidemiological aspects of the 'urutu' lance-headed viper (*Bothrops alternatus*) bite in a Brazilian hospital. *Tropical Medicine and Parasitology*, v. 45, p. 243-245, 1994.

BENNETT, J. S.; BERGER, B. W.; BILLINGS, P. C. The structure and function of platelet integrins. *Journal of Thrombosis and Haemostasis*, v.7, p.200-205, 2009. https://doi.org/10.1111/j.1538-7836.2009.03378.x

BERGMEIER, W. et al. Rhodocytin (aggretin) activates platelets lacking α2β1 integrin, glycoprotein VI, and the ligand-binding domain of glycoprotein Iba. *The Journal of Biological Chemistry*, v. 276, p. 25121-25126, 2001. https://doi.org/10.1074/jbc.M103892200

BILGRAMI, S. et al. Crystal structure of schistatin, a disintegrin homodimer from saw-scaled viper (*Echis carinatus*) at 2.5 A ° resolution. *Journal of Molecular Biology*, v. 341, p. 829-837, 2004. https://doi.org/10.1016/j.jmb.2004.06.048

BJARNASON, J. B.; FOX, J. W. Hemorrhagic Metalloproteinases from snake venoms. *Pharmacology & Therapeutics*, v. 62, p. 325-372, 1994. https://doi.org/10.1016/0163-7258(94)90049-3

BJARNASON, J. B.; TU, A. T. Hemorrhagic toxins from the western diamondback rattlesnake (*Crotalus atrox*) venom: isolation and characterization of five toxins and the role of zinc in hemorrhagic toxin. *Biochemistry*, v. 17, p. 3395-3404, 1978. https://doi.org/10.1021/bi00609a033

BOCHNER, R. The international view of envenoming in Brazil: myths and realities. *Journal of Venomous Animals and Toxins including Tropical Diseases*, v. 19, p. 1-10, 2013.
https://doi.org/10.1186/1678-9199-19-29

BOCHNER, R.; STRUCHINER, C. J. Snake bite epidemiology in the last 100 years in Brazil: a review. *Caderno de Saúde Pública*, v. 19, p. 7-16, 2003.
https://doi.org/10.1590/S0102-311X2003000100002

BODE, W.; GOMIS-RUTH, F. X.; STOCKLER, W. Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins', *FEBS Letters*, v. 331, p. 134-140, 1993.
https://doi.org/10.1016/0014-5793(93)80312-I

BRASIL. Ministério da Saúde. Manual de diagnóstico e tratamento de acidentes por animais peçonhentos. *Ministério da Saúde, Fundação Nacional de Saúde*. 2. ed. Brasília: Fundação Nacional da Saúde, 2001.

BRASIL. Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de Vigilância Epidemiológica. Guia de vigilância epidemiológica. *Ministério da Saúde, Secretaria de Vigilância em Saúde, Departamento de Vigilância Epidemiológica*. 7. ed. Brasília: Ministério da Saúde, 2009.

BRASIL. Ministério da Saúde. Secretaria de Vigilância em Saúde. Guia de Vigilância em Saúde. *Ministério da Saúde, Secretaria de Vigilância em Saúde*. Brasília: Ministério da Saúde, 2014.

BRASIL. *Ministério da Saúde. Sistema de Informação de Agravos de Notificação (Sinan). Acidente por animais peçonhentos.* Disponível em: <http://www2.datasus.gov.br/DATASUS/index.php>. Acesso em: 01 mar. 2017.

BRAUD, S.; BON, C.; WISNER, A. Snake venom proteins acting on hemostasis. *Biochimie*, v. 82, p. 851-859, 2000.
https://doi.org/10.1016/S0300-9084(00)01178-0

BURKE, J. E.; DENNIS, E. A. Phospholipase A2 biochemistry. *Cardiovascular Drugs Therapy,* v.1, p. 49-59, 2009.
https://doi.org/10.1007/s10557-008-6132-9

CALVETE, J. J. et al. The disulfide bond pattern of catrocollastatin C, a disintegrin/cysteine-rich protein isolated from *Crotalus atrox* venom. *Protein Science*, v. 9, p. 1365-1373, 2000a.

CALVETE, J. J. et al. Disulfide-bond patternand molecular modelling of the dimeric disintegrin EMF-10, a potent and selective integrin alpha5beta1 antagonist from *Eristocophis macmahoni* venom. *Biochemical Journal*, v. 345, p. 573-581, 2000b.
https://doi.org/10.1042/bj3450573

CALVETE, J. J. et al. Snake venom disintegrins: novel dimeric disintegrins and structural diversification by disulphide bond engineering. *Biochemical Journal,* v. 372, p. 725-734, 2003.
https://doi.org/10.1042/bj20021739

CALVETE, J. J. et al. Snake venom disintegrins: evolution of structure and function. *Toxicon*, v. 45, p. 1063-1074, 2005.
https://doi.org/10.1016/j.toxicon.2005.02.024

CALVETE, J. J. et al. Venoms, venomics, antivenomics. *FEBS Letters*, v. 583, p. 1736-1743, 2009.
https://doi.org/10.1016/j.febslet.2009.03.029

CALVETE, J. J. The continuing saga of snake venom disintegrins. *Toxicon*, v. 62, p. 40-49, 2013.
https://doi.org/10.1016/j.toxicon.2012.09.005

CAMEY, K. U.; VELARDE, D. T.; SÁNCHEZ, E. F. Pharmacological characterization and neutralization of the venoms used in the production of bothropic antivenom in Brazil. *Toxicon*, v. 40, p. 501-509, 2002.
https://doi.org/10.1016/S0041-0101(01)00245-8

CAMPOS, L. B. et al. In vitro comparison of enzymatic effects among Brazilian *Bothrops* spp. venoms. *Toxicon*, v. 76, p. 1-10, 2013.
https://doi.org/10.1016/j.toxicon.2013.08.063

CARDOSO, D. F; YAMAGUCHI, I. K.; SILVA, A. M. M. Produção de soros antitoxinas e perspectivas de modernização por técnicas de biologia molecular. In: CARDOSO, J. L. C. et al. *Animais Peçonhentos no Brasil: Biologia, clínica e terapêutica dos acidentes*. 2. ed. São Paulo: Sarvier, 2009, p.81-95.

CARON, E. J. et al. Apparent marked reduction in early antivenom reactions compared to historical controls: was it prophylaxis or method of administration? *Toxicon,* v. 54, p. 79-83, 2009.
https://doi.org/10.1016/j.toxicon.2009.06.001

CASTRO, H. C. et al. Snake venom thrombin-like enzymes: from reptilase to now. *Cellular and Molecular Life Sciences,* p. 843-856, 2004.
https://doi.org/10.1007/s00018-003-3325-z

CASTRO, H. C. et al. Platelets: still a therapeutical target. *Jornal Brasileiro de* Patologia *e Medicina Laboratorial*, v. 42, p. 321-332, 2006.
https://doi.org/10.1590/S1676-24442006000500004

CHAN, Y. S. et al. Snake venom toxins: toxicity and medicinal applications. *Applied Microbiology* and *Biotechnology*, v. 14, p. 6165-1181, 2016.
https://doi.org/10.1007/s00253-016-7610-9

CHIPPAUX, J. P. Epidemiology of envenomations by terrestrial venomous animals in Brazil based on case reporting: from obvious facts to contingencies. *Journal of Venomous Animals and Toxins including Tropical Diseases*, v. 21, p. 1-17, 2015. https://doi.org/10.1186/s40409-015-0011-1

CHIPPAUX, J. P.; WILLIAMS, V.; WHITE, J. Snake venom variability: Methods of study, results and interpretation. *Toxicon*, v. 29, p. 1279-1303, 1991. https://doi.org/10.1016/0041-0101(91)90116-9

CISCOTTO, P. et al. Antigenic, microbicidal and antiparasitic properties of an l-amino acid oxidase isolated from *Bothrops jararaca* snake venom. *Toxicon*, v. 53, p. 330-341, 2009. https://doi.org/10.1016/j.toxicon.2008.12.004

CLELAND, J. G. Preventing atherosclerotic events with aspirin. *British Medical Journal*, v. 324, p. 103-5, 2002. https://doi.org/10.1136/bmj.324.7329.103

CLEMETSON, K. J. Snaclecs (snake C-type lectins) that inhibit or activate platelets by binding to receptors. *Toxicon*, v. *56*, p. 1236-1246, 2010. https://doi.org/10.1016/j.toxicon.2010.03.011

CLEMETSON, K. J.; LU, Q.; CLEMETSON, J. M. Snake venom proteins affecting platelets and their applications to anti-thrombotic research. *Current Pharmaceutical Design*, v. 13, p. 2887-2892, 2007. https://doi.org/10.2174/138161207782023702

COLLER, B. S. et al. Platelet morphology, biochemistry and function**.** In: LITCHTMAN, M. A.; BEUTLER, E.; KIPPS, T. J.; SELIGSOHN, U.; KAUSHANSKY, K.; PRCHAL, J. T. *Williams Hematology*. 8ª Edição. The McGraw-Hill Companies, 2010, pp. 1587-1664.

COSTA, H. C.; BÉRNILS, R. S. Répteis brasileiros: Lista de espécies 2015. *Herpetologia Brasileira*, v. 4, p. 75-93, 2015.

COSTA, J. O. et al. Bhalternin: Functional and structural characterization of a new thrombin-like enzyme from *Bothrops alternatus* snake venom. *Toxicon*, v.55, p.1365-1377, 2010. https://doi.org/10.1016/j.toxicon.2010.02.014

COSTA, T. R. et al. Snake venom L-amino acid oxidases: an overview on their antitumor effects. *Journal of Venomous Animals and Toxins including Tropical Diseases*, v. 20, p. 1-7, 2014. https://doi.org/10.1186/1678-9199-20-23

CUNHA, E. M; MARTINS, O. A. Principais compostos químicos presente nos venenos de cobras dos gêneros B*othrops* e *Crotalus* – uma revisão. *Revista Eletrônica de Educação e Ciência*, v. 2, p. 21-26, 2012.

DALE, G. L., et al. Stimulated platelets use serotonin to enhance their retention of procoagulant proteins on the cell surface. *Nature,* v. 415, p. 175-179, 2002. https://doi.org/10.1038/415175a

DE LUCA, M. et al. Jararhagin and jaracetin: novel snake venom inhibitors of the integrin collagen receptor $\alpha_2\beta_1$. *Biochemical and Biophyscal Research Communications*, v. 206, p. 570-576, 1995. https://doi.org/10.1006/bbrc.1995.1081

DENNIS, E. A. et al. Phospholipase A2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. *Chemical Reviews*, v. 10, p. 6130-6185, 2011. https://doi.org/10.1021/cr200085w

DODD, R. B.; DRICKAMER, K. Lectin-like proteins in model organisms: implications for evolution of carbohydrate-binding activity. *Glycobiology*, v. 5, p. 71-79, 2001. https://doi.org/10.1093/glycob/11.5.71R

DORSAM, R. T.; KUNAPULI, S. P. Central role of the P2Y(12) receptor in platelet activation. *The Journal of Clinical Investigation*, v. 113, p. 340-345, 2004. https://doi.org/10.1172/JCI20986

DRICKAMER, K. Two distinct classes of carbohydrate recognition domains in animal lectins**.** *The Journal of Biological Chemistry*, v. 263, p. 9557-9560, 1988.

DU, X. Y.; CLEMETSON, K. J. Snake venom L-amino acid oxidases.*Toxicon*, v. 40, p. 659-665, 2002. https://doi.org/10.1016/S0041-0101(02)00102-2

DUMÉRIL, A. M. C.; BIBRON, G.; DUMÉRIL, A. H. A. *Erpétologie générale ou histoire naturelle compléte des reptiles*. 7. ed. Paris: Roret, 1854, p.1834-1854.

DUTTA, S.; GOGOI, D.; MUKHERJEE, A. K. Anticoagulant mechanism and platelet deaggregation property of a non-cytotoxic, acidic phospholipase A2 purified from Indian cobra (*Naja naja*) venom: Inhibition of anticoagulant activity by low molecular weight heparin. *Biochimie*, v. 10, p. 93-106, 2015. https://doi.org/10.1016/j.biochi.2014.12.020

EBLE, J.A. Matrix biology meets toxinology. *Matrix Biology*, v. 29, p. 239-247, 2010. https://doi.org/10.1016/j.matbio.2010.01.004

ESCALANTE, T. et al. Key events in microvascular damage induced by snake venom hemorrhagic metalloproteinases. *Journal of Proteomics,* v. 74, p. 1781-1794, 2011. https://doi.org/10.1016/j.jprot.2011.03.026

FALCÃO, F. J. A. et al. Receptores plaquetários $P2Y_{12}$: importância na intervenção coronariana percutânea. *Arquivos Brasileiros de Cardiologia*, v. 101, p. 277-282, 2013.

FAURE, G.; GOWDA, V. T.; MAROUN, R. C. Characterization of a human coagulation factor Xa-binding site on Viperidae snake venom phospholipases A2 by affinity binding studies and molecular bioinformatics. *BMC Structural Biology,* v. 7, p. 82-89, 2007.
https://doi.org/10.1186/1472-6807-7-82

FOX, J. W. A brief review of the scientific history of several lesser-known snake venom proteins: l-Amino acid oxidases, hyaluronidases and phosphodiesterases. *Toxicon*, v. 62, p. 75-82, 2013.
https://doi.org/10.1016/j.toxicon.2012.09.009

FOX, J. W.; SERRANO, S. M. T. Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases, *Toxicon*, v.45, p. 969-985, 2005.
https://doi.org/10.1016/j.toxicon.2005.02.012

FOX, J. W.; SERRANO, S. M. T. Insights into and speculations about snake venom metalloproteinase (SVMP) synthesis, folding and disulfide bond formation and their contribution to venom complexity. *FEBS Journal*, v. 275, p. 3016-3030, 2008.
https://doi.org/10.1111/j.1742-4658.2008.06466.x

FOX, J. W.; SERRANO, S. M. T. Timeline of key events in snake venom metalloproteinase research. *Journal of Proteomics*, v. 72, p. 200-209, 2009.
https://doi.org/10.1016/j.jprot.2009.01.015

FRANÇA, F. O. S.; MÁLAQUE, C. M. S. A. Acidente Botrópico. In: CARDOSO, J. L. C. et al. *Animais peçonhentos no Brasil: Biologia, clínica e terapêutica dos acidentes*. 2. ed. São Paulo: Sarvier, 2009, p. 81-95.

FUKUDA, K. et al. The snake venom protein botrocetin acts as a biological brace to promote dysfunctional platelet aggregation. *Nature Structure & Molecular Biology*, v. 12, p. 152-159, 2005.
https://doi.org/10.1038/nsmb892

FULY, A. L. et al. Signal transduction pathways involved in the platelet aggregation induced by a D-49 phospholipase A2 isolated from *Bothrops jararacussu* snake venom. *Biochimie*, v. 86, 731-739, 2004.
https://doi.org/10.1016/j.biochi.2004.07.001

FURTADO, M. F. D. et al. Antigenic cross-reactivity and immunogenicity of *Bothrops* venoms from snakes of the Amazon region. *Toxicon*, v. 55, p. 881-887, 2010.
https://doi.org/10.1016/j.toxicon.2009.12.014

GACHET, C. ADP receptors of platelets and their inhibition. *Thrombosis and Haemostasis*, v. 86, p. 222-232, 2001.

GALE, A. J. Current understanting of hemostasis. *Toxicologic Pathology,* v. 39, p. 273- 280, 2011.
https://doi.org/10.1177/0192623310389474

GARTNER, T. K.; STOCKER, K.; WILLIAMS, D. C. Thrombolectin: a lectin isolated from *Bothrops atrox* venom. *FEBS Letters*, v. 117, p. 13-16, 1980.
https://doi.org/10.1016/0014-5793(80)80902-1

GAWAZ, M. Role of platelets in coronary thrombosis and reperfusion of ischemic myocardium. *Cardiovascular Research,* v.61, p.498-511, 2004.
https://doi.org/10.1016/j.cardiores.2003.11.036

GEIGER, J. Inhibitors of platelet signal transduction as antiaggregatory drugs. *Expert Opinion on Investigational Drugs*, v. 10, p. 865-90, 2001.
https://doi.org/10.1517/13543784.10.5.865

GIORDANO, A. et al. Effects Of Glycoprotein IIb/IIIa Antagonists: Anti Platelet Aggregation And Beyond. *Current Drug Metabolism*, v. 17, p. 194-203, 2016.
https://doi.org/10.2174/1389200217666151211121112

GIRISH, K. S.; KEMPARAJU, K. Overlooked issues of snakebite management: time for strategic approach. *Current Topics in Medicinal Chemistry,* v. 11, p. 2494-2508, 2011.
https://doi.org/10.2174/156802611797633393

GRAEME, J.; HANKEY, J.; EIKELBOOM, W. Aspirin resistance. *Lancet*, v. 367, 606-17, 2006.
https://doi.org/10.1016/S0140-6736(06)68040-9

GUO, C. et al. Past decade study of snake venom L-amino acid oxidase. *Toxicon*, v. 60, p. 302-311, 2012.
https://doi.org/10.1016/j.toxicon.2012.05.001

GUTIÉRREZ J. M. et al. Skeletal muscle necrosis and regeneration after injection of BaH1, a hemorrhagic metalloproteinase isolated from the venom of the snake *Bothrops asper*. *Experimental and Molecular Pathology*, v. 62, p. 28-41, 1995.
https://doi.org/10.1006/exmp.1995.1004

GUTIÉRREZ, J. M. et al. Experimental pathology of local tissue damage induced by *Bothrops asper* snake venom. *Toxicon*, v. 54, p. 958-975, 2009.
https://doi.org/10.1016/j.toxicon.2009.01.038

GUTIÉRREZ, J. M. et al. Snakebite envenoming from a global perspective: Towards an integrated approach. *Toxicon*, v. 56, p. 1223-1235, 2010.
https://doi.org/10.1016/j.toxicon.2009.11.020

GUTIÉRREZ, J. M.; ESCALANTE, T.; RUCAVADO, A. Experimental pathophysiology of systemic alterations induced by *Bothrops asper* snake venom. *Toxicon*, v. 54, p. 976-987, 2009. https://doi.org/10.1016/j.toxicon.2009.01.039

GUTIÉRREZ, J. M.; LOMONTE, B. Local tissue damage induced by *Bothrops* snake venoms: a review. *Memórias Instituto Butantan,* v. 51, p. 211-223, 1989.

GUTIÉRREZ, J. M.; RUCAVADO, A. Snake venom metalloproteinases: Their role in the pathogenesis of local tissue damage. *Biochimie*, v. 82, p. 841-850, 2000. https://doi.org/10.1016/S0300-9084(00)01163-9

HAMAKO, J. et al. Purification and characterization of bitiscetin, a novel von Willebrand factor modulator protein from *Bitis arietans* snake venom. *Biochemical and Biophysical Research Communications,* v. 226, p. 273-279, 1996. https://doi.org/10.1006/bbrc.1996.1345

HAMAKO, J. et al. Purification and characterization of kauthiagin, a von Willebrand factor-binding and–cleaving metalloproteinase from *Naja Kaouthia* cobra venom. *Thrombosis and Haemostasis*, v. 80, p. 499-505, 1998.

HARDY, M. C.; COCHRANE, J.; ALLAVENA, R. E. Venomous and Poisonous Australian Animals of Veterinary Importance: A Rich Source of Novel Therapeutics. *BioMed Research International,* p. 1-12, 2014. https://doi.org/10.1155/2014/671041

HARRISON, P. Platelet function analysis. *Blood reviews*, p. 19, 111-119, 2005.

HARRISON, R. A. et al. Snake Envenoming: A Disease of Poverty. *PLoS Neglected Tropical Diseases*, v. 3, p. e569, 2009. https://doi.org/10.1371/journal.pntd.0000569

HERRERA, C. et al. Systemic effects induced by the venom of the snake *Bothrops caribbaeus* in a murine model. *Toxicon***,** v. 63, p. 19-31, 2012. https://doi.org/10.1016/j.toxicon.2012.10.023

HICKMAN, C. P.; ROBERTS, L. S.; LARSON, A. *Princípios integrados de zoologia.* 11. ed. Rio de Janeiro: Guanabara Koogan, 2004.

HITE, L. A., et al. Sequence of a cDNA clone encoding the zinc metalloproteinase hemorrhagic toxin e from *Crotalus atrox:* evidence for signal, zymogen, and disintegrin-like structures. *Biochemistry,* v. 31, p. 6203-6211. 1992. https://doi.org/10.1021/bi00142a005

HOGE, A. R. Preliminary account on Neotropical Crotalinae (Serpentes: Viperidae). *Memórias do Instituto de Butantan*, v. 32, p. 109-184, 1966.

HORII, K.; BROOKS, M. T.; HERR, A. B. Convulxin forms a dimer in solution and can bind eight copies of glycoprotein VI: implications for platelet activation. *Biochemistry*, v. 48, p. 2907-2914, 2009. https://doi.org/10.1021/bi801820q

HUANG, M. Z.; GOPALAKRISHNAKONE, P.; KINI, R. M. Role of enzymatic activity in the antiplatelet effects of a phospholipase A2 from *Ophiophagus hannah* snake venom. *Life Sciences*, v. 61, p. 2211-2217, 1997. https://doi.org/10.1016/S0024-3205(97)00923-5

HUANG, T. F.; CHANG, J. C.; TENG, C. M. Antiplatelet protease, kistomin, selectively cleaves human glycoprotein Ib. *Biochimica et Biophysica Acta*, v. 1158, p. 293-299, 1993. https://doi.org/10.1016/0304-4165(93)90028-7

IBBOTSON, T.; MCGAVIN, J. K.; GOA, K. L. Abciximab: an updated review of its therapeutic use in patients with ischaemic heart disease undergoing percutaneous coronary revascularisation. *Drugs*, v. 63, p. 1121-1163, 2003. https://doi.org/10.2165/00003495-200363110-00014

ISRAELS, S. J. et al. Platelet Disorders in Children: A Diagnostic Approach. *Pediatric Blood & Cancer*, v. 56, p. 975-983, 2011. https://doi.org/10.1002/pbc.22988

IZIDORO, L. F. M. et al. Snake Venom L-Amino Acid Oxidases: Trends in Pharmacology and Biochemistry. *BioMed Research International*, p. 1-19, 2014. https://doi.org/10.1155/2014/196754

JACKSON, J. The evolution of venom-delivery systems in snakes. *Zoological Journal of the Linnean Society*, v. 137, p. 337-354, 2003. https://doi.org/10.1046/j.1096-3642.2003.00052.x

JIA, L. G. et al. Function of disintegrin-like/cysteine-rich domains of atrolysin A. Inhibition of platelet aggregation by recombinant protein and peptide antagonists. *The Journal of Biological Chemistry*, v. 272, p. 13094-13102, 1997. https://doi.org/10.1074/jbc.272.20.13094

JOHNSON, E. K.; KARDONG, K. V.; OWNBY, C. L. Observations on white and yellow venoms from an individual southern Pacific rattlesnake (*Crotalus viridis helleri*). *Toxicon*, v. 25, p. 1169-1180, 1987. https://doi.org/10.1016/0041-0101(87)90135-8

JURK, K.; KEHREL, B. E. Platelets: Physiology and Biochemistry. *Seminars in Thrombosis and Hemostasis*, v. 31, p. 381-392, 2005. https://doi.org/10.1055/s-2005-916671

KAMIGUTI, A. S. Platelets as targets of snake venom metalloproteinases. *Toxicon*, v.45, p.1041-1049, 2005. https://doi.org/10.1016/j.toxicon.2005.02.026

KAMIGUTI, A. S.; HAY, C. R. M.; ZUZEL, M. Inhibition of collagen-induced platelet aggregation as the result of cleavage of $\alpha_2\beta_1$-integrin by the snake venom metalloproteinase jarararhagin. *Biochemical Journal*, v. 320, p. 635-641, 1996. https://doi.org/10.1042/bj3200635

KANAJI, S. et al. Convulxin binds to native, human glycoprotein Iba. *The Journal of Biological Chemistry*, v. 278, p. 39452-39460, 2003. https://doi.org/10.1074/jbc.M300199200

KANG, T. S. et al. Enzymatic toxins from snake venom: structural characterization and mechanism of catalysis. *FEBS Journal,* v. 278, p. 4544-4576, 2011. https://doi.org/10.1111/j.1742-4658.2011.08115.x

KASTURIRATNE, A. et al. The global burden of snakebite: a literature analysis and modelling based on regional estimates of envenoming and deaths. *PLoS Medicine*, v. 5, p. e218, 2008. https://doi.org/10.1371/journal.pmed.0050218

KERKKAMP, H. M. I.; CASEWELL, N. R.; VONK, F. J. Evolution of the Snake Venom Delivery System. In: GOPALKRISHNAKONE, P.; MALHOTRA, A. *Evolution of Venomous Animals and Their Toxins*. Dordrecht: Springer, p. 1-11, 2015. https://doi.org/10.1007/978-94-007-6727-0_11-1

KINI, R. M. Excitement ahead: structure, function and mechanism of snake venom phospholipase A2 enzymes. *Toxicon*, v. 42, p. 827-840, 2003. https://doi.org/10.1016/j.toxicon.2003.11.002

KINI, R. M. Platelet aggregation and exogenous factors from animal sources. *Current Drug Targets Cardiovascular & Haematological Disorders*, v. 4, p. 301-325, 2004. https://doi.org/10.2174/1568006043335835

KINI, R. M. Anticoagulant proteins from snake venoms: structure, function and mechanism. *Biochemical Journal*, v. 397, p. 377-387, 2006. https://doi.org/10.1042/BJ20060302

KINI, R. M.; EVANS, H. J. Effects of snake venom proteins on blood platelets. *Toxicon*, v. 28, p. 1387-1422, 1990. https://doi.org/10.1016/0041-0101(90)90155-Z

KINI, R. M.; EVANS, H. J. Structural domains in venom proteins: evidence that metalloproteinases and nonenzymatic platelet aggregation inhibitors (disintegrins) from snake venoms are derived by proteolysis from a common precursor. *Toxicon,* v. 30, p. 265-293, 1992. https://doi.org/10.1016/0041-0101(92)90869-7

KOH, C. Y.; KINI, R. M. From snake venom toxins to therapeutics – Cardiovascular examples. *Toxicon*, v. 59, p. 497-506, 2012. https://doi.org/10.1016/j.toxicon.2011.03.017

KOH, D. C. I; ARMUGAM, A.; JEYASEELAN, K. Snake venom components and their applications in biomedicine. *Cellular and Molecular Life Sciences,* v. 63, p. 3030-3041, 2006. https://doi.org/10.1007/s00018-006-6315-0

KOMMOJU, P. R.; MACHEROUX, P.; GHISLA, S. Molecular cloning, expression and purification of L-amino acid oxidase from the Malayan pit viper *Calloselasma rhodostoma*. *Protein Expression and Purification*, v. 52, p. 89-95, 2007. https://doi.org/10.1016/j.pep.2006.09.016

LAING, G. D. et al. Characterisation of a purified phospholipase A2 from the venom of the Papuan black snake (*Pseudechis papuanus*). *Biochimica et Biophysica Acta,* v. 1250, p. 137-143, 1995. https://doi.org/10.1016/0167-4838(95)00081-5

LAING, G. D.; MOURA-DA-SILVA, A. M. Jararhagin and its multiple effects on hemostasis. *Toxicon*, v. 45, p. 987-996, 2005. https://doi.org/10.1016/j.toxicon.2005.02.013

LAM, S. K.; NG, T. B. Lectins: production and practical applications. *Applied Microbiology and Biotechnology*, v. 89, p. 45-55, 2011. https://doi.org/10.1007/s00253-010-2892-9

LI, R. et al. L-amino acid oxidase from *Naja atra* venom activates and binds to human platelets. *Acta Biochimica et Biophysica Sinica*, v. 40, p. 19-26, 2008. https://doi.org/10.1111/j.1745-7270.2008.00372.x

LI, Z. Y.; YU, T. F.; LIAN, E. C. Purification and characterization of l-amino acid oxidase from king cobra (*Ophiophagus hannah*) venom and its effects on human platelet aggregation. *Toxicon*, v. 32, p. 1349-1358, 1994. https://doi.org/10.1016/0041-0101(94)90407-3

LIU, C. Z.; HUANG, T. F. Crovidisin, a collagen-binding protein isolated from snake venom of *Crotalus viridis*, prevents platelet-collagen interaction. *Archives of Biochemistry and Biophysics*, v. 337, p. 291-299, 1997. https://doi.org/10.1006/abbi.1996.9787

LOMONTE, B. et al. Isolation of a glactose-biding lectin from the venom of the snake *Bothrops godmani* (Godman's pit viper). *Toxicon*, v. 28, p. 75-81, 1990. https://doi.org/10.1016/0041-0101(90)90008-U

LU, Q. et al. Snake venom C-type lectins interacting with platelet receptors. Structure-function relationships and effects on haemostasis. *Toxicon*, v. 45, p. 1089-1098, 2005. https://doi.org/10.1016/j.toxicon.2005.02.022

MACHADO, A. S. et al. Hemorrhagic stroke related to snakebite by *Bothrops* genus: a case report. *Revista da Sociedade Brasileira de Medicina Tropical*, v. 43, 602-604, 2010.
https://doi.org/10.1590/S0037-86822010000500029

MAITA, N. et al. Crystal structure of von Willebrand factor A1 domain complexed with snake venom, bitiscetin: insight into glycoprotein Ib a binding mechanism induced by snake venom proteins. *The Journal of Biological Chemistry,* v. 278, p. 37777-37781, 2003.
https://doi.org/10.1074/jbc.M305566200

MAMEDE, C. C. N. et al. Comparative analysis of local effects caused by *Bothrops alternatus* and *Bothrops moojeni* snake venoms: enzymatic contributions and inflammatory modulations. *Toxicon*, v. 117, p. 37-45, 2016.
https://doi.org/10.1016/j.toxicon.2016.03.006

MARCINKIEWICZ, C. Pharmacological impact of snake venom compounds. *Current Pharmaceutical Design*, v. 13, p. 2851-2922, 2007.
https://doi.org/10.2174/138161207782023720

MARCINKIEWICZ, C. et al. Isolation and characterization of EMS16, a C-lectin type protein from *Echis multisquamatus* venom, a potent and selective inhibitor of the a2b1 integrin. *Biochemistry,* v. 39, p. 9859-9867, 2000.
https://doi.org/10.1021/bi000428a

MARKLAND, F. S. Snake venoms and the hemostatic system. *Toxicon*, v. 36, p. 1749-1800, 1998.
https://doi.org/10.1016/S0041-0101(98)00126-3

MARKLAND, F.; SWENSON, S. Snake venom metalloproteinases. *Toxicon*, v. 62, p. 3-18, 2013.
https://doi.org/10.1016/j.toxicon.2012.09.004

MARSH, N.; WILLIAMS, V. Practical applications of snake venom toxins in haemostasis. *Toxicon*, v. 45, p. 1171-1181, 2005.
https://doi.org/10.1016/j.toxicon.2005.02.016

MARTINS, M.; MARQUES, O. A. V.; SAZIMA, I. Ecological and phylogenetic correlates of feeding habitats in Neotropical pitvipers of the genus *Bothrops*. In: SCHUETT, G.; HÖGGREN, M.; GREENE, H. W. *Biology of the Vipers*. Carmel: Indiana Biological Sciences Press, 2002, p. 307-328.

MATSUI, T.; FUJIMURA, Y.; TITANI, K. Snake venom proteases affecting hemostasis and thrombosis. *Biochimica et Biophysica Acta,* v. 1477, p. 146-156, 2000.
https://doi.org/10.1016/S0167-4838(99)00268-X

MATSUI, T. et al. Binding Site on Human von Willebrand Factor of Bitiscetin, a Snake Venom-Derived Platelet Aggregation Inducer. *Biochemistry,* v. 41, p. 7939-7946, 2002.
https://doi.org/10.1021/bi020004b

MATSUI, T.; HAMAKO, J. Structure and function of snake venom toxins interacting with human von Willebrand factor. *Toxicon* , v. 45, p. 1075-1087, 2005.
https://doi.org/10.1016/j.toxicon.2005.02.023

MCCLEARY, R. J. R.; KINI, R. M. Non-enzymatic proteins from snake venoms: A gold mine of pharmacological tools and drug leads. *Toxicon*, v. 62, p. 56-74, 2012.
https://doi.org/10.1016/j.toxicon.2012.09.008

MCLANE, M. A. et al. Viper venom disintegrins and related molecules. *Proceedings of the Society for Experimental Biology and Medicine*, v. 219, p. 109-119, 1998.
https://doi.org/10.3181/00379727-219-44322

MELGAREJO, A. R. Serpentes peçonhentas do Brasil. In: CARDOSO, J. L. C. et al. *Animais peçonhentos no Brasil: biologia, clínica e terapêutica dos acidentes*. 2 ed. São Paulo: Sarvier, p. 42-70, 2009.

MENEZES, M. C. et al. Sex-based individual variation of snake venom proteome among eighteen *Bothrops jararaca* siblings. *Toxicon*, v. 47, p. 304-312, 2006.
https://doi.org/10.1016/j.toxicon.2005.11.007

MOREIRA L. et al. Pathological changes induced by BaH1, a hemorrhagic proteinase isolated from *Bothrops asper* (terciopelo) snake venom, on mouse capillary blood vessels. *Toxicon*, v. 32, p. 977-987, 1994.
https://doi.org/10.1016/0041-0101(94)90376-X

MORITA, T. Structures and functions of snake venom CLPs (C-type lectin-like proteins) with anticoagulant-, procoagulant-, and platelet-modulating activities. *Toxicon*, v. 45, p. 1099-1114, 2005.
https://doi.org/10.1016/j.toxicon.2005.02.021

MOUNIER, C. M.; BON, C.; KINI, R. M. Anticoagulant venom and mammalian secreted phospholipases A2: protein- versus phospholipid dependent mechanism of action. *Haemostasis*, v. 31, p. 279-287, 2001.

MOURA-DA-SILVA, A. M.; BUTERA, D.; TANJONI, I. Importance of Snake Venom Metalloproteinases in Cell Biology: Effects on Platelets, Inflammatory and Endothelial Cells. *Current Pharmaceutical Design*, v. 13, p. 2893-2905, 2007.
https://doi.org/10.2174/138161207782023711

MOURA-DA-SILVA, A. M. et al. Processing of Snake Venom Metalloproteinases: Generation of Toxin Diversity and Enzyme Inactivation. *Toxins*, v. 8, p. 2-15, 2016.
https://doi.org/10.3390/toxins8060183

MOUSTAFA, I. M. et al. A. Crystal structure of LAAO from *Calloselasma rhosdostom*a with an L-phenylalanine substrate: Insights into structure and mechanism. *Journal Molecular Biology*, v. 364, p. 991-1002, 2006. https://doi.org/10.1016/j.jmb.2006.09.032

NAUMANN, G. B. et al. Cytotoxicity and inhibition of platelet aggregation caused by an l-amino acid oxidase from *Bothrops leucurus* venom. *Biochimica et Biophysica Acta*, v. 1810, p. 683-694, 2011. https://doi.org/10.1016/j.bbagen.2011.04.003

NAVDAEV, A. et al. Echicetin, a GPIb-binding snake C-type lectin from *Echis carinatus*, also contains a binding site for IgMkappa responsible for platelet agglutination in plasma and inducing signal transduction. *Blood*, v. 97, p. 2333-2341, 2001. https://doi.org/10.1182/blood.V97.8.2333

NAVDAEV, A. et al. Echicetin Coated Polystyrene Beads: A Novel Tool to Investigate GPIb-Specific Platelet Activation and Aggregation. PlosOne 9, e93569- e93569, 2014. https://doi.org/10.1371/journal.pone.0093569

NIESWANDT, B.; WATSON, S. P. Platelet collagen interaction: is GPVI the central receptor? *Blood*, v. 102, p. 449-461, 2003. https://doi.org/10.1182/blood-2002-12-3882

NIEWIAROWSKI, S. et al. Disintegrins and other naturally occurring antagonists of platelet fibrinogen receptors. *Seminars in Hematology*, v. 31, p. 289-300, 1994.

NISHIDA, S. et al. Purification and characterization of bothrombin, a fibrinogen clotting serine protease from the venom of *Bothrops jararaca. Biochemistry*, v. 33, p. 1843-1849, 1994. https://doi.org/10.1021/bi00173a030

NOGUEIRA, C.; SAWAYA, R.J.; MARTINS, M. Ecology of the Pitviper, *Bothrops moojeni*, in the Brazilian Cerrado. *Journal of Herpetology*, v. 37, p. 653-659, 2003. https://doi.org/10.1670/120-02A

NUNES, E. S. et al. Purification of a lectin with antibacterial activity from *Bothrops leucurus* snake venom. *Comparative Biochemistry and Physiology - Part B,* v. 159, p. 57-63, 2011. https://doi.org/10.1016/j.cbpb.2011.02.001

OBERT, B. et al. Characterization of bitiscetin-2, a second form of bitiscetin from the venom of *Bitis arietans* : comparison of its binding site with the collagen-binding site on the von Willebrand factor A3-domain. *Journal of Thrombosis and Haemostasis*, v. 4, p. 1596-1601, 2006. https://doi.org/10.1111/j.1538-7836.2006.01994.x

OKUDA, D.; KOIKE, H.; MORITA, T. A new gene structure of the disintegrin

family: a subunit of dimeric disintegrin has a short coding region. *Biochemistry*, v. 41, p. 14248-14254, 2002.
https://doi.org/10.1021/bi025876s

OLIVEIRA, F. et al. Biochemical and functional characterization of BmooSP, a new serine protease from *Bothrops moojeni* snake venom. *Toxicon*, v. 111, p. 130-138, 2016.
https://doi.org/10.1016/j.toxicon.2016.01.055

OSHIKAWA, K.; TERADA, S. Ussuristatin 2, a novel KGD bearing disintegrin from *Agkistrodon ussuriensis* venom**.** *Biochemical Journal,* v. 125, p. 31-35, 1999.
https://doi.org/10.1093/oxfordjournals.jbchem.a022264

OWNBY, C. L. et al. Lysine 49 phospholipase A2 proteins. *Toxicon*, v. 37, p. 411-445, 1999.
https://doi.org/10.1016/S0041-0101(98)00188-3

PANUNTO, P. C. et al. biological activities of a lectin from *Bothrops jararacussu* snake venom. *Toxicon*, v. 47, p. 21-31., 2006.
https://doi.org/10.1016/j.toxicon.2005.08.012

PEREIRA-BITTENCOURT, M. C.; GAGLIARDI, A. R.; COLLINS, D. C. The effect of a lectin from the venom of the snake *Bothrops jararacussu* on tumor cell proliferation. *Anticancer Research*, v. 19, p. 4023-4025, 1999.

PINHO, F. M. O.; PEREIRA, I. D. Ofidismo. *Revista da Associação Médica Brasileira*, v. 47, p. 24-29, 2001.
https://doi.org/10.1590/S0104-42302001000100026

POLGAR, J. et al. Platelet activation and signal transduction by convulxin, a C-type lectin from *Crotalus durissus terrificus* (tropical rattlesnake) venom via the p62/GPVI collagen receptor. *The Journal of Biological Chemistry*, v. 272, p. 13576-13583, 1997.
https://doi.org/10.1074/jbc.272.21.13576

QUEIROZ, L. S.; PETTA, C. A. Histopatological changes caused by venom of Urutu snake (*Bothrops alternatus*) in mouse skeletal muscle. *Revista do Instituto de Medicina Tropical de São Paulo*, v. 26, p. 247-253, 1984.
https://doi.org/10.1590/S0036-46651984000500004

RAMOS, O. H. P.; SELISTRE-DE-ARAÚJO, H. S. Snake venom metalloproteases - structure and function of catalytic and disintegrin domains. *Comparative Biochemistry and Physiology Part C*, v. 142, p. 328-346, 2006.
https://doi.org/10.1016/j.cbpc.2005.11.005

RIBEIRO, L. A.; JORGE, M. T. Acidente por serpentes do gênero *Bothrops*: Série de 3.139 casos. *Revista da Sociedade Brasileira de Medicina Tropical,* v. 30, p. 475-480, 1997.
https://doi.org/10.1590/S0037-86821997000600006

ROCHA, M. M. T.; FURTADO, M. F. D. Individual characterization of *Bothrops alternatus* Duméril, Bibron & Duméril venoms, according to their geographic distribution in Brazil (Serpentes, Viperidae). *Revista Brasileira de Zoologia*, v. 22, p. 383-393, 2005.
https://doi.org/10.1590/S0101-81752005000200012

RODRIGUES, R. S. et al. Structural and functional properties of Bp-LAAO, a new L-amino acid oxidase isolated from *Bothrops pauloensis* snake venom. *Biochimie* , v. 91, p. 490–501, 2009.
https://doi.org/10.1016/j.biochi.2008.12.004

RUGGERI, Z. M. Platelets in atherothrombosis. *Nature Medicine*, v. 8, p. 1227-1234, 2002.
https://doi.org/10.1038/nm1102-1227

RUGGERI, Z. M; MENDOLICCHIO, G. L. Interaction of von Willebrand factor with platelets and the vessel wall. *Hämostaseologie*, v. 3, p. 211-224, 2015.
https://doi.org/10.5482/HAMO-14-12-0081

RUSSELL, F. E. Venomous animal injuries. *Current Problems in Pediatrics*, v. 3, p. 3-47, 1973.
https://doi.org/10.1016/S0045-9380(73)80035-0

SAJEVIC, T.; LEONARDI, A.; KRIZAJ, I. Haemostatically active proteins in snake venoms. *Toxicon*, v. 57, p. 627 - 645, 2011.
https://doi.org/10.1016/j.toxicon.2011.01.006

SAKURAI, Y. et al. Inhibition of human platelet aggregation by L-amino acid oxidase purified from *Naja naja* kaouthia venom. *Toxicon*, v. 39, p. 1827-1833, 2001.
https://doi.org/10.1016/S0041-0101(01)00133-7

SAMEL, M. et al. Isolation and characterization of an apoptotic and platelet aggregation inhibiting l-amino acid oxidase from *Vipera berus berus* (common viper) venom. *Biochimica et Biophysica Acta*, v. 1764, p. 707-714, 2006.
https://doi.org/10.1016/j.bbapap.2006.01.021

SANTOS, B. F. et al. Interaction of viper venom serine peptidases with thrombin receptors on human platelets. *FEBS Letters*, v. 477, p. 199-202, 2000.
https://doi.org/10.1016/S0014-5793(00)01803-2

SANTOS-FILHO, N. A. et al. A new acidic myotoxic, anti-platelet and prostaglandin I2 inductor phospholipase A2 isolated from *Bothrops moojeni* snake venom. *Toxicon*, v. 52, p. 908-917, 2008.
https://doi.org/10.1016/j.toxicon.2008.08.020

SANZ, L. et al. Molecular cloning of disintegrins from *Cerastes vipera* and *Macrovipera lebetina* transmediterranea venom gland cDNA libraries. Insight into

the evolution of the snake venom's integrin inhibition system. *Biochemical Journal*, v. 395, p. 385-392, 2006.
https://doi.org/10.1042/BJ20051678

SCARBOROUGH, R. M. et al. Barbourin. A GPIIb-IIIa-specific integrin antagonist from the venom of *Sistrurus M. barbouri*. *Journal of Biological Chemistry,* v. 266, p. 9359-9362, 1991.

SCHULZE, H.; SHIVDASANI, R. A. Mechanisms of thrombopoiesis. *Journal of Thrombosis and Haemostasis*, v. 3, n. 8, p. 1717-24, 2005.
https://doi.org/10.1111/j.1538-7836.2005.01426.x

SERRANO, S. M. T. The long road of research on snake venom serine proteinase. *Toxicon*, v. 62, p. 19-26, 2013.
https://doi.org/10.1016/j.toxicon.2012.09.003

SERRANO, S. M. T.; MAROUN, R. C. Snake venom serine proteinases: sequence homology vs. substrate specificity, a paradox to be solved. *Toxicon*, v. 45, p. 1115-1132, 2005.
https://doi.org/10.1016/j.toxicon.2005.02.020

SHAH, I. et al. Eptifibatide: The evidence for its role in the management of acute coronary syndromes. *Core Evidence*, v. 4, p. 49-65, 2009.

SHIMOKAWA, K. et al. Sequence and biological activity of catrocollastatin-C: a disintegrin-like/cysteine rich two-domain protein from *Crotalus atrox* venom. *Archives of Biochemistry and Biophysics*, v. 343, p. 35-43, 1997.
https://doi.org/10.1006/abbi.1997.0133

SILVA, A. S. T. et al. *Soros e Vacinas*. 2. ed. São Paulo: Instituto Butantan, 2013, 60 p.

SILVEIRA, L. B. et al. Isolation and expression of a hypotensive and anti-platelet acidic phospholipase A2 from *Bothrops moojeni* snake venom. *Journal of Pharmaceutical and Biomedical Analysis*, v. 73, p. 35-43, 2013.
https://doi.org/10.1016/j.jpba.2012.04.008

SLAGBOOM, J. et al. Haemotoxic snake venoms: their functional activity, impact on snakebite victims and pharmaceutical promise. *British Journal of Haematology*, p. 1-13, 2017.
https://doi.org/10.1111/bjh.14591

SOARES, A. M.; FONTES, M. R. M.; GIGLIO, J. R. Phospholipases A2 myotoxins from *Bothrops* snake venoms: structure-function relationship. *Current Organic Chemistry*, v. 8, p. 1677-1690, 2004.
https://doi.org/10.2174/1385272043369610

SOTELO-CRUZ, N.; GÓMEZ-RIVERA, N. A retrospective review of rattlesnake bites in 100 children. *Minerva Pediatrica*, v. 69, p. 121-128, 2017.

SOUZA, D. H. et al. Isolation and structural characterization of a cytotoxic L-amino acid oxidase from *Agkistrodon contortrix laticinctus* snake venom: preliminary crystallographic data. *Archives of Biochemistry and Biophysics*, v. 368, p. 285-290, 1999.
https://doi.org/10.1006/abbi.1999.1287

SOUZA, L. L. et al. Determination of Toxic Activities in *Bothrops* spp. Snake Venoms Using Animal-Free Approaches: Correlation Between In Vitro Versus In Vivo Assays. *Toxicological Sciences*, v. 147, p. 458-465, 2015.
https://doi.org/10.1093/toxsci/kfv140

STÁBELI, R. G. et al. *Bothrops moojeni* myotoxin-II, a Lys49-phospholipase A2 homologue: an example of function versatility of snake venom proteins. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* v.142, p.371-381, 2006.
https://doi.org/10.1016/j.cbpc.2005.11.020

STASSEN, J. M.; ARNOUT, J.; DECKMYN, H. The hemostatic system. *Current Medicinal Chemistry*, v. 11, n. 17, p. 2245-60, 2004.
https://doi.org/10.2174/0929867043364603

SUN, M. Z. et al. Biochemical, functional and structural characterization of Akbu-LAAO: a novel snake venom L-amino acid oxidase from *Agkistrodon blomhoffii ussurensis. Biochimie*, v. 92, p. 343–349, 2010.
https://doi.org/10.1016/j.biochi.2010.01.013

TAKEYA, H. et al. Coagulation factor X activating enzyme from Russell's viper venom (RVV-X). A novel metalloproteinase with disintegrin (platelet aggregation inhibitor)-like and C-type lectin-like domains. *The Journal of Biological Chemistry*, v. 267, p. 14109-14117, 1992.

TAN, C.H. et al. Genus Calliophis of Asiatic coral snakes: a deficiency of venom cross-reactivity and neutralization against seven regional elapid antivenoms. *Toxicon*, v. 121, p. 130-133, 2016.
https://doi.org/10.1016/j.toxicon.2016.09.003

TEIXEIRA, S. S. et al. Molecular characterization of an acidic phospholipase A2 from *Bothrops pirajai* snake venom: Synthetic-peptide identifies its anti-platelet region. *Archives of Toxicology*, v. 85, p. 1219-1233, 2011.
https://doi.org/10.1007/s00204-011-0665-6

TENG, C. M.; CHEN, Y. H.; OUYANG, C. Biphasic effect on platelet aggregation by phospholipase a purified from *Vipera russellii* snake venom. *Biochimica et Biophysica Acta*, v. 772, p. 393-402, 1984.
https://doi.org/10.1016/0005-2736(84)90156-1

TOKARNIA, C. H.; PEIXOTO, P. V. A importância dos acidentes ofídicos como causa de mortes em bovinos no Brasil. *Pesquisa Veterinária Brasileira*, v. 26, p. 55-68, 2006.
https://doi.org/10.1590/S0100-736X2006000200001

TONISMAGI, K. et al. l-Amino acid oxidase from *Vipera lebetina* venom: isolation, characterization, effects on platelets and bacteria. *Toxicon*, v. 48, p. 227-237, 2006.
https://doi.org/10.1016/j.toxicon.2006.05.004

TORRES, A. F. C. et al. Antibacterial and antiparasitic effects of *Bothrops marajoensis* venom and its fractions: phospholipase A2 and l-amino acid oxidase. *Toxicon*, v. 55, p. 795-804, 2010.
https://doi.org/10.1016/j.toxicon.2009.11.013

UETZ, P.; FREED, P; HOSEK, J. *The Reptile Database.* Disponível em: <http://www.reptile-database.org/>. Acesso em: 21 fev. 2017.

USAMI, Y. et al. Primary structure of two-chain botrocetin, a von Willebrand factor modulator purified from the venom of *Bothrops jararaca*. *Proceedings of the National Academy of Sciences*, v. 90, p. 928-932, 1993.
https://doi.org/10.1073/pnas.90.3.928

USAMI, Y. et al. A 28-kDa protein with disintegrin-like-like structure (jararhagin-C) purified from *Bothrops jararaca* venom inhibits collagen and ADP-induced platelet aggregation. *Biochemical and Biophyscal Research Communications*, v. 201, p. 331-339, 1994.
https://doi.org/10.1006/bbrc.1994.1706

VALENTIN, E.; LAMBEAU, G. Increasing molecular diversity of secreted phospholipases A(2) and their receptors and binding proteins.
Biochimica et Biophysica Acta, v. 1488, p. 59-70, 2000.
https://doi.org/10.1016/S1388-1981(00)00110-4

VARGAS, L. J. et al. Cloning and characterization of an antibacterial L-amino acid oxidase from *Crotalus durissus cumanensis* venom. *Toxicon*, v. 64, p. 1-11, 2013.
https://doi.org/10.1016/j.toxicon.2012.11.027

VITT, L. J.; CALDWELL, J. P. *Herpetology: An Introductory Biology of Amphibians and Reptiles*. 4. ed. San Diego: Elsevier, 2013.

WANG, W. J.; HUANG, T. F. Purification and characterization of a novel metalloproteinase acurhagin, from *Agkistrodon acutus* venom. *Thrombosis and Haemostasis*, v. 87, p. 641-650, 2002.

WARD, C. M. et al. Mocarhagin, a novel cobra venom metalloproteinase, cleaves the platelet von Willebrand factor receptor glycoprotein Ib alpha. Identification of the sulphated tyrosine/anionic sequence Tyr-276-Glu-282 of glycoprotein Ib alpha

as a binding site for von Willebrand factor and alpha thrombin. *Biochemistry*, v. 35, p. 4929-4938, 1996.
https://doi.org/10.1021/bi952456c

WARREL, D. A. Snakebites in Central and South America: epidemiology, clinical features, and clinical management. In: CAMPBELL, J. A.; LAMAR, W. W. *The venomous reptiles of the Western Hemisphere.* Nova York: Cornell University Press, 2004, p.709-715.
https://doi.org/10.1016/S0140-6736(09)61754-2

WARRELL, D. A. Snake bite. *The Lancet*, v. 375, p. 77-88, 2010.

WEI, P. et al. Clinical effects of treatment with Tirofiban on patients with high-risk NSTE-ACS after PCI. *European Review for Medical and Pharmacological Sciences*, v. 20, p. 1356-1359, 2016.

WEIS, W. I.; DRICKAMER, K. Structural basis of lectin-carbohydrate recognition. *Annual Reviews in Biochemistry*, v. 65, p. 441-473, 1996.
https://doi.org/10.1146/annurev.bi.65.070196.002301

WEN, F. H. Soroterapia. In: CARDOSO, J. L. C. et al. *Animais Peçonhentos no Brasil: Biologia, clínica e terapêutica dos acidentes*. 2. ed. São Paulo: Sarvier, 2009, p. 81-95.

WORLD HEALTH ORGANIZATION (WHO). *Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins*. Geneva: World Health Organization Press, 2010.

WORLD HEALTH ORGANIZATION (WHO). *Neglected Tropical Diseases. The Global Burden of Snakebite: A Literature Analysis and Modelling Based on Regional Estimates of Envenoming and Deaths.* Disponível em: <http://www.who.int/neglected diseases/integrated media snakebite>. Acesso em: 2 mar. 2017.

WU, W. B.; PENG, H. C.; HUANG, T. F. Crotalin, a vWF and GP Ib cleaving metalloproteinase from venom of *Crotalus atrox*. *Thrombosis and Haemostasis*, v. 86, p. 1501-1511, 2001.

YAMADA, D.; MORITA, T. Purification and characterization of a Ca^{2+}-dependent prothrombin activator, multactivase, from the venom of *Echis multisquamatus*. *The Journal of Biochemistry*, v. 122, p. 991-997, 1997.
https://doi.org/10.1093/oxfordjournals.jbchem.a021862

YIP, J. et al. Primary Platelet Adhesion Receptors. *IUBMB Life*, v. 57, p. 103-108, 2005.
https://doi.org/10.1080/15216540500078962

ZELENSKY, A. N.; GREADY, J. E. The C-type lectin-like domain superfamily. *FEBS Journal*, v. 272, p. 6179-6217, 2005.

https://doi.org/10.1111/j.1742-4658.2005.05031.x

ZHONG, S. R. et al. Purification and characterization of a new l-amino acid oxidase from *Daboia russellii siamensis* venom. *Toxicon*, v. 54, p. 763-771, 2009. https://doi.org/10.1016/j.toxicon.2009.06.004

ZHOU, Q.; DANGELMAIER, C.; SMITH, J. B. The hemorrhagin catrocollastatin inhibits collagen-induced platelet aggregation by binding to collagen *via* its disintegrin-like domain. *Biochemical and Biophyscal Research Communications*, v. 219, p. 720-726, 1996. https://doi.org/10.1016/j.toxicon.2009.06.004

ZHOU, Q.; SMITH, J. B.; GROSSMAN, M. H. Molecular cloning and expression of catrocollastatin, a snake-venom protein from *Crotalus atrox* (western diamondback rattlesnake) which inhibits platelet adhesion to collagen. *Biochemical Journal*, v. 307, p. 411-417, 1995. https://doi.org/10.1042/bj3070411

CAPÍTULO II

BaltPAi: A peptide platelet aggregation inhibitor from *Bothrops alternatus* snake venom

(Research article)

BaltPAi: A peptide platelet aggregation inhibitor from *Bothrops alternatus* snake venom

Bruna Barbosa de Sousa[1,5], Carla Cristine N. Mamede[2,5], Déborah Fernanda C. Pereira[1], Edigar Henrique Vaz Dias[1], Mariana Santos Matias[1], Mayara Ribeiro de Queiroz[5], Luciano Paulino Silva[3], Fábio de Oliveira[1,4,5]*

[1]Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia, 38400-902 Uberlândia, MG, Brazil

[2]Instituto de Ciências Agrárias, Universidade Federal de Uberlândia, 38400-902 Uberlândia, MG, Brazil

[3]Laboratório de Nanobiotecnologia (LNANO), Embrapa Recursos Genéticos e Biotecnologia, 70770-917, Brasília, DF, Brazil

[4]Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia, 38400-902 Uberlândia, MG, Brazil

[5]Instituto Nacional de Ciência e Tecnologia em Nano-Biofarmacêutica (N-Biofar), 31270-901 Belo Horizonte, MG, Brazil.

**Corresponding author:* Av. Pará, 1720, Bairro Umuarama; CEP 38400-902 - Uberlândia, MG - Brazil. E-mail address: fabio@ufu.br

Abstract

Snake venoms are complex mixtures of biologically active peptides and proteins. A number of them interact with components of the human hemostatic system affecting platelet aggregation and/or promoting physiological and biochemical changes in specific tumor cells interfering with angiogenesis, proliferation, survival, adhesion and migration of tumor cells. In this study, we identified, for the first time, a peptide from *B. alternatus* snake venom that inhibits collagen-induced platelet aggregation, which was named BaltPAi (platelet aggregation inhibitor from *Bothrops alternatus* snake venom). In addition, BaltPAi also presented cytotoxic effect on human cervix epitheloid carcinoma and human prostate adenocarcinoma cell lines. The results presented here suggest that the BaltPAi is similar of a protein fragment present in the C-terminal region of snake venom phospholipase A2 enzymes. This peptide has potential clinical usefulness for the development of novel antithrombotic drugs and in cancer therapy since peptides may be easily synthesized via solid-phase chemistry or expressed in a recombinant form.

Keywords: Snake venom; *Bothrops alternatus*; Antiplatelet; Antitumor

1 Introduction

Snake venoms are complex mixtures of enzymatic and non-enzymatic toxins with neurotoxic, cytotoxic, cardiotoxic, myotoxic and others activities (Chan et al., 2016; Pal et al., 2002; Sajevic, Leonardi, Krizaj, 2011). The ability of some snake venom toxins to cause toxicity is associated with their high specificity and affinity for cells and tissues (Vyas et al., 2013). Because of these properties, snake venom toxins have attracted interest of biochemists and pharmacologists as potential pharmaceutical agents (Mccleary and Kini, 2012). Over the last few decades, several hundreds of proteins and peptides from snake venoms have been purified and characterized as antiplatelet (Koh and Kini, 2012; Sajevic, Leonardi, Krizaj, 2011; Queiroz et al., 2014a; Queiroz et al., 2014b; Queiroz et al., 2017), antitumoral (Calderon et al., 2014; Costa et al., 2017; Ebrahim et al., 2016; Silva et al., 2015), antibacterial (Sulca et al., 2017; Wen et al., 2013), antiviral (Muller et al., 2012; Muller et al., 2014), antiparasitic (Borges et al., 2016; Passero et al., 2007) and antifungal agents (Cavalcante et al., 2017; Yamane et al., 2013).

Toxins from snake venoms may selectively modulate platelet function, either promoting or inhibiting platelet aggregation by targeting GPIb-IX-V, $\alpha_2\beta_1$, GPVI, $\alpha_{IIb}\beta_3$ and others ligands, such as von Willebrand factor (vWF) and collagen (Andrews and Berndt, 2000; Chang et al., 2017; Chen et al. 2011; Queiroz et al., 2017; Sajevic, Leonardi, Krizaj, 2011). Antiplatelet toxins appear to be useful tools for investigating the mechanisms involved in hemostasis. These toxins have been widely used as molecular tools for the development of diagnostic tests and new therapeutic agents or drugs for the treatment of thrombotic disorders (Chan et al., 2016; Koh and Kini, 2012; Mccleary and Kini, 2012).

Snake venom toxins can also act towards tumor cells. The ability of some of these toxins to have an inhibitory effect on tumor cells makes them potential candidates for the cancer treatment and this fact has been known for a long time (DeWys, Kwaan, Bathina, 1976). These toxins show antitumor activity by directly killing the tumor cells, inhibiting tumor angiogenesis or suppressing tumor growth (Calderon et al., 2014; Chan et al., 2016; Jain and Kumar, 2012; Sobrinho et al. 2016).

In the present study, we identified and characterized a peptide from *Bothrops alternatus* venom that potently inhibits the platelet aggregation and the proliferation of human cervix epitheloid carcinoma (HeLa) and human prostate adenocarcinoma (PC-3) cells.

2 Material and Methods

2.1 Material

B. alternatus venom was purchased from Bioagents Serpentarium (Batatais, SP, Brazil). Acrylamide, ammonium bicarbonate, ammonium persulphate, bromophenol blue, ethylenediaminetetracetic acid (EDTA), glycine, *β*-mercaptoethanol, *N,N'*-methylene-*bis*-acrylamide, sodium dodecyl sulphate (SDS), *N,N,N',N'*-tetramethylethylenediamine (TEMED), Tris and Roswell Park Memorial Institute-1640 (RPMI) medium were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chromatographic media (DEAE-Sephacel, Sephadex G-75 and source 15 RPC ST 4.6/100 columns) were purchased from GE Healthcare Technologies (Uppsala, Sweden). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) and dimethyl sulfoxide (DMSO) were purchased from Amresco. All agonists used in the platelet aggregation assays (collagen from equine tendon and adenosine diphosphate (ADP) were purchased from Helena Laboratories (Beaumont, Texas, USA). HeLa (human cervix epitheloid carcinoma), PC-3 (human prostate adenocarcinoma) and VERO (african green monkey kidney) cells line were kindly provided by Laboratory of Nanobiotechnology of the Federal University of Uberlândia. All other reagents used were of analytical grade.

2.2 Human blood

Human blood was obtained from 10 voluntary donors aged between 18 and 65, weighing more than 50 kg, no signs and symptoms of disease, malnutrition or dehydration, and they declared no use of any medication that interferes with hemostasis or use of illicit drugs or alcohol in the last 24 h before the experiment. The experiments were performed in accordance with current guidelines for human research established by the Committee for Ethics in Human Research of the

Federal University of Uberlândia (CEP/UFU), Minas Gerais, Brazil (Protocol number 1.627.982/2016).

2.3 Fractionation of *B. alternatus* crude venom

B. alternatus crude venom was fractionated using the methodology previously described by Bernardes et al. (2008) with modifications. Crude venom (400 mg) was dissolved in 50 mmol/L of AMBIC (ammonium bicarbonate buffer) (pH 7.8) and clarified by centrifugation at 10,000 *g* for 10 min. The supernatant solution was applied onto a DEAE-Sephacel column (2.5 × 20 cm) previously equilibrated with 50 mmol/L of AMBIC (pH 7.8). Elution was carried out at a flow rate of 20 mL/h with a linear concentration gradient of AMBIC (50 mmol/L - 1.0 mol/L). Fractions with 3.0 mL/tube were collected and their absorbances were recorded at a wavelength of 280 nm on a spectrophotometer (BioSpec-Mini Shimadzu Biotech, Japan). Then a molecular exclusion chromatography on a Sephadex G-75 column and a reverse-phase HPLC chromatography were performed. The Sephadex G-75 column (1.0 × 100 cm) was previously equilibrated with 50 mmol/L AMBIC (pH 7.8). The samples were eluted with 50 mmol/L of AMBIC at a flow rate of 20 mL/h. Fractions with 3.0 mL/tube were collected and their absorbance was recorded at a wavelength of 280 nm. The reverse-phase chromatography was performed in a source 15 RPC ST 4.6/100 column using the ÄKTApurifier™ HPLC system. The column was equilibrated with solvent A (0.065% trifluoroacetic acid) and eluted with a linear concentration gradient from 0 to 100% of solvent B (0.050% trifluoroacetic acid containing acetonitrile) at a flow rate of 1.0 mL/min. Absorbance was monitored at wavelength of 280 nm and fraction of 1.0 mL/tube were collected. This last step of fractionation resulted a fraction capable of inhibiting the platelet aggregation, which was denominated RP.

2.4 Protein analysis

The dosages of protein solutions were determined by the method previously described by Bradford (1976), using bovine serum albumin as standard. The concentration of peptide solutions was performed using a UV absorption method

that calculates concentration from absorbance at 214 nm, using a BioSpec-mini spectrophotometer (Shimadzu Biotech, Japan).

2.5 Platelet aggregation assay

Platelet aggregation assays were performed in human platelet-rich plasma (PRP) and measured using an automated four-channel Aggregometer (AggRAM™ version 1.1, Helena Laboratories, USA) as described by Queiroz et al. (2014a) with modifications. Human blood collected in sodium citrate was centrifuged at 100 *g* for 12 min at room temperature to obtain PRP. Platelet-poor plasma (PPP) was obtained from the residue by centrifugation of citrated blood at 1,000 *g* for 15 min. Aggregation was triggered with collagen (10 µg/mL) or ADP (20 µM). The effect on collagen-induced platelet aggregation was tested immediately after adding the RP fraction (15, 10 and 5 µg) to PRP. In turn, the effect on ADP-induced platelet aggregation was tested after incubating the RP fraction (15 µg) with PRP for 15 min at 37°C. One hundred percent aggregation was expressed as the percentage absorbance relative to PPP aggregation. Control experiments were performed using only agonists. All experiments were carried out in triplicate.

2.6 Cell culture

HeLa, PC-3 and VERO cell lines were maintained as a monolayer culture in RPMI 1640 supplemented with 10% bovine fetal serum (BFS), 25 mM HEPES, 100 U/mL penicillin, 100 µg streptomycin, 2 mM L-glutamine and 3 mM sodium bicarbonate and maintained in a humidified incubator containing 5% CO_2 at 37°C.

2.7 Cytotoxicity assay

The cytotoxicity assays of RP fraction towards HeLa, PC-3 and VERO cultures were evaluated by the colorimetric MTT assay, according to the manufacturer's recommendations, with slight modifications. The cells (2×10^4 cells/well) were seeded into a 96-well plate for adhesion. After 24 h of incubation (37°C) the cells were treated with *B. alternatus* crude venom (50 µg/mL) (positive control) or different concentrations of RP fraction (100, 50, 25 and 12.5 µg/mL) diluted with RPMI medium. The untreated cells served as negative control. After 24 h, MTT solution was added to each well at a final concentration of 2 mg/mL and

the plates were incubated at 37°C for 1 h. The crystal formazan was dissolved by addition of 100 µL of DMSO to each well. The absorbance was detected at 560 nm in a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, USA). All experiments were carried out in triplicate. The cytotoxicity rate was calculated as follows: Cytotoxicity (%) = (1 − absorbance of the treated wells)/(absorbance of the control wells) × 100%.

2.8 Statistical Analysis

Statistical significant values were compared to controls by two-way ANOVA followed by Bonferroni's test using the software Prism 5.0 (GraphPad Software Inc., USA). Data are presented a mean ± standard error (n ¼ 3). Differences with p values of less than 5% were considered significant ($p < 0.05$).

2.9 Mass spectrometry analysis

RP fraction was subjected to analysis on a MALDI-TOF/TOF mass spectrometer (ultrafleXtreme, Bruker Daltonics, Germany), in positive and reflector mode, after external calibration using a standard mixture of peptides. All detected ions were subjected to MS/MS fragmentation. MS/MS fragmentation was carried out using the LIFT mode (Suckau et al., 2003). Peptides identification was performed by manual interpretations through *De novo* method by identifying all major fragmentation series. The primary structures of all sequenced peptides were compared with the sequences of other related proteins/peptides using BLAST (Basic Local Alignment Search) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3 Results and discussion

Snake venom toxins have been shown to possess a wide spectrum of biological activities because of their high affinity and selectivity for receptors, ion channels or plasma proteins (Calderon et al., 2014; Chan et al., 2016; McCleary and Kini, 2012). The diversity of these toxins makes them excellent models from which new therapeutic agents may be developed (Dhananjaya and Sivashankari, 2015; Koh and Kini, 2012; Vyas et al., 2013). Snake venom toxins with antiplatelet or antitumor activities usually belong to metalloproteinase (DeClerck, 2000; Zhu et

al., 2010), serine proteinase (Nishida et al., 1994; Shibuya et al., 1990), phospholipase A_2 (PLA_2) (Cummings, 2007; Santos-Filho et al., 2008), L-amino acid oxidase (SV-LAAO) (Samel et al., 2006; Sun et al., 2010), C-type lectin (Lu et al., 2004; Nunes et al., 2012) or disintegrin (Kauskot et al., 2008; Markland et al., 2001) families. In the present work, we identified and characterized, for the first time, a peptide from *B. alternatus* snake venom with antiplatelet and cytotoxic activities on HeLa and PC-3 cell lines.

The fractionation of the *B. alternatus* snake venom was carried out using three chromatographic steps including ion-exchange chromatography on a DEAE-Sephacel column, molecular exclusion chromatography on a Sephadex G-75 column and reverse-phase HPLC chromatography in a source 15 RPC ST 4.6/100 column, resulting in the purification of an antiplatet fraction, named RP (results not shown). Our results showed that 10 µg of this fraction, immediately inhibited approximately 55% of collagen-induced platelet aggregation, whereas 15 µg completely inhibited the aggregation immediately after adding collagen (Fig. 1A). Moreover, when this fraction was incubated with human PRP for 3 min prior to the addition of collagen the aggregation was completely inhibited even at low doses (5 µg), demonstrating its potent antiplatelet effect (data not shown). In addition, RP fraction had no effect on ADP-induced platelet aggregation even when it was pre-incubated with PRP for 15 min at 37°C (Fig. 1B).

Collagen is the most thrombogenic component of the subendothelial matrix and plays an essential role in thrombus formation providing a substrate for platelet adhesion (Nieswandt et al., 2001). A large number of collagen receptors have been identified in platelets, including $\alpha_2\beta_1$ integrin and GPVI (Deckmyn et al., 2012). A current model of platelet adhesion suggests that the first step in platelet recruitment to collagen in high shear stress (*e.g.* stenosed arteries) occurs indirectly by binding of platelet GPIb-IX-V to collagen-bound vWF (Savage, Cattaneo, Ruggeri, 2001). The vWF-GPIb binding is insufficient for stable adhesion, but it is important to recruit other platelets and facilitates the direct binding of platelets to collagen with the major collagen receptors, GPVI (Nieswandt and Watson, 2003). The interaction mediated by the GPVI upregulate the activity of $\alpha_2\beta_1$ integrins which in turn promotes subsequent $\alpha_2\beta_1$-collagen binding (Nieswandt et al., 2001; Ozaki, Suzuki-Inoue, Inoue, 2009). The high

affinity binding of $\alpha_2\beta_1$ to collagen strengthens firm adhesion and induces platelet activation with agonists release such as adenosine diphosphate (ADP) and thromboxane A_2 (TXA_2). These secreted agonists promote the activation of $\alpha_{IIb}\beta_3$ integrin (GPIIb/IIIa) that bind to fibrinogen and link adjacent platelets inducing thrombus formation (Jurk and Kehrel, 2005).

Snake venom toxins that are involved in platelet aggregation inhibition could be used to design antithrombotic agents, especially toxins targeting collagen receptors, since they have the advantage of blocking the initial processes of the thrombogenic reaction (Andrews and Bernt, 2000; Clemetson, Lu, Clemetson, 2007; Manon-Jensen, Kjeld, Karsdal, 2016). Several different platelet aggregation inhibitors that interferes with the interaction between collagen and its receptors have been isolated from snake venom (Andrews and Berndt, 2000; Markland, 1998; Wijeyewickrema, Berndt, Andrews, 2005). This inhibition may occur by cleaving (Hsu, Wu, Huang, 2008; Sanchez et al., 2016) or binding to these ligands (Kumar et al., 2011; Wang, Shih, Huang, 2005; Wang, 2007). Jararhagin, a well know P-III SVMP from *B. jararaca* acts as a collagen receptor antagonist. Its platelet aggregation inhibition is mediated through its binding to platelet α_2-subunit, followed by proteolysis of the β_1-subunit (Kamiguti, Hay, Zuzel, 1996; Paine et al., 1992). Catrocollastatin is also an P-III SVMP isolated from *Crotalus atrox* that inhibits collagen-induced platelet aggregation, however it exerts its effect by directly binding to collagen (Zhou, Smith, Grossman, 1995; Zhou, Dangelmaier, Smith, 1996). On the other hand, the snake venom C-type lectin from *Bitis gabonica rhinoceros,* Rhinocetin, targets $\alpha_2\beta_1$ integrin blocking its activity (Vaiyapuri et al., 2012). Other examples are: Acurhagin (Wang, Shih, Huang, 2005), Trowaglerix (Chang et al., 2017) and AAV1 (Wang, 2007) that inhibit collagen-induced platelet aggregation by blocking platelet GPVI; Flavocetin-A (Arlinghaus and Eble, 2013) and NN-PF3 (Kumar et al., 2011) that bind to $\alpha_2\beta_1$ integrin; and Kistomin (Hsu, Wu, Huang, 2008) that inhibits platelet aggregation through its proteolytic activity on GPVI. Our results suggest that the RP fraction interacts with the collagen receptors ($\alpha_2\beta_1$ or GPVI), potentially inhibiting the platelet aggregation. In addition, we rule out a possible cleavage of collagen or one of its receptors since RP fraction does not exhibit proteolytic activity (data not shown).

A number of toxins from snake venoms also may bind specifically to cancer cell membranes, affecting the migration, adhesion, proliferation and invasion of these cells, suggesting their promising use as antitumor agents (Calderon et al., 2014; Chan et al., 2016; Jain and Kumar, 2012; Vyas et al., 2013). To evaluate the cytotoxic effect of RP fraction, MTT assays were performed using both tumor cell lines HeLa and PC-3, and one non-tumoral cell line, VERO. Our results showed that the RP fraction induces significant cytotoxicity toward HeLa and PC-3 cells, both tumor cell lines, in a dose-dependent manner. RP fraction (100 µg/mL) presented approximately 90% of cytotoxicity toward HeLa cell line 24 h after treatment, and approximately 50% of cytotoxicity toward PC-3 cell line under the same conditions. Surprisingly, RP fraction (100 µg/mL) showed a little cytotoxic effect (≈20%) on the non-tumoral cell line (VERO) 24 h after treatment (Fig. 2). These results showed that the cytotoxicity induced by the RP fraction on the tumor cell lines tested, is higher than the cytotoxicity on the non-tumoral cell line tested, suggesting that this toxin may preferentially target these tumor cells.

Snake venom toxins may inhibit cell proliferation and promote cell death by different means: induction of apoptosis and/or necrosis (Ali et al., 2000; Silva et al., 2015; Torii, Naito, Tsuruo, 1997; Wu et al., 2013); generation of H_2O_2 (Iijima, Kisugi, Yamazaki, 2003; Naumann et al., 2011; Samel et al., 2006); decreasing or increasing the expression of proteins that control cell cycle (Cura et al., 2002; Gomes et al., 2007); causing damage to cell membranes (Cummings, 2007; Dubovskii and Utkin, 2015; Sobrinho et al., 2016) or targeting specific integrins (Chung et al., 2003; Kamiguti et al., 1997; Macedo, Fox, Castro, 2015; Marcinkiewicz et al., 2003; Sarray et al. 2001; Sarray et al., 2007; Yang et al., 2005).

Integrins imply an important class of cell surface adhesion receptors critically involved in cell-cell and cell-matrix interactions. They contribute to important processes such as survival, proliferation, angiogenesis, migration and invasion (Calderon et al., 2014; Gould et al., 1990; Yang et al., 2005). Thus, integrin antagonists provide a special interest as possible candidate targets for the development of antiangiogenic and antimetastatic cancer therapies (Brooks et al., 2010; Kamiguti, Zuzel, Theakston, 1998; Macedo, Fox, Castro, 2015; Selistre-de-Araujo et al., 2010). Particularly the subfamilies β_1 and β_3 have been identified as

major functional adhesion receptors on tumor cells playing a key role in their proliferation and migration (Hou et al., 2016; Lundstrom et al., 1998; Marcinkiewicz et al., 2003; Morini et al., 2000; Pluijm et al., 1997; Trikha, Clerck, Markland, 1994). Results presented in this work suggest that the RP fraction may target $\alpha_2\beta_1$ integrin on tumor cells interfering with the adhesion of them to the extracellular matrix since this fraction supposedly interacts with the $\alpha_2\beta_1$ platelet integrin. However, further studies are needed to better understand its mechanism of action regarding its inhibitory activity in cancer cell proliferation.

In order to identify which peptide is responsible for the antiplatelet and cytotoxicity activities, RP fraction was analyzed by MALDI-TOF mass spectrometry and the ions corresponding to its peptides were fragmented by MS/MS and *De novo* sequenced. MALDI-TOF mass spectrometry analysis showed that RP fraction is composed of peptides with molecular mass range from 900 to 2,200 Da (results not shown). The peptides were fragmented (MS/MS) to determine their amino acid sequences and the sequences found were subject to BLAST searches. Among them, two sequences aroused our interest. The first one, when subjected to BLAST search, showed 100% identity to a fragment of a phospholipase A_2 from snake venom already described. In turn, the second one also showed 100% identity with the metalloprotease isolated from *Bothrops moojeni* venom.

Similar to the RP fraction, the phospholipase A_2 is also described as an antiplatelet and cytotoxic snake venom toxin. This toxin inhibits collagen-induced platelet aggregation in a dose-dependent manner and inhibits the proliferation of different cell lines. Thus, we suggest that the small the sequence identified in this work, which was named BaltPAi (platelet aggregation inhibitor from *Bothrops alternatus* snake venom), is responsible for the antiplatelet and cytotoxic activities of this phospholipase A_2. In addition, previous studies performed in our laboratory showed that the metalloprotease has no inhibitory effect on platelet aggregation (results not shown).

Small peptides have attracted the attention and interest of pharmaceutical companies that search for new drug leads due to their simpler structures and therapeutic potential. Moreover, the peptides can be easily synthesized via solid-phase chemistry or expressed in a recombinant form. These properties make them

particularly suitable probes for the development of pharmacological drugs (Chan et al., 2016; Duggan and Tuck, 2015; Mccleary and Kini, 2012).

In summary, in this work, we first identified a peptide from *B. alternatus* snake venom with potent antiplatelet activity on collagen-induced platelet aggregation and specific cytotoxic activity on tumor cell lines (HeLa and PC-3). This peptide may be of great importance to medicine since it has therapeutic potential for the development of antithrombotic drugs as well as applications in cancer therapy.

Conflict of interest

The authors declare that there is no conflict of interests.

Acknowledgements

The authors gratefully acknowledge the financial support of Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Figures

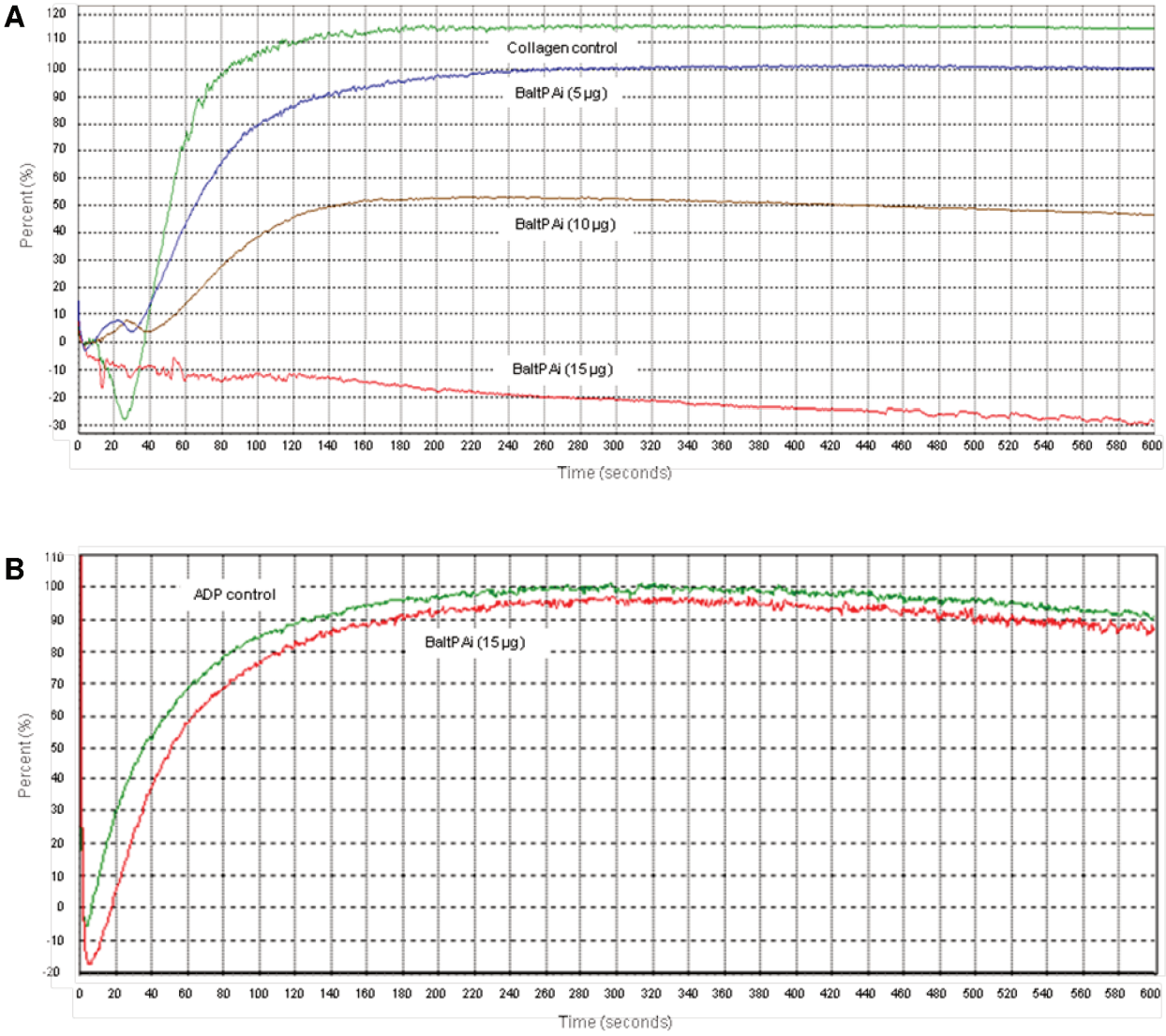

Fig. 1: Platelet aggregation assays. (A) Effect of RP fraction on collagen-induced platelet aggregation: aggregation was triggered with collagen (10 µg/mL) immediately after adding different doses of RP (15, 10 and 5 µg) to human PRP. (B) Effect of RP on ADP-induced platelet aggregation: aggregation was triggered with ADP (20 µM) after incubating RP fraction (15 µg) with human PRP for 15 minutes at 37ºC. Platelet aggregation was recorded for 10 minutes in an automated four-channel Aggregometer (AggRAM^{TM} version 1.1) (Helena Laboratories, USA). Results were expressed as an increase in light transmission,

where PPP represents the maximum response (100%). Control experiments were performed in the absence of the RP fraction.

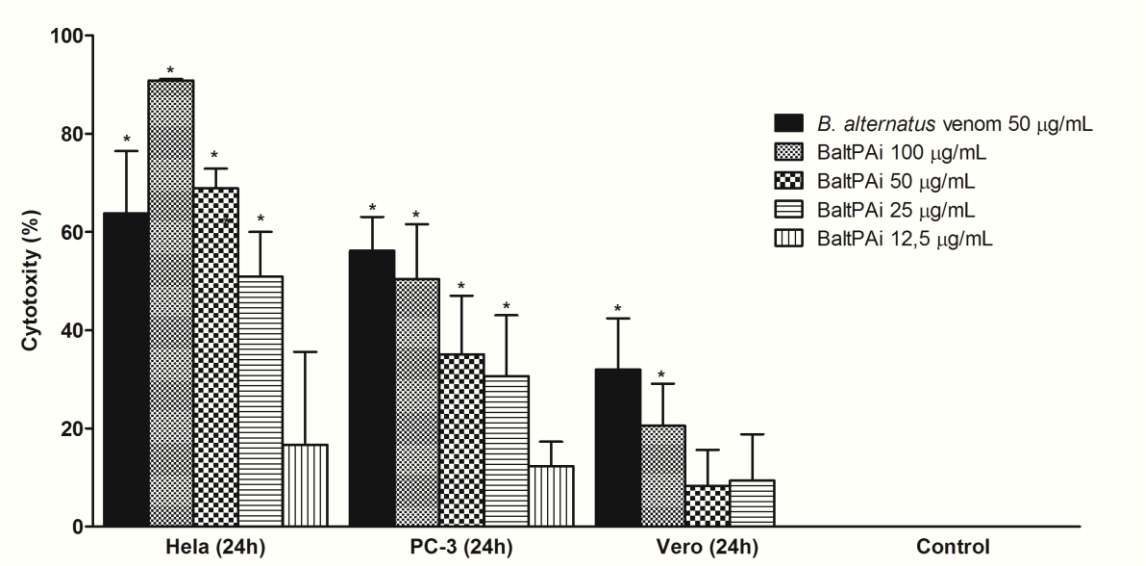

Fig. 2: Analysis of RP fraction cytotoxicity towards HeLa, PC-3 and VERO cell lines by MTT assay. Cell lines were treated with different concentrations (100, 50, 25 and 12.5 μg/mL) of RP for 24 h. These results are representative of at least 3 independent experiments. Data show the mean ± standard deviation (S. D.). *Statistically significant difference ($p<0.05$) compared to control.

References

Ali, S. A., Stoeva, S., Abbasi, A., Alam, J. M., Kayed, R., Faigle, M., Neumeister, B., Voelter, W., 2000. Isolation, structural, and functional characterization of an apoptosis-inducing L-amino acid oxidase from leaf-nosed viper (*Eristocophis macmahoni*) snake venom. Archives of Biochemistry and Biophysics 384, 216-226.

Andrews, R. K., Berndt, M.C., 2000. Snake venom modulators of platelet adhesion receptors and their ligands. Toxicon 38, 775-791.

Arlinghaus, F. T., Eble, J. A., 2013. The collagen-binding integrin α2β1 is a novel interaction partner of the *Trimeresurus flavoviridis* venom protein flavocetin-A. The Journal of Biological Chemistry 288, 947-955.

Bernardes, C. P., Santos-Filho, N. A., Costa, T. R., Gomes, M. S., Torres, F. S., Costa, J., Borges, M. H., Richardson, M., Santos, D. M., Pimenta, A. M. C., Homsi-Brandeburgo, M. I., Soares, A. M., Oliveira, F., 2008. Isolation and structural characterization of a new fibrin(ogen)olytic metalloproteinase from *Bothrops moojeni* snake venom. Toxicon 15, 574-584.

Borges, I. P., Castanheira, L. E., Barbosa, B. F., de Souza, D. L., da Silva, R. J., Mineo, J. R., Tudini, K. A., Rodrigues, R. S., Ferro, E. A., de Melo Rodrigues, V., 2016. Anti-parasitic effect on *Toxoplasma gondii* induced by BnSP-7, a Lys49-phospholipase A2 homologue from *Bothrops pauloensis* venom. Toxicon 119, 84-91.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72, 248–54.

Brooks, S. A., Lomax-Browne, H. J., Carter, T. M., Kinch, C. E., Hall, D. M. S., 2010. Molecular interactions in cancer cell metastasis. Acta Histochemica 112, 3-25.

Calderon, L. A., Sobrinho, J. C., Zaqueo, K. D., de Moura, A. A., Grabner, A. N., Mazzi, M. V., Marcussi, S., Nomizo, A., Fernandes, C. F. C., Zuliani, J. P., Carvalho, B. M. A., da Silva, S. L., Stábeli, R. G., Soares, A. M., 2014. Antitumoral Activity of Snake Venom Proteins: New Trends in Cancer Therapy. BioMed Research International, 1-19.

Cavalcante, C. S., Falcão, C. B., Fontenelle, R. O., Andreu, D., Rádis-Baptista, G., 2017. Anti-fungal activity of Ctn[15-34], the C-terminal peptide fragment of crotalicidin, a rattlesnake venom gland cathelicidin. The Journal of antibiotics 70, 231-237.

Chan, Y. S., Cheung, R. C., Xia, L., Wong, J. H., Ng, T. B., Chan, W. Y., 2016. Snake venom toxins: toxicity and medicinal applications. Applied Microbiology and Biotechnology, 1-17.

Chang, C. H., Chung, C. H., Tu, Y. S., Tsai, C. C., Hsu, C. C., Peng, H. C., Tseng, Y. J., Huang, T. F., 2017. Trowaglerix Venom Polypeptides As a Novel Antithrombotic Agent by Targeting Immunoglobulin-Like Domains of Glycoprotein VI in Platelet. Arteriosclerosis, Thrombosis and Vascular Biology 37, 1307-1314.

Chen, Z., Wu, J., Zhang, Y., Yang, X., Yu, G., Zhu, S., Lee, W., Lu, Q., Zhang, Y., 2011. A novel platelet glycoprotein Ib-binding protein with human platelet aggregation-inhibiting activity from *Trimeresurus jerdonii* venom. Toxicon 57, 672-679.

Chung, K., Kim, S., Han, K., Sohn, Y. D., Chang, S. I., Baek, K. H., Jang, Y., Kim, D. S., Kang, I. C., 2003. Inhibitory effect of salmosin, a Korean snake venom-derived disintegrin, on the integrin αvmediated proliferation of SK-Mel-2 human melanoma cells. Journal of Pharmacy and Pharmacology 55, 1577-1582.

Clemetson, K. J., Lu, Q., Clemetson, J. M., 2007. Snake venom proteins affecting platelets and their applications to anti-thrombotic research. Current Pharmaceutical Design 13, 2887-2892.

Costa, T. R., Menaldo, D. L., Zoccal, K. F., Burin, S. M., Aissa, A. F., Castro, F. A., Faccioli, L. H., Greggi Antunes, L. M., Sampaio, S. V., 2017. CR-LAAO, an L-amino acid oxidase from *Calloselasma rhodostoma* venom, as a potential tool for developing novel immunotherapeutic strategies against cancer. Scientific reports, 1-12.

Cummings, B. S., 2007. Phospholipase A2 as targets for anti-cancer drugs. Biochemical Pharmacology 74, 949-959.

Cura, J. E., Blanzaco, D. P., Brisson, C., Cura, M. A., Cabrol, R., Larrateguv, L., Mendez, C., Sechi, J. C., Silveira, J. S., Theiller, E., Roodt, A. R., Vidal, J. C., 2002. Phase I and pharmacokinetics study of crotoxin (cytotoxic PLA2, NSC-624244) in patients with advanced cancer. Clinical Cancer Research 8, 1033-1041.

Deckmyn, H., Meyer, S. F., Broos, K., Vanhoorelbeke, K., 2012. Inhibitors of the Interactions Between Collagen and Its Receptors on Platelets. Antiplatelet Agents 210, 311-337.

DeClerck, Y. A., 2000. Interactions between tumour cells and stromal cells and proteolytic modification of the extracellular matrix by metalloproteinases in cancer. European Journal of Cancer 36, 1258-1268.

DeWys, W. D., Kwaan, H. C., Bathina, S., 1976. Effect of defibrination on tumor growth and response to chemotherapy. Cancer Research 36, 3584-3587.

Dhananjaya, B. L., Sivashankari, P. R., 2015. Snake venom derived molecules in tumor angiogenesis and its application in cancer therapy; an overview. *C*urrent Topics in Medicinal Chemistry 15, 649-657.

Dubovskii, P. V, Utkin, Y. N., 2015. Antiproliferative activity of cobra venom cytotoxins. Current Topics in Medicinal Chemistry 15, 638-648.

Duggan, P. J., Tuck, K. L. Bioactive mimetics of conotoxins and other venom peptides. Toxins 7, 4175-4198.

Ebrahim, K., Vatanpour, H., Zare, A., Shirazi, F. H., Nakhjavani, M., 2016. Anticancer Activity a of Caspian Cobra (*Naja naja oxiana*) snake Venom in Human Cancer Cell Lines Via Induction of Apoptosis. Iranian Journal of Pharmaceutical Research, 101-112.

Gomes, A., Choudhury, S. R., Saha, A., Mishra, R., Giri, B., Biswas, A. K., 2007. A heat stable protein toxin (drCT-I) from the Indian viper (*Daboia russelli russelli*) venom having antiproliferative, cytotoxic and apoptotic activities. Toxicon 49, 46-56.

Gould, R. J., Polokoff, M. A., Friedman P. A., Huang, T. F., Holt, J. C., Cook, J. J., Niewiarowski, S., 1990. Disintegrins: a family of integrin inhibitory proteins from viper venoms (43129B). Proceedings of the Society for Experimental Biology and Medicine 195, 168-171.

Hou, S., Isaji, T., Hang, Q., Im, S., Fukuda, T., Gu, J., 2016. Distinct effects of β1 integrin on cell proliferation and cellular signaling in MDA-MB-231 breast cancer cells. Scientific Reports 6, 18430.

Hsu, C. C., Wu, W. B., Huang, T. F., 2008. A snake venom metalloproteinase, kistomin, cleaves platelet glycoprotein VI and impairs platelet functions. Journal of Thrombosis and Haemostasis 6, 1578-1585.

Iijima, R., Kisugi, J., Yamazaki, M., 2003. L-amino acid oxidase activity of an antineoplastic factor of a marine mollusk and its relationship to cytotoxicity. Developmental and Comparative Immunology 27, 505-512.

Jain, D., Kumar, S., 2012. Snake venom: a potent anticancer agent. Asian Pacific Journal of Cancer Prevent 13, 4855-4860.

Jurk, K., Kehrel, B. E., 2005. Platelets: Physiology and Biochemistry. *S*eminars inThrombosis and Hemostasis 31, 381-392.

Kamiguti, A., Hay, C. R. M., Zuzel, M., 1996. Inhibition of collagen-induced platelet aggregation as the result of cleavage of a2b1-integrin by the snake venom metalloproteinase jararhagin. Biochemical Journal 320, 635-641.

Kamiguti, A. S., Markland, F. S., Zhou, Q., Laing, G. D., Theakston, R. D. G., Zuzel, M., 1997. Proteolytic cleavage of the β1 subunit of platelet α2β1 integrin by the metalloproteinase jararhagin compromises collagen-stimulated phosphorylation of pp72(syk). The Journal of Biological Chemistry 272, 32599-32605.

Kamiguti, A. S., Zuzel, M., Theakston, R. D. G., 1998. Snake venom metalloproteinases and disintegrins: interactions with cells. Brazilian Journal of Medical and Biological Research 31, 853-862.

Kauskot, A., Cominetti, M. R., Ramos, O. H., Bechyne, I., Renard, J. M., Hoylaerts, M. F., Crepin, M., Legrand, C., Selistre-de-Araujo, H. S., Bonnefoy, A., 2008. Hemostatic effects of recombinant DisBa-01, a disintegrin from *Bothrops alternatus*. Frontiers in Bioscience 13, 6604-6616.

Koh, C. Y., Kini, R. M., 2012. From snake venom toxins to therapeutics - Cardiovascular examples. Toxicon 59, 497-506.

Kumar, M. S. Girish, K. S., Vichwanath, B. S., Kemparaju, K., 2011. The metalloprotease, NN-PF3 from *Naja naja* venom inhibits platelet aggregation primarily by affecting α2β1 integrin. Annals of Hematology *5, 569-577.*

Lu, Q., Navdaev, A., Clemetson, J. M., Clemetson, K. J., 2004. GPIb is involved in platelet aggregation induced by mucetin, a snake C-type lectin protein from Chinese habu (*Trimeresurus mucrosquamatus*) venom. Thrombosis and Haemosasis 91, 1168-1176.

Lundstrom, A., Holmbom, J., Lindgvist, C., Nordstrom, T., 1998. The role of alpha2 beta1 and alpha3 beta1 integrin receptors in the initial anchoring of MDA-MB-231 human breast cancer cells to cortical bone matrix. Biochemical and Biophysical Research Communications 29, 735-740.

Macedo, J. K. A., Fox, J. W., Castro, M. S., 2015. Disintegrins from Snake Venoms and their Applications in Cancer Research and Therapy. Current Protein & Peptide Science 16, 532-548.

Manon-Jensen, T., Kjeld, N. G., Karsdal, M. A., 2016. Collagen-mediated hemostasis. Journal of Thrombosis and Haemostasis 14, 438-448.

Marcinkiewicz, C., Weinreb, P. H., Calvete, J. J. Kisiel,, D. G., Mousa, S. A., Tuszynski, G. P. Lobb, R. R., 2003. Obtustatin: a potent selective inhibitor of α1β1 integrin *in vitro* and angiogenesis *in vivo.* Cancer Research 63, 2020-2023.

Markland, F. S., 1998. Snake venom and the hemostatic system. Toxicon 36, 1749-1800.

Markland, F. S., Shieh, K., Zhou, Q., Golubkov, V., Sherwin, R. P., Richters, V., Sposto, R., 2001. A novel snake venom disintegrin that inhibits human ovarian cancer dissemination and angiogenesis in an orthotopic nude mouse model. Haemostasis 31, 183-191.

McCleary, R. J., Kini, R. M., 2012. Non-enzymatic proteins from snake venoms: A gold mine of pharmacological tools and drug leads. Toxicon, 1-19.

Morini, M., Mottolese, M., Ferrari N., Ghiorzo, F., Buglioni, S., Mortarini, R., Noonan, D. M., Natali, P. G. Albini, A., 2000. The alpha 3 beta 1 integrin is associated with mammary carcinoma cell metastasis, invasion, and gelatinase B (MMP-9) activity. International Journal of Cancer 87, 336-342.

Muller, V. D., Russo, R. R., Cintra, A. C., Sartim, M. A., Alves-Paiva, R. M., Figueiredo, L. T., Sampaio, S. V., Aquino, V. H., 2012. Crotoxin and phospholipases A2 from *Crotalus durissus terrificus* showed antiviral activity against dengue and yellow fever viruses. Toxicon 59, 507-515.

Muller, V. D., Soares, R. O., dos Santos, N. N. Jr., Trabuco, A. C., Cintra, A. C., Figueiredo, L. T., Caliri, A., Sampaio, S. V., Aquino, V. H., 2014. Phospholipase A2 isolated from the venom of *Crotalus durissus terrificus* inactivates dengue virus and other enveloped viruses by disrupting the viral envelope. PlosOne 9, e112351- e112351.

Naumann, G. B., Silva, L. F., Silva, L. Faria, G., Richardson, M., Evangelista, K., 2011. Citotoxicity and inhibition of platelet aggregation caused by an L-amino acid oxidase from *Bothrops leucurus* venom. Biochimica et Biophysica Acta 1810, 683-694.

Nieswandt, B., Brakebusch, C., Bergmeier, W., Schulte, V., Bouvard, D., Mokhtari-Nejad, R., Lindhout, T., Heemskerk, J. W. M., Zirngibl, H., Fassler, R., 2001. Glycoprotein VI but not $\alpha_2\beta_1$ integrin is essential for platelet interaction with collagen. The EMBO Journal 20, 2120-2130.

Nieswandt, B., Watson, S. P., 2003. Platelet collagen interaction: is GPVI the central receptor? Blood 102, 449-461.

Nishida, S., Fujimura, Y., Miura, S., Ozaki, Y., Usami, Y., Suzuki, M., Titani, K., Yoshida, E., Sugimoto, M., Yoshioka, A., Fukui, H., Ozaki, Y., Usami, Y., 1994. Purification and characterization of bothrombin, a fibrinogen clotting serine protease from the venom of *Bothrops jararaca*. Biochemistry 33, 1843-1849.

Nunes, E. S., Souza, M. A., Vaz, A. F., Silva, T. G., Aguiar, J. S., Batista, A. M., Guerra, M. M., Guarnieri, M. C., Coelho, L. C. Correia, M. T., 2012. Cytotoxic effect and apoptosis induction by *Bothrops leucurus* venom lectin on tumor cell lines. Toxicon 59, 667-671.

Ozaki, Y., Suzuki-Inoue, K., Inoue, O., 2009. Novel interactions in platelet biology: CLEC-2/podoplanin and laminin/GPVI. Journal of Thrombosis and Haemostasis 7, 191-194.

Paine, M. J. I., Desmond, H. P., Theakston, R. D. G., Cramption, J. M., 1992. Purification, cloning, and molecular weight characterization of a high molecular weight hemorrhagic metalloproteinase, jararhagin, from *Bothrops jararaca* venom. The Journal of Biological Chemistry 267, 22869-22876.

Pal, S. K., Gomes, A., Dasgupta, S. C., Gomes, A. 2002. Snake venom as therapeutic agents: from toxin to drug development. Indian Journal of Experimental Biology 40, 1353-1358.

Passero, L. F. D., Tomokane, T. Y., Corbett, C. E. P., Laurenti, M. D., Toyama, M. H., 2007. Comparative studies of the antileishmanial activity of three *Crotalus durissus* ssp. venoms. Parasitology Research 101, 1365-1371.

Pluijm, V. D., Vloedgraven, H., Papapoulos, S., Lowick, C., Grzesik, K., Kerr, J., Robey, P. G., 1997. Attachment characteristics and involvement of integrins in adhesion of breast cancer cell lines to extracellular bone matrix components. Laboratory Investigation 77, 665-75.

Queiroz, M. R., Mamede, C. C. N., Fonseca, K. C., Morais, N. C., Sousa, B. B., Santos-Filho, N. A., Beletti, M. E., Arantes, E. C., Stanziola, L., Oliveira, F., 2014a. Rapid purification of a new pi class metalloproteinase from *Bothrops moojeni* venom with antiplatelet activity. BioMed Research International, 1-12.

Queiroz, M. R., Mamede, C. C. N., Morais, N. C. G., Fonseca, K. C., Sousa, B. B., Migliorini, T. M., Pereira, D. F. C., Stanziola, L., Calderon, L. A., Simões-Silva, R., Soares, A. M., Oliveira, F., 2014b. Purification and characterization of BmooAi: a new toxin from *Bothrops moojeni* snake venom that inhibits platelet aggregation. BioMed Research International, 1-7.

Queiroz, M. R., Sousa, B. B., Pereira, D. F. C., Mamede, C. C. N., Matias, M. S., Morais, N. C. G., Costa, J. O., Oliveira, F., 2017. The role of platelets in hemostasis and the effects of snake venom toxins on platelet function. Toxicon 133, 33-47.

Sajevic, T., Leonardi, A., Krizaj, I., 2011. Haemostatically active proteins in snake venoms. Toxicon 57, 627-645.

Samel, M., Vija, H., Ronnholm, G., Siigur, J., Kalkkinen, N., Siigur, E., 2006. Isolation and characterization of an apoptotic and platelet aggregation inhibiting l-amino acid oxidase from *Vipera berus berus* (common viper) venom. Biochimica et Biophysica Acta 1764, 707-714.

Sanchez, E. F., Richardson, M., Gremski, L. H., Veiga, S. S., Yarleque, A., Niland, S. Lima, A. M., Estevao-Costa, M. I., Eble, J. A., 2016. A novel fibrinolytic metalloproteinase, barnettlysin-I from *Bothrops barnetti* (Barnett´s pitviper) snake venom with anti-platelet properties. Biochimica et Biophysica Acta 3, 542-56.

Santos-Filho, N. A., Silveira, L. B., Oliveira, C. Z., Bernardes, C. P., Menaldo, D. L., Fuly, A. L., Arantes, E. C., Sampaio, S. V., Mamede, C. C., Beletti, M. E., de Oliveira, F., Soares, A. M., 2008. A new acidic myotoxic, anti-platelet and prostaglandin I2 inductor phospholipase A2 isolated from *Bothrops moojeni* snake venom. Toxicon 52, 908-917.

Sarray, S., Srairi, N., Luis, J., Marvaldi, J., Ayeb, M. E., Marrakchi, N., 2001. Lebecetin, a C-lectin protein from the venom of *Macrovipera lebetina* that inhibits platelet aggregation and adhesion of cancerous cells. Haemostasis 31, 177-182.

Sarray, S., Delamarre, E., Marvaldi, J., Ayeb, M. E., Marrakchi, N., Luis, J., 2007. Lebectin and lebecetin, two C-type lectins from snake venom, inhibit α5β1 and αv-containing integrins. Matrix Biology 26, 306-313.

Savage, B., Cattaneo, M., Ruggeri, Z. M., 2001. Mechanisms of platelet aggregation. Current Opinion in Hematology 8, 270–276.

Selistre-de-Araujo, H. S., Pontes, C. L. S., Montenegro, C. F., Martin, A. C. B. M., 2010. Snake venom disintegrins and cell migration. Toxins 2, 2606-2621.

Shibuya, M., Niitani, H., Aoyama, A., Kawachi, S., Nukariva, N., Baba, M., Iizuka, K., Sakai, S., Ohtsuka, M., 1990. Antimetastatic effect of defibrinogenation with batroxobin depends on the natural killer activity of host in mice. Journal of Cancer Research and Clinical Oncology 116, 168-172.

Silva, C. P., Costa, T. R., Paiva R. M. A., Cintra, A. C. O., Menaldo, D. L., Antunes, L. M. G., Sampaio, S. V., 2015. Antitumor potential of the myotoxin BthTX-I from *Bothrops jararacussu* snake venom: evaluation of cell cycle alterations and death mechanisms induced in tumor cell lines. Journal of Venomous Animals and Toxins including Tropical Diseases, 21-44.

Silveira, L. B., Marchi-Salvador, D. P., Santos-Filho, N. A., Silva Jr., F. P., Marcussi, S., Fuly, A. L., Nomizo, A., Silva, S. L., Stábeli, R. G., Arantes, E. C., Soares, A. M., 2013. Isolation and expression of a hypotensive and anti-platelet acidic phospholipase A2 from *Bothrops moojeni* snake venom. Journal of Pharmaceutical and Biomedical Analysis 73, 35-43.

Sobrinho, J. C., Simões-Silva, R., Holanda, R. J., Alfonso, J., Gomez, A. F., Zanchi, F. B., Moreira-Dill, L. S., Grabner, A. N., Zuliani, J. P., Calderon, L. A., Soares, A. M., 2016. Antitumoral Potential of Snake Venom Phospholipases A2 and Synthetic Peptides. Current Pharmaceutical Biotechnology 17, 1201-1212.

Suckau, D., Resemann, A., Schuerenberg, M., Hufnagel, P., Franzen, J., Holle, A., 2003. A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. Analytical and Bioanalytical Chemistry 376, 952-965.

Sulca, M. A., Remuzgo, C., Cárdenas, J., Kiyota, S., Cheng, E., Bemquerer, M. P., Machini, M. T., 2017. Venom of the Peruvian snake Bothriopsis oligolepis: Detection of antibacterial activity and involvement of proteolytic enzymes and C-type lectins in growth inhibition of Staphylococcus aureus. Toxicon 134, 30-40.

Sun, M. Z., Guo, C., Tian, Y., Chen, D., Greenaway, F. T., Liu, S., 2010. Biochemical, functional and structural characterization of Akbu-LAAO: a novel snake venom l-amino acid oxidase from *Agkistrodon blomhoffii ussurensis*. Biochimie 92, 343-349.

Torii, S., Naito, M., Tsuruo, T., 1997. Apoxin I, a novel apoptosis inducing factor with L-amino acid oxidase activity purified from western diamondback rattlesnake venom. The journal of Biological Chemistry 272, 9539-9542.

Trikha, M., Clerck, Y. A., Markland, F. S., 1994. Contortrostatin, a snake venom disintegrin, inhibits β1 integrin- mediated human metastatic melanoma cell adhesion and blocks experimental metastasis. Cancer Research 54, 4993-4998.

Vaiyapuri, S., Hutchinson, E. G., Ali, M. S., Dannoura, A., Stanley, R. G., Harrison, R. A., Bicknell, A. B., Gibbins J. M., 2012. Rhinocetin, a Venom-derived Integrin-specific Antagonist Inhibits Collagen-induced Platelet and Endothelial Cell Functions. The Journal of Biological Chemistry 287, 26235-26244.

Vyas, V. K., Brahmbhatt, K., Bhatt, H., Parmar, U., 2013. Therapeutic potential of snake venom in cancer therapy: current perspectives. Asian Pacific Journal Tropical of Biomedicine 3, 156-162.

Wang, W. J., Shih, C. H., Huang, T. F., 2005. Primary structure and antiplatelet mechanism of a snake venom metalloproteinase, acurhagin, from *Agkistrodon acutus* venom. Biochimie 87, 1065-1077.

Wang, W. J., 2007. Purification and functional characterization of AAV1, a novel P-III metalloproteinase, from formosan *Agkistrodon acutus* venom. Biochimie 89, 105-115.

Wen, Y. L., Wu, B. J., Kao, P. H., Fu, Y. S., Chang, L. S., 2013. Antibacterial and membrane-damaging activities of b-bungarotoxin B chain. Journal of Peptide Science 19, 1-8.

Wijeyewickrema, L. C., Berndt, M. C., Andrews, R. K., 2005. Snake venom probes of platelet adhesion receptors and their ligands. Toxicon 45, 1051-1061.

Wu, M., Ming, W., Tang, Y., Zhou, S., Kong, T., Dong, W., 2013. The anticancer effect of cytotoxin 1 from *Naja atra* Cantor venom is mediated by a lysosomal cell death pathway involving lysosomal membrane permeabilization and cathepsin B release. The American Journal of Chinese Medicine 41, 643-663.

Yamane, E. S., Bizerra, F. C., Oliveira, E. B., Moreira, J. T., Rajabi, M., Nunes, G. L. de Souza, A. O., da Silva, I. D., Yamane, T., Karpel, R. L., Silva, P. I., Havashi, M. A., 2013. Unraveling the antifungal activity of a South American rattlesnake toxin crotamine. Biochimie 95, 231-240.

Yang, R. S., Tang, C. H., Chuang, W. J., Huang, T. H., Peng,, H. C., Huang, T. F., Fu, W. M., 2005. Inhibition of tumor formation by snake venom disintegrin. Toxicon 45, 661-669.

Zhou, Q., Smith, J. B., Grossman, M. H.,1995. Molecular cloning and expression of catrocollastatin, a snake-venom protein from *Crotalus atrox* (western

diamondback rattlesnake) which inhibits platelet adhesion to collagen. Biochemical Journal 307, 411-417.

Zhou, Q., Dangelmaier, C., Smith, J. B., 1996. The hemorrhagin catrocollastatin inhibits collagen-induced platelet aggregation by binding to collagen via its disintegrin-like domain. Biochemical and Biophysical Research Communications 219, 720-726.

Zhu, L., Yuan, C., Chen, Z., Wang, W., Huang, M., 2010. Expression, purification and characterization of recombinant jerdonitin, a P-II class snake venom metalloproteinase comprising metalloproteinase and disintegrin domains. Toxicon 55, 375-380.

CAPÍTULO III

BmooPAF: a new platelet-activating factor from *Bothrops moojeni* snake venom

(Research article)

BmooPAF: a new platelet-activating factor from *Bothrops moojeni* snake venom

Bruna Barbosa de Sousa[1,4], Mayara Ribeiro de Queiroz[4], Tamires Lopes Silva[2], Carla Cristine N. Mamede[3,4], Déborah Fernanda C. Pereira[1], Mariana Santos Matias[1], Edigar Henrique Vaz Dias[1], Fábio de Oliveira[1,2,4*]

[1]Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia, 38400-902 Uberlândia, MG, Brazil

[2]Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia, 38400-902 Uberlândia, MG, Brazil

[3]Instituto de Ciências Agrárias, Universidade Federal de Uberlândia, 38400-902 Uberlândia, MG, Brazil

[4]Instituto Nacional de Ciência e Tecnologia em Nano-Biofarmacêutica (N-Biofar), 31270-901 Belo Horizonte-MG, Brazil.

**Corresponding author:* Av. Pará, 1720, Bairro Umuarama; CEP 38400-902 - Uberlândia, MG - Brazil. E-mail address: fabio@ufu.br

Abstract

A number of snake venom proteins that interfere on platelet aggregation have been isolated. However, there are no reports in the literature of small-mass peptides interfering in aggregation. In the present study, we identified and characterized, for the first time, a peptide from *Bothrops moojeni* snake venom, which was named BmooPAF, that potentially induces platelet aggregation without affecting *in vitro* cell viability. Monoclonal antibodies (anti-integrin α2b and anti-GP1BA) showed a significant inhibitory effect on BmooPAF-induced platelet aggregation. On the other hand, anti-GPVI antibody showed no effect on platelet function. These findings, associated with molecular docking, indicate that BmooPAF induces platelet aggregation via binding to the GPIbα platelet receptor leading to $\alpha_{IIb}\beta_3$ integrin activation. BmooPAF could be of interest as a new tool for basic research on platelet plug formation and diagnostic use for some platelet disorders.

Keywords: *Bothrops moojeni*; Snake venom; Platelet-activating factor

1 Introduction

Snake venoms contain a complex mixture of non-enzymatic and enzymatic compounds that selectively modulate platelet adhesion, activation and aggregation through a specific effect upon the adhesion receptors or their ligands (Andrews and Berndt, 2000; Hamako et al., 1996; Hooley et al., 2008; Horii, Brooks, Herr, 2009; Kanaji et al., 2003; Lambert et al., 2008; Lu et al., 2004; Lu et al., 2005; Marcinkiewicz et al., 2000; Polgar et al., 1997; Serrano et al., 2006; Tanjoni et al., 2010; Usami et al., 1993; Wang, Shih, Huang, 2005). The components that interfere with platelet function are divided into two main categories: those that inhibit platelet aggregation (Della-Casa et al., 2011; Jakubowski et al., 2013; Naumann et al., 2011; Queiroz et al., 2014a,b; Silveira et al., 2013) and those that activate it (Andrews et al., 2001; Fully et al., 2004; Rodrigues et al., 2009; Vilca-Quispe et al., 2010). Both components attract researchers: the first by medical interest, as a tool for the development of novel therapeutic agents to prevent and treat thrombotic disorders and the latter as a tool for the diagnosis of some disorders including von Willebrand and Bernard-Soulier diseases (Braud, Bon, Wisner, 2000; Hutton and Warrell, 1993; Sajevic, Leonardi, Krizaj, 2011).

The mechanism of action of snake venom toxins that induce platelet aggregation has not been well elucidated. Some snake venom serine proteases (SVSPs) activate platelet aggregation due to the hydrolysis of protease-activated receptors expressed in human platelets, similar to thrombin (Santos et al., 2000). Studies have also shown that other SVSPs, as well as some C-type lectins and some snake venom metalloproteases (SVMPs) may activate platelet aggregation by interacting with specific platelet receptors (Andrews et al 2001; Chen et al., 2011; Chung, Peng, Huang, 2001; Clemetson, Lu, Clemetson, 2005; Clemetson, 2010; Du et al., 2001; Hirotsu et al., 2001; Lu et al., 2004). The L-amino acid oxidases from snake venoms (SV-LAAOs) may act as platelet aggregation inducers by a currently unknown mechanism. Some studies suggest that the H_2O_2 produced by the enzymatic action of this toxin may be responsible for the platelet aggregation activation, since the released H_2O_2 leads to the synthesis of thromboxane A_2 (TXA_2), a platelet aggregation inducer (Abdelkafi-Koubaa et al., 2014; Izidoro et al., 2006; Li, Yu, Lian, 1994; Rodrigues et al., 2009; Stábeli et al.

2007; Wei et al., 2003). The phospholipases A_2 (PLA_2s) can activate platelet aggregation by cleaving the platelet membrane phospholipids, thereby releasing arachidonic acid and its metabolites, such as TXA_2 (Markland,1998; Mounier et al., 1994).

To date, there are no published reports about small-mass peptides from snake venom able to activate platelet aggregation. In this study we identified and characterized, for the first time, a peptide from *Bothrops moojeni* venom that induces platelet aggregation.

2 Material and Methods

2.1 Material

B. moojeni venom was purchased from Bioagents Serpentarium (Batatais, SP, Brazil). Acrylamide, ammonium bicarbonate, ammonium persulphate, bromophenol blue, ethylenediaminetetracetic acid (EDTA), glycine, *β*-mercaptoethanol, *N*,*N'*-methylene-*bis*-acrylamide, sodium dodecyl sulphate (SDS), *N*,*N*,*N'*,*N'*-tetramethylethylenediamine (TEMED), Tris, monoclonal anti-GP1BA, anti-GPVI (ab1), monoclonal anti-integrin α2b (CD41) and Roswell Park Memorial Institute-1640 (RPMI) medium were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chromatographic media (DEAE-Sephacel and Sephadex G-75) were purchased from GE Healthcare Technologies (Uppsala, Sweden). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) and dimethyl sulfoxide (DMSO) were purchased from Amresco. Collagen from equine tendon used in the platelet aggregation assays was purchased from Helena Laboratories (Beaumont, Texas, USA). HeLa (human cervix epitheloid carcinoma), PC-3 (human prostate adenocarcinoma) and VERO (african green monkey kidney) cells line were kindly provided by Laboratory of Nanobiotechnology of the Federal University of Uberlândia. All other reagents used were of analytical grade.

2.2 Human blood

The experiments were performed in accordance with current guidelines for human research established by the Committee for Ethics in Human Research of the Federal University of Uberlândia (CEP/UFU), Minas Gerais, Brazil (Protocol

number 1.627.982/2016). Blood was obtained by blood donation from 10 individuals who were invited to participate in the research as volunteer donors. The criteria for selection of donor volunteers included: absence of signs or symptoms of disease, malnutrition or dehydration, aged between 18 - 65 years, weight more than 50 kg, no use of any medication that interferes with hemostasis, no use of illicit drugs, no alcohol consumption in the last 24 h preceding the experiment and no hemostasis disorders.

2.3 Fractionation of *B. moojeni* crude venom

Crude venom from the *B. moojeni* snake was fractionated using the methodology previously described by Bernardes et al. (2008) with modifications. Crude venom (400 mg) was dissolved in 50 mmol/L AMBIC (ammonium bicarbonate buffer) (pH 7.8) and clarified by centrifugation at 10,000 × *g* for 10 min. The supernatant solution was fractionated on a DEAE-Sephacel column (2.5 x 20 cm) with an increasing concentration gradient (50 mmol/L - 1.0 mol/L) of AMBIC, and on a Sephadex G-75 column (1.0 x 100 cm). This last step of fractionation resulted in a fraction capable of inducing the platelet aggregation, which was named DG. Chromatographs were carried out at a flow rate of 20 mL/h and fractions with 3.0 mL/tube were collected. All peaks were monitored by measuring absorbance at 280 nm on a BioSpec-Mini spectrophotometer (Shimadzu Biotech, Japan).

2.4 Protein analysis

The dosages of protein solutions were determined by the method previously described by Bradford (1976), using bovine serum albumin as standard. The concentration of peptide solutions was performed using a UV absorption method that calculates concentration from absorbance at 214 nm, using a BioSpec-mini spectrophotometer (Shimadzu Biotech, Japan).

2.5 Platelet aggregation assay

Platelet aggregation assays were performed in human platelet-rich plasma (PRP) and measured using an automated four-channel Aggregometer (AggRAM™ version 1.1, Helena Laboratories, USA) as described by Queiroz et al. (2014a)

with modifications. Aggregation was triggered with collagen (10 µg/mL) or different doses of DG fraction (10, 30 and 50 µg). The effect of DG-induced platelet aggregation was also tested after heating the toxin (50 µg) for 15 min at 100ºC prior adding to PRP. In some assays, antibodies (monoclonal anti-GP1BA, anti-GPVI (ab1) and monoclonal anti-integrin α2b (CD41)) at a final concentration of 25 µg/mL were pre-incubated with PRP for 5 min at 37°C prior to the addition of the DG fraction (50 µg). One hundred percent aggregation was expressed as the percentage absorbance relative to platelet-poor plasma (PPP) aggregation. All experiments were carried out in triplicate.

2.6 Cell culture

HeLa, PC-3 and VERO cell lines were maintained as a monolayer culture in RPMI 1640 medium supplemented with 10% bovine fetal serum (BFS), 25 mM HEPES, 100 U/mL penicillin, 100 µg streptomycin, 2 mM L-glutamine and 3 mM sodium bicarbonate and maintained in a humidified incubator containing 5% CO_2 at 37°C.

2.7 Cell viability assay

Cell viability assays were evaluated by the colorimetric MTT assay, according to the manufacturer's recommendations, with slight modifications. HeLa, PC-3 and VERO cells (2 x 10^4 cells/well) were seeded into a 96-well plate for adhesion. After 24 h of incubation (37ºC) the cells were treated with *B. moojeni* crude venom (100 µg/mL) (positive control) or different concentrations of DG fraction (100, 50, 25 and 12.5 µg/mL) diluted with RPMI medium. The untreated cells served as negative control. After 24 h MTT solution was added to each well at a final concentration of 2 mg/mL and the plates were incubated at 37°C for 1 h. The crystal formazan was dissolved by addition of 100 µL of DMSO to each well. The absorbance was detected at 560 nm in a micro-plate reader (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, USA). All experiments were carried out in triplicate. Cell survival rate was calculated as (absorbance of the treated wells)/(absorbance of the control wells) × 100%.

2.8 Statistical Analysis

Statistical significant values were compared to controls by two-way ANOVA followed by Bonferroni's test using the software Prism 5.0 (GraphPad Software Inc., USA). Data are presented a mean ± standard error (n ¼ 3). Differences with p values of less than 5% were considered significant ($p < 0.05$).

2.9 Mass spectrometry analysis

DG fraction was subjected to peptide mass fingerprinting on a MALDI-TOF/TOF mass spectrometer (ultrafleXtreme, Bruker Daltonics, Germany), in positive and reflector mode, after external calibration using a standard mixture of peptides. All detected ions were subjected to MS/MS fragmentation. MS/MS fragmentation was carried out using the LIFT mode (Suckau et al., 2003). Mass spectra were analyzed using flexAnalysis and Biotools software (Bruker Daltonics). Protein identification was performed using Mascot software (Perkins et al., 1999). The searches were performed against the NCBI database (Johnson et al., 2008) with 100 ppm mass tolerance and fragmentation mass tolerance of 0.5 Da. The search parameters were restricted to Chordata and allowed one missed cleavage, S-pyridylethylation of cysteines as fixed modification and oxidation of methionine as variable modification.

2.10 Bioinformatic analysis

2.10.1 Peptides structure prediction

The first step to evaluate the possible peptide involved in platelet activation and GPIbα interaction was to predict the peptides structure using PEP-FOLD3 (http://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::PEP-FOLD3). In sequence, the given structure was checked in the MolProbity platform (http://molprobity.biochem.duke.edu/) through the construction of Ramachandran plot. The PeptideRanker was used to evaluate the possible probability of biological activity of the peptide.

2.10.2 GPIbα structure

The sequence of GPIbα was found in SWISS-PROT (accession number P07359.2) and the glycoprotein structure was modelled using SWISS-MODEL (https://swissmodel.expasy.org/). The 1P9A crystal was used as reference and the GPIbα structure was validated trough PDBsum (http://www.ebi.ac.uk/thornton-srv/databases/cgi-in/pdbsum/GetPage.pl?pdbcode=index.html).

2.10.3 Molecular docking

A rigid molecular docking between each peptide and the glycoprotein was performed in Cluspro One platform (https://cluspro.bu.edu/login.php). This molecular docking approach utilizes FFT (Fast Fourier Transform) correlation approach for execute the rigid body docking using the clustering of the generated structures for identification of the largest cluster that possibly can represent the model of the complex based on the RMSD (Root Mean Square Deviation) and the refinement of selected structures. The only structure refining was done through minimizing the Charm energy in the program.

Following the molecular docking, the viability of the GPIbα-peptide complexes were checked. Firstly, we checked the complex structures by PDBsum that generates a Ramachandran plot. Secondly, the interface area average (ΔASA) which indicates the changes of surface area upon protein complex formation, and the normalized B factor average (avgΣB) which is measured to quantify the vibrational motion of an atom, were calculated by PDBePISA (http://www.ebi.ac.uk/pdbe/pisa/) and ResQ (http://zhanglab.ccmb.med.umich.edu/ResQ/), respectively as described by Liu, Li, Li (2014) to distinguish true protein interactions from crystal packing contacts. Finally, the interacting residues between the peptide and GPIbα were evaluated in LIGPLOT.

3 Results and Discussion

Snake venoms are known to contain a number of molecules that interfere on hemostasis. Many of them, such as SVMPs, SVSPs, PLA_2, SV-LAAOs, C-type lectins and disintegrins may inhibit or activate platelet aggregation (Queiroz et al.,

2017 for review). In the present work, we describe the identification and characterization of a platelet-activating peptide from *B. moojeni* snake venom.

B. moojeni crude venom was fractionated using two-step procedure including ion-exchange chromatography on a DEAE-Sephacel column and molecular exclusion chromatography on a Sephadex G-75 column (results not shown). The fraction (DG) obtained by the molecular exclusion chromatography was subjected on platelet aggregation assays. Surprisingly, our results showed that the DG fraction was able to activate the platelet aggregation in a dose-dependent manner (Fig. 1A). This fraction (50 µg) activates the platelet aggregation in human PRP similarly to collagen-induced platelet aggregation (10 µg/mL) (approximately 100% of platelet aggregation), one of the most potent platelet agonist (Manon-Jensen, Kjeld, Karsdal, 2016). Lower doses (10 and 30 µg of DG fraction) activated approximately 28 and 50%, respectively, of platelet aggregation. In addition, the three-dimensional structure of the peptide appears to be important for the platelet aggregation activation since when preheated (for 15 min at 100°C) its effect on platelets was completely lost (Fig. 1B).

In order to identify the platelet membrane receptors involved in DG-induced platelet aggregation, the following antibodies were used: monoclonal anti-GP1BA (specific for GPIbα), anti-GPVI (ab1) (specific for GPVI) and monoclonal anti-integrin α2b (CD41) (specific for α_{IIb}). GPIbα represents the major functional subunit of GPIb-V-IX complex and is a specific receptor for von Willebrand factor (vWF) whereas GPVI is the major signaling receptor for collagen on platelets (Matsui and Hamako, 2005; Nieswandt and Watson, 2003). α_{IIb} integrin undergoes proteolytic cleavage releasing two disulfide linked chains, 114 kDa (heavy) and 22 kDa (light), which associate with β_3 to form the major fibrinogen receptor in activated platelets ($\alpha_{IIb}\beta_3$ integrin, also known as GPIIb/IIIa) (Bennett, 2005). All of these receptors are critical for platelet aggregation and defects in any them lead to coagulation disorders (Harrison, 2005).

Our results strongly suggest that platelet aggregation induced by the DG fraction is directly associated with vWF and fibrinogen receptors. This fraction loses its effect on platelets when these receptors were neutralized by their respective antibodies (monoclonal anti-GP1BA and monoclonal anti-integrin α2b). On the other hand, collagen receptors are not involved in the DG-induced platelet

aggregation since the anti-GPVI antibody did not interfere with aggregation (Fig. 2C). The vWF-GPIbα complex plays an essential role in mediating the hemostatic response of platelets to injured blood vessels, functioning as the primary binding between platelets and the subendothelial matrix under high shear forces (*e.g.* stenosed arteries) (Berndt, Metharom, Andrews, 2014; Clemetson, 2012; Gardiner and Andrews, 2014; Kulkarni et al., 2000). This binding is insufficient for stable adhesion because of its low affinity for interaction and rapid dissociation rate. However, it is important to recruit platelets at the injury site and to enable the interaction of other receptors for the thrombogenic surface, as the binding of GPVI to collagen, resulting in an irreversible adhesion (Nieswandt et al., 2001; Nieswandt and Watson, 2003). These interactions trigger intracellular signals that promote platelet activation and release of different agonists (*e.g.* adenosine diphosphate (ADP) and TXA_2), which in turn mediate the recruitment of additional platelets and promote the activation of the $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) receptor. This receptor binds platelet-platelet through fibrinogen or vWF and, finally, forms the platelet plug (Jurk and Kehrel, 2005; Kamiguti, 2005; Offermanns, 2006; Varga-Szabo, Pleines, Nieswandt, 2008). As DG-induced platelet aggregation was inhibited by anti-GP1BA and anti-integrin α2b monoclonal antibodies, we can suggest that DG fraction have peptide(s) that acts as platelet aggregation inducers because of its interaction with the GPIbα platelet receptor, which, in turn, culminates in the $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) integrin activation.

We performed some assays to test the DG fraction ability to reduce the viability of some cells. For this, the viability of HeLa, PC-3 and VERO cell lines were evaluated after treatment with DG fraction (100, 50, 25 and 12.5 µg/mL) by MTT assays. Our results showed that even at high concentration (100 µg/mL) the DG fraction did not significantly reduce the viability of the tested cell lines 24 h after treatment (Fig. 2).

In order to identify which peptide(s) is(are) responsible for the platelet aggregation activation, DG fraction was analyzed by MALDI-TOF mass spectrometry. MALDI-TOF mass spectrometry analysis showed that DG fraction is composed of peptides with molecular mass range from 650 to 2,000 Da (results not shown). In addition, the major peptides were fragmented (MS/MS) and their amino acid sequences were determined. All sequenced peptides were submitted

to a molecular docking study to verify the possible interaction with GPIbα. Among them, the molecular docking demonstrated that only one peptide interacts with GPIbα, which was named BmooPAF (platelet-activating factor from *Bothrops moojeni* snake venom). Furthermore, according to Mooney et al. (2012), the Peptide Ranker analysis demonstrated that BmooPAF is the main peptide to possess a favorable score for developing biological activity (score of 0.97 of 1).

In our study, we used the Cluspro One platform for molecular docking studies (BmooPAF/GPIbα interaction). This platform is widely used for evaluate protein-protein, protein-DNA and protein-peptide interactions (Jiang et al., 2013; Rakhmetov et al., 2015). As result, the molecular docking between all sequenced peptides and GPIbα resulted in the formation of 7 or 10 clusters between each peptide and the receptor. To select the best interaction model, firstly, a Ramachandran plot was done using PDBsum. The complex formed by BmooPAF and GPIbα presented the best G-score (average = -0.34) (Fig. 3A and B). Then, these interactions were confirmed by measurement of ΔASA and avgΣB. We obtained an ΔASA of 11830.31 $Å^2$ (Fig. 3D) and the avgΣB was bellow -10 (Fig. 3C). The Ramachandran plot of BmooPAF-GPIbα interaction was compatible with a viable structure according to the parameters established by De Beer et al. (2014). Furthermore, the literature shows that a threshold of ΔASA at 856 $Å^2$ is able to distinguish crystal packing contacts from homodimers with high accuracy (Ponstingl, Henrick, Thornton, 2000) and a cutoff of ΔASA at 650 $Å^2$ has been shown to provide low error rates to identify true protein-ligand interactions. Even more, avgΣB bellow -10 is a reasonable predictor of true complex interactions (Liu, Li, Li, 2014). Therefore, these results suggest that BmooPAF is the possible GPIbα linker and this interaction is not an artifact.

The LIGPLOT analysis demonstrated that the ASP79, ASP122, TYR146, LYS148, ASP191 and GLN248 amino acid residues on GPIbα are major target of BmooPAF (results not shown). Another important point is that our analysis demonstrated mainly non-bonded contacts and hydrogen bonds. This is in accordance with previous studies which show that the interaction between GPIbα and vWF is formed mainly by non-bonded contacts and hydrogen bonds (Dumas et al., 2004; Huizinga et al., 2002; Uff et al., 2002). The GPIbα residues that were found to interact with BmooPAF are part of the binding domain between GPIbα

and vWF (Dumas et al., 2004; Uff et al., 2002). This domain is important since it has been shown that the mutation in this interaction site changes the affinity of GPIbα-vWF complex leading to platelet-type and type 2B von Willebrand diseases (Dumas et al., 2004; Huizinga et al., 2002). In addition, the interaction of the OS1 inhibitor, a peptide isolated from a cysteine-constrained phage display library, to this domain has been shown to be a target for inhibit platelet aggregation and thrombus formation (McEwan, Andrews, Emsley, 2009).

A number of snake venom proteins that activate platelet aggregation specifically modulating the vWF-GPIbα interaction have already been isolated. Many of these proteins belong to one of two major protein families, the C-type lectins and serine proteinases families (Andrews and Berndt, 2000; Matsui and Hamako, 2005). An example is the serine proteinase bothrombin from *B. jararaca* venom, which activates platelet aggregation by binding to the GPIbα receptor. Similar to BmooPAF, bothrombin-induced platelet activity was completely inhibited by anti-GPIbα monoclonal antibody (Nishida et al., 1994; Serrano and Maroun, 2005). Other examples are the C-type lectins aggretin, mucetin, bilinexin and alboluxin that target specific GPIbα sites leading to platelet aggregation activation (Chung, Peng, Huang, 2001; Du et al., 2001; Du et al., 2002; Lu et al., 2004).

These snake venom proteins are suitable probes for basic research on platelet plug formation mediated by GPIb-V-IX receptor and for subsidiary diagnostic use for some platelet disorders (Matsui and Hamako, 2005). The C-type lectin alboaggregin-B from *T. albolabris* venom, that also binds to GPIbα platelet receptor, has been used to quantitate vWF platelet receptors and to detect vWF-GPIbα-dependent disorders (Kowalska et al., 1998; Peng, Lu, Kirby, 1991; Yoshida et al., 1993, 1995).

Interestingly, in this work we identified a peptide, BmooPAF, that potentially induces platelet aggregation. Among the snake venom components, peptides, in particular, are being extensively studied after the discovery of their diverse pharmacological properties and simpler structures. They represent a huge and undiscovered source of new therapeutic leads (Chan et al., 2016; Kaas and Craik, 2015).

In summary, BmooPAF-induced platelet aggregation was inhibited in the presence of anti-GP1BA and anti-integrin α2b (CD41) monoclonal antibodies.

These results, associated with molecular docking, indicate that BmooPAF may activate platelets by interacting with GPIbα leading to $\alpha_{IIb}\beta_3$ integrin activation. BmooPAF provides a potentially useful tool for studying the mechanism of plug formation and for use as diagnostic tools of some platelet disorders. Further investigations about this peptide are necessary to detail its platelet activation mechanisms and molecular characteristics as well as to clarify some of the unresolved questions concerning the role of the GPIb-V-IX complex in platelet adhesion and activation.

Conflict of interest

The authors declare that there is no conflict of interests.

Acknowledgements

The authors gratefully acknowledge the financial support of Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Figures

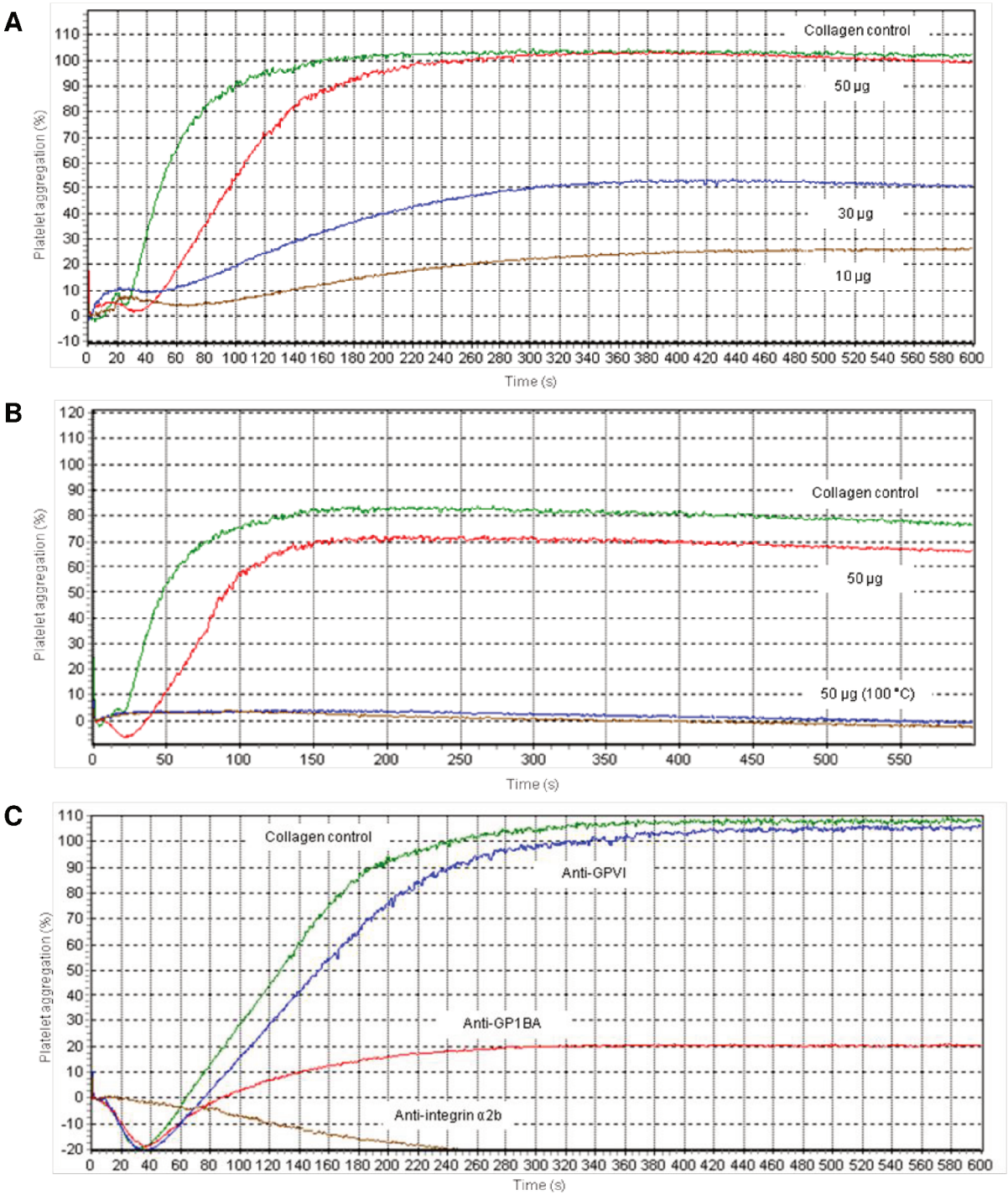

Fig. 1: Platelet aggregation assays. (A) Effect of DG fraction on platelet aggregation: aggregation was triggered with collagen (10 µg/mL) or different doses of DG fraction (10, 30 and 50 µg) on PRP. (B) Effect of preheated DG fraction on platelet aggregation: aggregation was triggered with collagen (10 µg/mL) or

preheated DG fraction (50 µg) to 100ºC for 15 min before addition to PRP. (C) Antibodies inhibition assays: monoclonal anti-GP1BA, anti-GPVI (ab1) and monoclonal anti-integrin α2b (CD41) antibodies (25 µg/mL) were pre-incubated with PRP for 5 min at 37°C prior to the platelet aggregation activation with DG fraction (50 µg). Control experiment was performed using only DG fraction (50 µg). Platelet aggregation was recorded for 10 min in an automated four-channel Aggregometer (AggRAM^{TM} version 1.1) (Helena Laboratories, USA). Results were expressed as an increase in light transmission, where PPP represents the maximum response (100%).

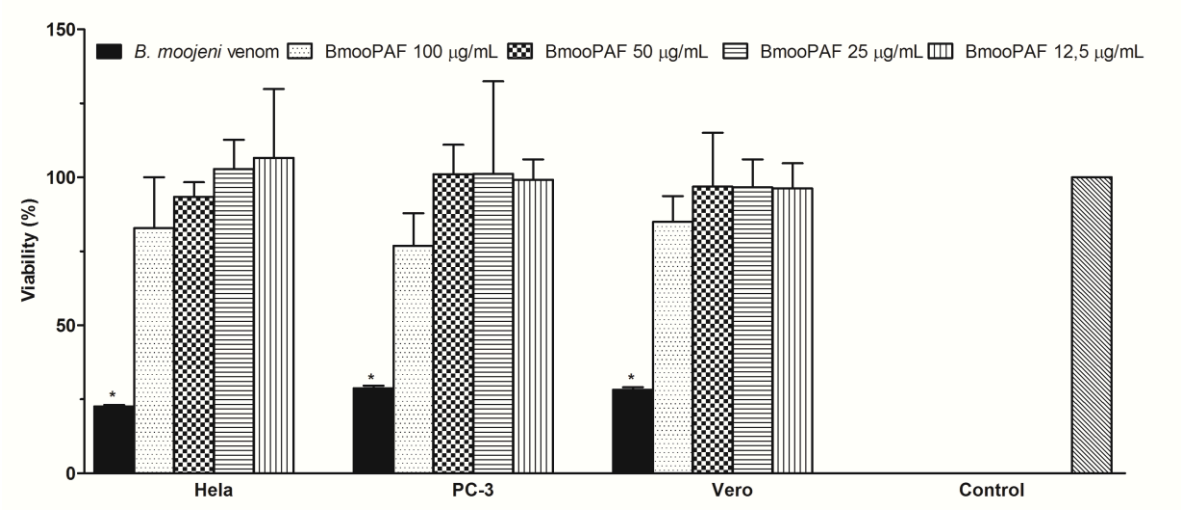

Fig. 2: Viability analysis of HeLa, PC-3 and VERO cell lines treated with DG fraction by MTT assay. Cell lines were treated with different concentrations (100, 50, 25 and 12.5 µg/mL) of DG for 24 h. The results are expressed as the percentage of growth of the treated cells compared to the untreated control cells. These results are representative of at least 3 independent experiments. Data show the mean ± standard deviation (S. D.). *Statistically significant difference ($p<0.05$) compared to control.

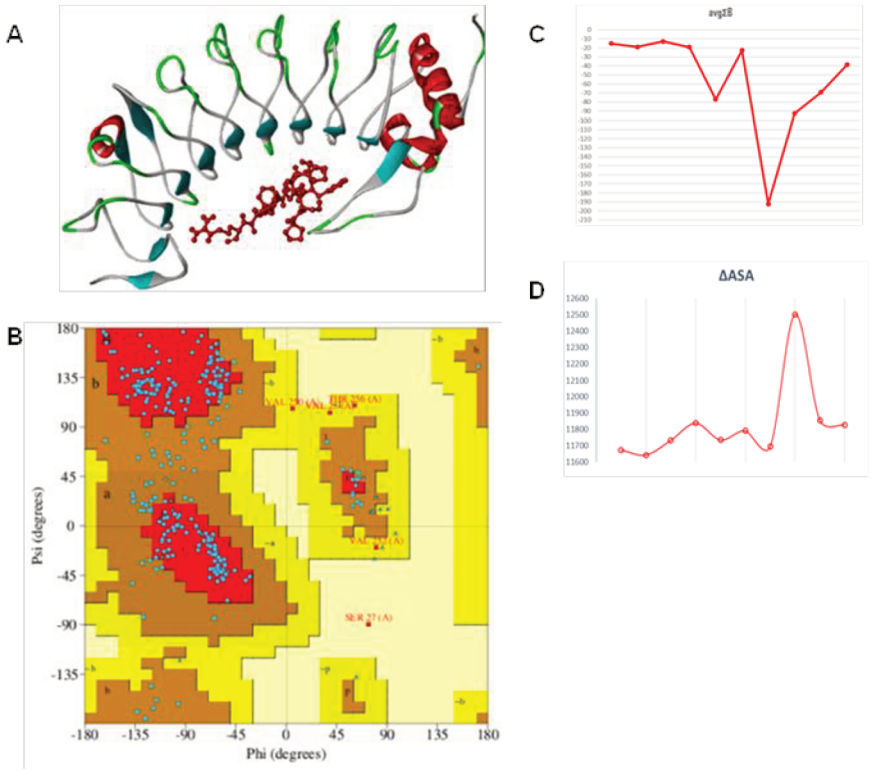

Fig. 3: Bioinformatic analysis. (A) Molecular docking between BmooPAF (in red) and GPIbα. (B) Ramachandran plot that is represented by different colouring/shading regions according to Morris et al. (1992) where the darkest areas (in red) correspond to the most favorable combinations of phi-psi values. (C) avgΣB through different points during interaction between BmooPAF and GPIbα. (D) ΔASA through different points during interaction between BmooPAF and GPIbα.

References

Abdelkafi-Koubaa, Z., Jebali, J., Othman, H., Morjen, M., Aissa, I., Zouari-Kesentini, R., Bazaa, A., Ellefi, A.A., Majdoub, H., Srairi-Abid, N., Gargouri, Y., El Ayeb, M., Marrakchi, N., 2014. A thermoactive L-amino acid oxidase from *Cerastes cerastes* snake venom: Purification, biochemical and molecular characterization. Toxicon 89, 32-44.

Andrews, R. K., Berndt, M.C., 2000. Snake venom modulators of platelet adhesion receptors and their ligands. Toxicon 38, 775-791.

Andrews, R. K., Gardiner, E. E., Asazuma, N., Berlanga, O., Tulasne, D., Nieswandt, B., Smith, A. I., Berndt, M. C., Watson, S. P., 2001. A novel viper venom metalloproteinase, alborhagin, is an agonist at the platelet collagen receptor GPVI. The Journal of Biological Chemistry 276, 28092-28097.

Bennett, J. S., 2005. Structure and function of the platelet integrin $\alpha_{IIb}\beta_3$. The Journal of Clinical Investigation 115, 3363-3369.

Bernardes, C. P., Santos-Filho, N. A., Costa, T. R., Gomes, M. S., Torres, F. S., Costa, J., Borges, M. H., Richardson, M., Santos, D. M., Castro Pimenta, A. M., Homsi-Brandeburgo, M. I., Soares, A. M., Oliveira, F., 2008. Isolation and structural characterization of a new fibrin(ogen)olytic metalloproteinase from *Bothrops moojeni* snake venom. Toxicon 51, 574-584.

Berndt, M. C., Metharom, P., Andrews, R. K., 2014. Primary haemostasis: newer insights. Haemophilia 20, 15-22.

Bradford, M. M., 1976. Rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-254.

Braud, S., Bon, C., Wisner, A., 2000. Snake venom proteins acting on hemostasis. Biochimie 82, 851-859.

Chan, Y. S., Cheung, R. C., Xia, L., Wong, J. H., Ng, T. B., Chan, W. Y., 2016. Snake venom toxins: toxicity and medicinal applications. Applied Microbiology and Biotechnology, 1-17.

Chen, Z. M., Wu, J. B., Zhang, Y., Yu, G. Y., Lee, W. H., Lu, Q. M., Zhang, Y., 2011. Jerdonuxin, a novel snaclec (snake C-type lectin) with platelet aggregation activity from *Trimeresurus jerdonii* venom. Toxicon 57, 109-116.

Chung, C. H., Peng, H. C., Huang, T. F., 2001. Aggretin, a C-type lectin protein, induces platelet aggregation via integrin α2β1 and GPIb in a phosphatidylinositol 3-kinase independent pathway. Biochemical and Biophysical Research Communications 285, 689-695.

Clemetson, K. J. 2010. Snaclecs (snake C-type lectins) that inhibit or activate platelets by binding to receptors. Toxicon 56, 1236-1246.

Clemetson, K. J., 2012. Plateles and primary haemostasis. Thrombosis Research 129, 220-224.

Clemetson, K. J., Lu, Q., Clemetson, J. M., 2005. Snake C-type lectin-like proteins and platelet receptors. Pathophysiology of Haemostasis and Thrombosis 34, 150-155.

De Beer, T. A., Berka, K., Thornton, J. M., Laskowski, R. A., 2014. PDBsum additions. Nucleic acids research 42, D292-D296.

Della-Casa, M. S., Junqueira-de-Azevedo, I., Butera, D., Clissa, P. B., Lopes, D. S., Serrano, S. M. T., Pimenta, D. C., Magalhães, G. S., Ho, P. L., Moura-da-Silva, A. M., 2011. Insularin, a disintegrin from *Bothrops insularis* venom: Inhibition of platelet aggregation and endothelial cell adhesion by the native and recombinant GST-insularin proteins. Toxicon 57, 125-133.

Du, X. Y., Navdaev, A., Clemetson, J. M., Magnenat, E., Wells, T. N., Clemetson, K. J., 2001. Bilinexin, a snake C-type lectin from *Agkistrodon bilineatus* venom agglutinates platelets via GPIb and alpha2beta1. Thrombosis and Haemostasis 86, 1277-1283.

Du, X. Y., Magnenat, E., Wells, T. N., Clemetson, K. J., 2002. Alboluxin, a snake C-type lectin from *Trimeresurus albolabris* venom is a potent platelet agonist acting via GPIb and GPVI. Thrombosis and Haemostasis 87, 692-698.

Dumas, J. J., Kumar, R., McDonagh, T., Sullivan, F., Stahl, M. L., Somers, W. S., Mosyak, L., 2004. Crystal Structure of the Wild-type von Willebrand Factor A1-Glycoprotein Ib Complex Reveals Conformation Differences with a Complex Bearing von Willebrand Disease Mutations. The Journal Of Biological Chemistry 279, 23327-23334.

Fuly, A. L., Soares, A. M., Marcussi, S., Giglio, J. R., Guimarães, J. A., 2004. Signal transduction pathways involved in the platelet aggregation induced by a D-49 phospholipase A_2 isolated from *Bothrops jararacussu* snake venom. Biochimie 86, 731-739.

Gardiner, E. E., Andrews, R. K., 2014. Structure and function of platelet receptors initiating blood clotting. Advances in Experimental Medicine and Biology 844 263-275.

Hamako, J., Matsui, T., Suzuki, M., Ito, M., Makita, K., Fujimura, Y., Ozeki, Y, Titani, K., 1996. Purification and characterization of bitiscetin, a novel von Willebrand factor modulator protein from *Bitis arietans* snake venom. Biochemical and Biophysical Research Communications 226, 273-279.

Harrison, P., 2005. Platelet function analysis. Blood reviews 19, 111-123.

Hirotsu, S., Mizuno, H., Fukuda, K., Qi, M. C., Matsui, T., Hamako, J., Morita, T., Titani, K., 2001. Crystal structure of bitiscetin, a von Willebrand factor dependent platelet aggregation inducer. Biochemistry 40, 13592-13597.

Hooley, E., Papagrigariou, E., Navdaev, A., Pandey, A. V., Clemetson, J. M., Clemetson K. J., Emsley, J., 2008. The crystal structure of the platelet activator aggretin reveals a novel (ab)2 dimeric structure. Biochemistry 47, 7831-7837.

Horii, K., Brooks, M. T., Herr, A. B., 2009. Convulxin forms a dimer in solution and can bind eight copies of glycoprotein VI: implications for platelet activation. Biochemistry 48, 2907-2914.

Huizinga, E. G., Tsuji, S., Romijn, R. A., Schiphorst, M. E., Groot, P. G., Sixma, J. J., Gros, P., 2002. Structures of glycoprotein Ibα and its complex with von Willebrand factor A1 domain. Science 297, 1176-1179.

Hutton, R. A., Warrell, D. A., 1993. Action of snake venom components on the haemostatic system. Blood reviews 7, 176-189.

Izidoro, L. F. M., Ribeiro, M. C., Souza, G. R., Sant'Ana, C. D., Hamaguchi, A., Homsi-Brandeburgo, M. I., Goulart, L. R., Beleboni, R. O., Nomizo, A., Sampaio, S. V., Soares, A. M., Rodrigues, V. M., 2006. Biochemical and functional characterization of an l-amino acid oxidase isolated from *Bothrops pirajai* snake venom. Bioorganic & Medicinal Chemistry 14, 7034-7043.

Jakubowski, P., Calvete, J. J., Eble, J. A., Lazarovici, P., Marcinkiewicz, C., 2013. Identification of inhibitors of $\alpha_2\beta_1$ integrin, members of C-lectin type proteins in *Echissochureki* venom. Toxicology and Applied Pharmacology 269, 34-42.

Jiang, S., Lu, L., Du, L., Debnath, A. K., 2013. Putative conformations of the receptor-binding domain in S protein of hCoV-EMC in complex with its receptor dipeptidyl peptidase-4. The Journal of Infection, 67, 156.

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., Madden, T. L., 2008. NCBI BLAST: a better web interface. Nucleic Acids Researchs 36, W5-W9.

Jurk, K., Kehrel, B. E., 2005. Platelets: Physiology and Biochemistry. *S*eminars in Thrombosis and Hemostasis 31, 381-392.

Kaas, Q., Craik, D. J., 2015. Bioinformatics-aided venomics. Toxins 7, 2159-2187.

Kamiguti, A. S., 2005. Platelets as targets of snake venom metalloproteinases. Toxicon 45, 1041-1049.

Kanaji, S., Kanaji, T., Furihata K., Kato, K., Ware, J. L., Kunicki, T. J., 2003. Convulxin binds to native, human glycoprotein Iba. The Journal of Biological Chemistry 278, 39452-39460.

Kowalska, M. A., Tan, L., Holt, J. C., Peng, M., Karczewski, J., Calvete, J. J., Niewiarowski, S., 1998. Alboaggregins A and B. Structure and interaction with human platelets. Thrombosis and Haemostasis 79, 609-613.

Kulkarni, S., Dopheide, S. M., Yap, C. L., Ravanat, C., Freund, M., Mangin, P., Heel, K. A., Street, A., Harper, I. S., Lanza, F., Jackson, S. P., 2000. A revised model of platelet aggregation. The Journal of Clinical Investigation 105, 783-791.

Lambert, L. J., Bobkov, A. A., Smith, J. W., Marassi, F. M., 2008. Competitive interactions of collagen and a jararhagin-derived disintegrin peptide with the integrin a2-I domain. The Journal of Biological Chemistry 283, 16665-16672.

Li, Z. Y., Yu, T. F., Lian, E. C. Y., 1994. Purification and characterization of L-amino
acid oxidase from king cobra (*Ophiophagus hannah*) venom and its effects on human platelet aggregation. Toxicon 32, 1349-1358.

Liu, Q., Li, Z., Li, J., 2014. Use B-factor related features for accurate classification between protein binding interfaces and crystal packing contacts. BMC bioinformatics 15, S3.

Lu, Q., Navdaev, A., Clemetson, J. M., Clemetson, K. J., 2004. GPIb is involved in platelet aggregation induced by mucetin, a snake C-type lectin protein from Chinese habu (*Trimeresurus mucrosquamatus*) venom. Thrombosis and Haemostasis 91, 1168-1176.

Lu, Q., Navdaev, A., Clemetson, J. M., Clemetson K. J., 2005. Snake venom C-type lectins interacting with platelet receptors. Structure–function relationships and effects on haemostasis. Toxicon 45, 1089-1098.

Manon-Jensen, T., Kjeld, N. G., Karsdal, M. A., 2016. Collagen-mediated hemostasis. Journal of Thrombosis and Haemostasis 14, 438-448.

Marcinkiewicz, C. Lobb, R. R., Marcinkiewicz, M. M., Daniel, J. L., Smith, J. B., Dangelmaier, C., Weinreb, P. H., Beacham, D. A., Niewiarowski, S., 2000. Isolation and characterization of EMS16, a C-lectin type protein from *Echis multisquamatus* venom, a potent and selective inhibitor of the a2b1 integrin. Biochemistry 39, 9859-9867.

Markland, F. S., 1998. Snake venoms and the hemostatic system. Toxicon 36, 1749-1800.

Matsui, T., Hamako, J., 2005. Structure and function of snake venom toxins interacting with human von Willebrand factor. Toxicon 45, 1075-1087.

McEwan, P. A., Andrews, R. K., Emsley, J., 2009. Glycoprotein Ibα inhibitor complex structure reveals a combined steric and allosteric mechanism of von Willebrand factor antagonism. Blood 114, 4883-4885.

Mooney, C., Haslam, N. J., Pollastri, G., Shields, D. C., 2012. Towards the improved discovery and design of functional peptides: common features of diverse classes permit generalized prediction of bioactivity. PloS one *7*, e45012.

Mounier, C., Vargaftig, B. B., Franken, P. A., Verheij, H. M., Bon, C., Touqui, L., 1994. Platelet secretory phospholipase A2 fails to induce rabbit platelet activation and to release arachidonic acid in contrast with venom phospholipases A2. Biochimica et Biophysica Acta 1214, 88-96.

Naumann, G. B., Silva, L. F., Silva, L., Faria, G., Richardson, M., Evangelista, K., Kohlhoff, M., Gontijo, C. M. F., Navdaev, A., Rezende, F. F., Eble, J. A., Sanchez, E. F., 2011. Cytotoxicity and inhibition of platelet aggregation caused by an l-amino acid oxidase from *Bothrops leucurus* venom.Biochimica et Biophysica Acta 1810, 683-694.

Nieswandt, B., Brakebusch, C., Bergmeier, W., Schulte, V., Bouvard, D., Mokhtari-Nejad, R., Lindhout, T., Heemskerk, J. W. M., Zirngibl, H., Fassler, R., 2001. Glycoprotein VI but not $\alpha_2\beta_1$ integrin is essential for platelet interaction with collagen. The EMBO Journal 20, 2120-2130.

Nieswandt, B., Watson, S. P., 2003. Platelet collagen interaction: is GPVI the central receptor? Blood 102, 449-461.

Nishida, S., Fujimura, Y., Miura, S., Yoshida, E., Sugimoto, M., Yoshioka, A., Fukui, H., Ozaki, Y., Usami, Y., 1994. Purification and characterization of bothrombin, a fibrinogen clotting serine protease from the venom of *Bothrops jararaca*. Biochemistry 33, 1843-1849.

Offermanns, S., 2006. Activation of platelet function through G protein–coupled receptors. Circulation research 99, 1293-1304.

Peng, M., Lu, W., Kirby, E. P., 1991. Alboaggregin-B: a new platelet agonist that binds to platelet membrane glycoprotein Ib. Biochemistry 30, 11529-11536.

Perkins, D. N., Pappin, D. J., Creasy, D. M., Cottrell, J. S., 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20, 3551-3567.

Polgar, J., Clemetson, J. M., Kehrel, B. E., Wiedemann, M., Magnenat, E. M., Wells, T. N., Clemetson, K. J., 1997. Platelet activation and signal transduction by convulxin, a C-type lectin from *Crotalus durissus terrificus* (tropical rattlesnake) venom via the p62/GPVI collagen receptor. The Journal of Biological Chemistry 272, 13576-13583.

Ponstingl, H., Henrick, K., Thornton, J. M., 2000. Discriminating between homodimeric and monomeric proteins in the crystalline state. Proteins 41, 47-57.

Queiroz, M. R., Mamede, C. C. N., Fonseca, K. C., Morais, N. C., Sousa, B. B., Santos-Filho, N. A., Beletti, M. E., Arantes, E. C., Stanziola, L., Oliveira, F., 2014a. Rapid purification of a new pi class metalloproteinase from *Bothrops moojeni* venom with antiplatelet activity. BioMed Research International, 1-12.

Queiroz, M. R., Mamede, C. C. N., Morais, N. C. G., Fonseca, K. C., Sousa, B. B., Migliorini, T. M., Pereira, D. F. C., Stanziola, L., Calderon, L. A., Simões-Silva, R., Soares, A. M., Oliveira, F., 2014b. Purification and characterization of BmooAi: a new toxin from *Bothrops moojeni* snake venom that inhibits platelet aggregation. BioMed Research International, 1-7.

Queiroz, M. R., Sousa, B. B., Pereira, D. F. C., Mamede, C. C. N., Matias, M. S., Morais, N. C. G., Costa, J. O., Oliveira, F., 2017. The role of platelets in hemostasis and the effects of snake venom toxins on platelet function. Toxicon 133, 33-47.

Rakhmetov, A., Lee, S. P., Grebinyk, D., Ostapchenko, L. and Chae, H. Z., 2015. Simulation of Peroxiredoxin II and Brain-type Creatine Kinase protein-protein interaction using the on-line docking server ClusPro 2.0. Journal of Applied Pharmaceutical Science 5, 11-16.

Rodrigues, R. S., Silva, J. F., França, J. B., Fonseca, F. P., Otaviano, A. R., Silva,H., Hamaguchi, A., Magro, A. J., Braz, A. S. K., Santos, J. I. S., Homsi-Brandeburgo, M. I., Fontes, M. R. M., Fuly, A. L., Soares, A. M., Rodrigues, V. M., 2009. Structural and functional properties of Bp-LAAO, a new l-amino acid oxidase isolated from *Bothrops pauloensis* snake venom. Biochimie 91, 490-501.

Sajevic, T., Leonardi, A., Krizaj, I., 2011. Haemostatically active proteins in snake venoms. Toxicon 57, 627-645.

Santos, B. F., Serrano, S. M., Kuliopulos, A., Niewiarowski, S., 2000. Interaction of viper venom serine peptidases with thrombin receptors on human platelets. FEBS Letters 477, 199-202.

Serrano, S. M., Maroun, R. C., 2005. Snake venom serine proteinases: sequence homology vs. substrate specificity, a paradox to be solved. Toxicon 45, 1115–1132.

Serrano, S. M., Kim, J., Wang, D., Draqulev, B., Shannon, J. D., Mann, H. H., Veit, G., Wagener, R., Koch, M., Fox, J. W., 2006. The cysteine-rich domain of snake venom metalloproteinases is a ligand for von Willebrand factor A domains: role in substrate targeting. The Journal of Biological Chemistry 281, 39746-39756.

Silveira, L. B., Marchi-Salvador, D. P., Santos-Filho, N. A., Silva, F. P., Marcussi, S., Fuly, A. L., Nomizo, A., Silva, S. L., Stábeli, R. G., Arantes, E. C., Soares, A. M., 2013. Isolation and expression of a hypotensive and anti-platelet acidic phospholipase A_2 from *Bothrops moojeni* snake venom. Journal of Pharmaceutical and Biomedical Analysis 73, 35-43.

Stábeli, R. G., Sant'Ana, C. D., Ribeiro, P. H., Costa, T. R., Ticli, F. K., Pires, M. G., Nomizo, A., Albuquerque, S., Malta-Neto, N. R., Marins, M., Sampaio, S. V., Soares, A. M., 2007. Cytotoxic L-amino acid oxidase from *Bothrops moojeni*: Biochemical and functional characterization. International Journal of Biological Macromolecules 41, 132-140.

Suckau, D. Resemann, A., Schuerenberg, M., Hufnagel, P., Franzen, J., Holle, A., 2003. A novel MALDI LIFTTOF/ TOF mass spectrometer for proteomics. Analytical and Bioanalytical Chemistry 376, 952-965.

Tanjoni, I., Evangelista, K., Della-Casa, M. S., Butera, D., Magalhaes, G. S., Baldo, C., Clissa, P. B., Fernandes, I., Eble, J., Moura-de-Silva, A. M., 2010. Different regions of the class P-III snake venom metalloproteinase jararhagin are involved in binding to a2b1 integrin and collagen. Toxicon 55, 1093-1099.

Uff, S., Clemetson, J. M., Harrison, T., Clemetson, K. J., Emslev, J., 2002. Crystal structure of the platelet glycoprotein Ibα N-terminal domain reveals an unmasking mechanism for receptor activation. Journal of Biological Chemistry 277, 35657-35663.

Usami, Y., Fujimura, Y., Suzuki, M., Ozeki, Y., Nishio, K., Fukui, H., Titani, K., 1993. Primary structure of two-chain botrocetin, a von Willebrand factor modulator purified from the venom of *Bothrops jararaca*. Proceedings of the National Academy of Sciences 90, 928-932.

Varga-Szabo, D., Pleines, I., Nieswandt, B. 2008. Cell Adhesion Mechanisms in Platelets. Arteriosclerosis, Thrombosis and Vascular Biology *2*8, 403-412.

Vilca-Quispe, A., Ponce-Soto, L. A., Winck, F. V., Marangoni, S., 2010, Isolation and characterization of a new serine protease with thrombin-like activity (TLBm) from the venom of the snake *Bothrops marajoensis*. Toxicon 55, 745-753.

Wang, W. J., Shih, C. H., Huang, T. F., 2005. Primary structure and antiplatelet mechanism of a snake venom metalloproteinase, acurhagin, from *Agkistrodon acutus* venom. Biochimie 87, 1065-1077.

Wei, J. F., Wei, Q., Lu, Q. M., Tai, H., Jin, Y., Wang, W. Y., Xiong, Y. L., 2003. Purification, characterization and biological activity of an L-amino acid oxidase from *Trimeresurus mucrosquamatus* venom. ABBS 35, 219-224.

Yoshida, E., Fujimura, Y., Miura, S., Sugimoto, M., Fukui, H., Narita, N., Usami, Y., Suzuki, M., Titani, K., 1993. Alboaggregin-B and botrocetin, two snake venom proteins with highly homologous amino acid sequences but totally distinct functions on von Willebrand factor binding to platelets. Biochemical and Biophysical Research Communications 191, 1386-1392.

Yoshida, E., Fujimura, Y., Ikeda, Y., Takeda, I., Yamamoto, Y., Nishikawa, K., Miyataka, K., Oonuki, M., Kawasaki, T., Katayama, M., 1995. Impaired high-shear-

stress-induced platelet aggregation in patients with chronic renal failure undergoing dialysis. British Journal of Haematology 89, 861-867.

ANEXO

Artigo publicado relacionado ao tema da tese:

A new platelet-aggregation-inhibiting factor isolated from *Bothrops moojeni* snake venom

Hindawi
BioMed Research International
Volume 2017, Article ID 4315832, 9 pages
https://doi.org/10.1155/2017/4315832

Research Article

A New Platelet-Aggregation-Inhibiting Factor Isolated from *Bothrops moojeni* Snake Venom

Bruna Barbosa de Sousa,[1,2] Carla Cristine Neves Mamede,[2,3] Mariana Santos Matias,[1] Déborah Fernanda da Cunha Pereira,[1] Mayara Ribeiro de Queiroz,[2] Edigar Henrique Vaz Dias,[1] Anielle Christine Almeida Silva,[4] Noelio Oliveira Dantas,[4] Júnia de Oliveira Costa,[1,5] and Fábio de Oliveira[2,6]

[1]*Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia, Uberlândia, MG, Brazil*
[2]*Instituto Nacional de Ciência e Tecnologia em Nano-Biofarmacêutica (N-Biofar), Belo Horizonte, MG, Brazil*
[3]*Instituto de Ciências Agrárias, Universidade Federal de Uberlândia, Monte Carmelo, MG, Brazil*
[4]*Instituto de Física, Universidade Federal de Uberlândia, Uberlândia, MG, Brazil*
[5]*Instituto Federal de Educação, Ciência e Tecnologia do Triângulo Mineiro, Campus Ituiutaba, Ituiutaba, MG, Brazil*
[6]*Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia, Uberlândia, MG, Brazil*

Correspondence should be addressed to Júnia de Oliveira Costa; juniacosta@iftm.edu.br and Fábio de Oliveira; foliveira@umuarama.ufu.br

Received 7 July 2017; Revised 12 September 2017; Accepted 24 September 2017; Published 1 November 2017

Academic Editor: Ji-Fu Wei

This work reports the purification and functional characterization of BmooPAi, a platelet-aggregation-inhibiting factor from *Bothrops moojeni* snake venom. The toxin was purified by a combination of three chromatographic steps (ion-exchange on DEAE-Sephacel, molecular exclusion on Sephadex G-75, and affinity chromatography on HiTrap™ Heparin HP). BmooPAi was found to be a single-chain protein with an apparent molecular mass of 32 kDa on 14% SDS-PAGE, under reducing conditions. Sequencing of BmooPAi by Edman degradation revealed the amino acid sequence LGPDIVPPNELLEVM. The toxin was devoid of proteolytic, haemorrhagic, defibrinating, or coagulant activities and induced no significant oedema or hyperalgesia. BmooPAi showed a rather specific inhibitory effect on ristocetin-induced platelet aggregation in human platelet-rich plasma, whereas it had little or no effect on platelet aggregation induced by collagen and adenosine diphosphate. The results presented in this work suggest that BmooPAi is a toxin comprised of disintegrin-like and cysteine-rich domains, originating from autolysis/proteolysis of PIII SVMPs from *B. moojeni* snake venom. This toxin may be of medical interest because it is a platelet aggregation inhibitor, which could potentially be developed as a novel therapeutic agent to prevent and/or treat patients with thrombotic disorders.

1. Introduction

Snake venoms comprise pharmacologically active proteins and peptides, both enzymatic and nonenzymatic, such as phospholipases A_2, metalloproteinases, serine proteinases, nucleotidases, L-amino acid oxidase, disintegrins, and C-type lectins [1–4]. Several snake venom metalloproteinases (SVMPs) have been isolated and characterized by their biological activities. These enzymes play a key role in the prominent local tissue damage and systemic alterations caused by snake venom. SVMPs induce haemorrhage, myonecrosis, skin damage, inflammation, and degradation of extracellular matrix components. In addition, some SVMPs affect platelet function, while others degrade blood clotting factors, potentiating the haemorrhagic effect [4–8].

SVMPs comprise a group of zinc-dependent enzymes of varying molecular mass, widely distributed in Viperidae venoms. They are synthesized as multidomain precursors and stored in the venom gland as inactive zymogens [7, 9–11]. SVMPs are classified into three major classes, PI, PII, and PIII, according to their size (molecular mass) and domain organization. PI SVMPs include small metalloproteinases with

only the metalloproteinase domain. PII SVMPs comprise medium-size proteinases composed of one metalloproteinase and one disintegrin domain. PIII SVMPs have an additional cysteine-rich domain following the disintegrin-like domain and, in some cases, a lectin-like domain. PII and PIII SVMPs are divided into several subclasses based on proteolytic processing. PII SVMPs can be processed into a metalloproteinase domain and a nonenzymatic disintegrin, and PIII SVMPs can also be degraded, releasing a stable fragment which corresponds to the disintegrin-like and cysteine-rich domains (dis-cys domain) [12, 13].

Several studies have investigated SVMPs as platelet aggregation inhibitors due to their specificity to platelet integrins [4, 7, 10, 14–18]. The disintegrin domain usually contains the RGD (Arg-Gly-Asp) or KGD (Lys-Gly-Asp) motifs in its inhibitory loop, which binds with a high degree of selectivity to the $\alpha_{IIb}\beta_3$ platelet integrin (also known as GPIIb/IIIa), blocking the last phase of platelet aggregation and clot formation [18, 19]. Therefore, SVMPs are considered potent platelet aggregation inhibitors [7]. Unlike the disintegrin domain, whose integrin-binding motif is well characterized, the dis-cys domain possesses the XXCD (X-X-Cys-Asp) motif, instead of the usual RGD, and its targets are not yet fully elucidated. The extant literature indicates that the biological activities attributed to this domain result from its ability to block the interaction of platelets with collagen [19].

In the present study, we describe the isolation and functional characterization of BmooPAi, a toxin comprised of the dis-cys domain, originating from autolysis/proteolysis of PIII SVMPs from *Bothrops moojeni* snake venom, which showed an inhibitory effect on platelet aggregation.

2. Materials and Methods

2.1. Material. Desiccated *B. moojeni* venom was purchased from Bioagents Serpentarium (Batatais, SP, Brazil). Acrylamide, ammonium bicarbonate, ammonium persulphate, azocasein, bromophenol blue, ethylenediaminetetracetic acid (EDTA), bovine fibrinogen, glycine, β-mercaptoethanol, N,N'-methylene-*bis*-acrylamide, sodium dodecyl sulphate (SDS), N,N,N',N'-tetramethylethylenediamine (TEMED), and Tris were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Molecular weight markers for electrophoresis and all chromatographic media (DEAE-Sephacel, Sephadex G-75, HiTrap Heparin HP and C2/C18 columns) were purchased from GE Healthcare Technologies (Uppsala, Sweden). All the agonists used in the platelet aggregation assays (collagen from equine tendon, adenosine diphosphate (ADP), and ristocetin) were purchased from Helena Laboratories (Beaumont, Texas, USA). All other reagents used were of analytical grade.

2.2. Animals. Male Swiss mice (20–25 g) and male Wistar rats (200–250 g) were provided by the Centro de Bioterismo e Experimentação Animal (CEBEA) at the Federal University of Uberlândia (Uberlândia-MG, Brazil). The animals were maintained under conditions of controlled temperature (22 ± 2°C) and light/dark cycle (12 hours) with free access to food and water. The experimental protocol was approved by the Committee for Ethics in Animal Experimentation of the Federal University of Uberlândia (CEUA/UFU), Minas Gerais, Brazil (Protocol number 108/12).

2.3. Human Blood. The experiments were performed in accordance with current guidelines for human research, established by the Committee for Ethics in Human Research of the Federal University of Uberlândia, Minas Gerais, Brazil (CEP/UFU, Protocol number 1.627.982/2016). Blood was obtained by blood donation from 10 individuals who were invited to participate in the research as volunteer donors. The criteria for the selection of donor volunteers included no signs or symptoms of disease, malnutrition, or dehydration; age between 18 and 65 years; weight more than 50 kg; no use of any medication that interferes with haemostasis; no use of illicit drugs; no alcohol consumption in the last 24 hours preceding the experiment; and no haemostasis disorders.

2.4. Isolation of BmooPAi. Protein isolation was carried out in three steps. The crude venom of *B. moojeni* (200 mg) was dissolved in 2.0 mL of 0.05 M ammonium bicarbonate buffer (pH 7.8) and clarified by centrifugation at 10,000 ×g for 10 min. The supernatant was applied to a DEAE-Sephacel column (2.5 × 20 cm) previously equilibrated with 0.05 M ammonium bicarbonate buffer (pH 7.8). Chromatography was carried out at a flow rate of 20 mL/h, with a linear concentration gradient of the same buffer (0.05–0.6 M), and fractions of 3.0 mL/tube were collected. All peaks were monitored by measuring absorbance at 280 nm on a spectrophotometer (BioSpec-Mini; Shimadzu Biotech, Japan). The seventh peak, designated D7, was pooled, lyophilised, and applied to a Sephadex G-75 column (1.0 × 100 cm) previously equilibrated with 0.05 M ammonium bicarbonate buffer (pH 7.8). The samples were eluted from this column with the same buffer, at a flow rate of 20 mL/h, and fractions of 3.0 mL/tube were collected. The second fraction, designated D7S2, was pooled, lyophilised, and submitted to the third step of separation using a HiTrap Heparin HP column (5 × 1 mL) in an ÄKTApurifier™ HPLC system, previously equilibrated with 20 mM Tris-HCl buffer (pH 7.0) containing 5 mM calcium chloride. The samples were eluted with an increasing concentration gradient of 20 mM Tris-HCl buffer (pH 7.0) containing 2.0 M sodium chloride. Elution was carried out at a flow rate of 30 mL/h; fractions of 1.0 mL/tube were collected and the absorbance was read at 280 nm. Isolated BmooPAi was concentrated in the major peak. To evaluate the degree of purity, isolated BmooPAi was passed through a reverse-phase C2/C18 column (4.6 × 100 mm) using the ÄKTApurifier HPLC system. The column was equilibrated with solvent A (0.1% trifluoroacetic acid) and eluted with a linear concentration gradient from 0 to 100% of solvent B (70% acetonitrile, 0.1% trifluoroacetic acid) at a flow rate of 0.3 mL/min. Absorbance was monitored at 280 nm.

2.5. Protein Analysis. The protein concentration was determined using the method established by Bradford [20]. Determination of the protein concentration was performed in triplicate and the absorbance was measured at 595 nm. The protein concentration (mg/μL) was determined from linear regression calculations based on the values obtained from

the standard curve. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed using 14% (w/v) gels. Electrophoresis was carried out at 20 mA/gel in Tris-glycine buffer (pH 8.3) containing 0.01% SDS. The molecular mass standard proteins used included phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Gels were stained with Coomassie blue R-250, 0.2% (w/v). The relative molecular mass of BmooPAi was estimated using Kodak 1D image analysis software.

2.6. N-Terminal Sequencing. The sequencing was performed in a membrane obtained from the purified protein in solution. The liquid sample was loaded onto an Applied Biosystems Prosorb™ device. The protocol for sample preparation using this device was followed, and the Prosorb membrane was loaded onto the instrument for sequence analysis.

2.7. Proteolytic Activity upon Fibrinogen. Fibrinogenolytic activity was assayed as previously described [21], with brief modifications. Samples containing 25 μL of bovine fibrinogen (3 mg/mL saline) were incubated with 20 μg of BmooPAi for 120 min at 37°C. The reaction was stopped by the addition of a denaturing buffer containing 10% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.2% (w/v) SDS, and 0.001% (w/v) bromophenol blue (pH 6.8). Reaction products were analysed using 14% (w/v) SDS-PAGE.

2.8. Proteolytic Activity upon Azocasein. Samples of 20 μg BmooPAi were supplemented with 200 μL of saline and incubated for 60 min with 800 μL of azocasein solution (1 mg/mL). The reaction was stopped by the addition of 200 μL of 15% trichloroacetic acid to precipitate the undegraded azocasein. The mixture was left standing for 20 min before centrifugation at 10,000 ×g for 10 min. The proteolytic activity was estimated by reading the absorbance of the clear supernatant at 405 nm. One unit of azocaseinolytic activity was defined as the amount of enzyme that produced an absorbance increase of 0.01 units.

2.9. Haemorrhagic Activity. Haemorrhagic activity was determined as previously described [22], with some modifications. Groups of three male Swiss mice were administered a dorsal skin subcutaneous injection of 50 μg BmooPAi diluted in 25 μL of saline. After three hours, animals were euthanized by an overdose of ketamine/xylazine, the skin was removed and the diameter of the haemorrhagic spot was measured on the inner surface. Control animals received the same volume of sterile saline.

2.10. Defibrinating Activity. Defibrinating activity was carried out as previously described [23], with slight modifications. Groups of three male Swiss mice were intraperitoneally injected with 50 μg BmooPAi (50 μg/100 μL saline). Control animals received the same volume of sterile saline. After one hour, mice were euthanized by an overdose of ketamine/xylazine and bled by cardiac puncture. Whole blood was placed in tubes and kept at 25–30°C. Activity was determined by measuring the time to the onset of blood clotting.

2.11. Coagulant Activity. Coagulant activity was assayed using bovine plasma. The plasma samples were mixed with 3.8% sodium citrate (9 : 1, v/v) and centrifuged at 2,500 ×g for 15 min to obtain platelet-rich plasma (PRP). BmooPAi (25 μg/25 μL), the same volume of saline (negative control), or 0.2 mol/L calcium chloride (positive control) was added to 200 μL of citrated bovine PRP at 37°C. Clotting activity was determined by measuring the time to fibrin clot onset by a coagulometer (CLO Timer).

2.12. Evaluation of Paw Oedema Formation. Groups of three male Wistar rats received an intraplantar injection of BmooPAi (50 μg/100 μL saline) into the subplantar region of one hind paw. An equal volume of sterile saline was injected into the contralateral paw. The volume of each paw was measured using a plethysmometer (model 7140; Ugo Basile, Italy) before and 1, 2, 3, 4, 5, 6, and 24 h after the injection. Results were calculated as the difference between each paw and expressed as the percentage increase in paw volume relative to the initial volume.

2.13. Evaluation of Hyperalgesia. Hyperalgesia was measured according to the paw pressure test, as previously described [24], with slight modifications. Groups of three male Wistar rats received an intraplantar injection of BmooPAi (50 μg/100 μL saline) into the right hind paw. An equal volume of sterile saline was injected into the left hind paw for control. The weight in grams (g) required to elicit a nociceptive response, that is, paw flexion, was determined as the nociceptive threshold. A cut-off value of 300 g was used to prevent damage to the paws. The nociceptive threshold was measured before and 1, 2, 3, 4, 5, 6, and 24 h after BmooPAi injection using a Randall-Selitto apparatus (EFF-440 model; Insight, Brazil). Results were calculated as the difference between each paw and expressed as the percentage decrease in nociceptive threshold relative to the initial threshold. To reduce stress, the rats were habituated to the apparatus one day before the experiment.

2.14. Platelet Aggregation Assay. Platelet aggregation assays were performed as previously described [25] in human PRP and measured using the automatic Aggregometer 4 channels (AggRAM™ version 1.1; Helena Laboratories, USA). Human blood collected in sodium citrate was centrifuged at 100 ×g for 12 min at room temperature to obtain PRP. Platelet-poor plasma (PPP) was obtained from the residue by centrifugation of citrated blood at 1,000 ×g for 15 min. Assays were carried out using 200 μL of PRP maintained at 37°C under continuous stirring, in siliconized glass cuvettes. Aggregation was triggered with collagen (10 μg/mL), ADP (20 μM), or ristocetin (1.5 mg/mL) immediately after adding BmooPAi (10, 25 and 50 μg) to human PRP. Finally, 100% aggregation was expressed as the percentage absorbance relative to PPP aggregation. Control experiments were performed using only platelet agonists. All experiments were carried out in triplicate.

3. Results and Discussion

In recent years, a number of snake venom proteins that interfere with platelet function have been identified. Most of these proteins belong to the SVMP family, and their effects are probably due to the presence of dis-cys domains which interact with specific protein molecules on platelet membrane surfaces [25–29]. In this work, we report the purification and functional characterization of BmooPAi, a platelet-aggregation-inhibiting factor, comprised of dis-cys domains processed from PIII SVMPs from *B. moojeni* snake venom.

The toxin was isolated using a three-step procedure, including ion-exchange, gel filtration, and affinity chromatographies. Other toxins have been purified using similar procedures, including BmooFIBMP-I [30] and BmooMPα-1 [31], both metalloproteinases purified from *B. moojeni* snake venom. *B. moojeni* crude venom (200 mg) was applied to ion-exchange chromatography on a DEAE-Sephacel column, which produced eight main protein peaks, designated D1 to D8 (Figure 1(a)). The D7 fraction represented around 5.5% (w/w) of the crude venom; it was submitted to gel filtration chromatography on a Sephadex G-75 column, resulting in four peaks, designated D7S1 to D7S4 (Figure 1(b)). The D7S2 fraction (~3.3%) was further fractionated over an affinity chromatography HiTrap Heparin HP column using the ÄKTApurifier HPLC system (Figure 1(c)). The nonadsorbed fraction (~1.7%) was able to inhibit platelet aggregation and it was designated BmooPAi (platelet aggregation inhibitor from *B. moojeni*). This fraction was devoid of proteolytic, haemorrhagic, defibrinating, or coagulant activities. In addition, BmooPAi did not cause hyperalgesia and oedematogenic responses in the hind paw when rats received a 50 μg intraplantar injection of the toxin. Thus, BmooPAi does not contribute to the local effects caused by *B. moojeni* envenomation.

The homogeneity of this sample was demonstrated by SDS-PAGE, showing a single protein band around 32 kDa in the presence or absence of the reducing agent β-mercaptoethanol (Figure 1(d)). The degree of purity of the isolated BmooPAi was confirmed by reverse-phase HPLC chromatography on a C2/C18 column, revealing a unique major peak (Figure 1(e)).

BmooPAi was subjected to N-terminal sequencing by Edman degradation. The first 15 amino acid residues from N-terminal sequencing were determined to be LGPDIVPPNELLEVM and were submitted to BLAST. The primary sequence of BmooPAi shared similarity with the middle of some PIII SVMPs, skipping the catalytic domain, and with some disintegrin or disintegrin-like proteins. Moojenin (GI: P0DKR0.1), Bothropasin (GI: 209870468), Jararhagin (GI: P30431.1), Leucurolysin-B (GI: P86092.1), Brevilysin-H6 (GI: P0C7B0.2), Atrolysin-A (GI: Q92043.1), and AaH-IV (GI: 255917952) are PIII SVMPs that showed homology with BmooPAi; Jarastatin (GI: Q0NZX5.1), Jararhagin-C (GI: P30431.1), Leucurogin (GI: P0DJ87.1), and Salmosin (GI: O93518.1) are disintegrin or disintegrin-like proteins homologous to BmooPAi (Figure 2).

PIII SVMPs comprise high molecular mass proteinases composed of a dis-cys domain following the proteinase domain. Some PIII SVMPs have an additional lectin-like domain linked to the others through a disulphide bond [12, 13]. SVMPs play an important role in haemostatic disorders. They are the primary factors responsible for haemorrhage [32]. PIII SVMPs are more able to induce haemorrhage than PI and PII classes due to the presence of the additional dis-cys domain which contributes to the proteolytic specificity of these enzymes, directing them to bind to critical components of the microvasculature [28, 33]. Subclass PIIIb SVMPs can undergo proteolysis/autolysis during secretion or under nonphysiological conditions *in vitro*, such as alkaline pH, low calcium, or the presence of reducing agents, generating intact biologically active polypeptides of ~30 kDa, which correspond to the dis-cys domain [13, 34].

Although BmooPAi showed high homology with many PIII SVMPs, it showed no haemorrhagic activity. These data suggest that this toxin originated from the proteolysis/autolysis of a PIII SVMP and comprises only the noncatalytic dis-cys domain. For this reason, BmooPAi was not able to induce haemorrhage due to the absence of the proteinase domain, preventing proteolysis of the capillary components.

Some PIII SVMPs homologous to BmooPAi, such as Bothropasin [35], Brevilysin-H6 [36], Leucurolysin-B [37], AaH-IV [38], and Jararhagin [39], can be cleaved through autoproteolytic events, releasing the dis-cys domain. According to the characteristics of BmooPAi, we suggest that this toxin is similar to Jararhagin-C (28 kDa) [40] and Acucetin (30 kDa) [41]. Both toxins are composed of the dis-cys domain with a molecular mass of approximately 30 kDa released through autoproteolysis of Jararhagin from *B. jararaca* [39] and AaH-IV from *Agkistrodon acutus* [38]. Similar to BmooPAi, Jararhagin-C and Acucetin do not induce haemorrhage. For this reason, we suggest that BmooPAi originates from the class of PIII SVMPs, as proposed by Fox and Serrano [13], in which the dis-cys domain has been processed from the proteinase domain.

The extant literature shows that several PIII SVMPs and processed dis-cys domains are able to interfere with platelet aggregation, including Jararhagin [39], Jararhagin-C [40], Alternagin-C [42], Leucurogin [43], and Leberagin-C [44]. Their targets on platelet membranes include the receptors GPIb-IX-V, $\alpha_{IIb}\beta_3$ integrin (GPIIb-IIIa), $\alpha_2\beta_1$ integrin, and GPVI and also their ligands, von Willebrand factor (vWF), fibrinogen, and/or collagen [4, 45]. In this work, BmooPAi was tested for its effect on platelet aggregation. Figure 3(a) shows that BmooPAi inhibited platelet aggregation induced by ristocetin in a dose-dependent manner, although it had little or no effect on platelet aggregation induced by ADP (Figure 3(b)) and collagen (Figure 3(c)). Our results showed that 10 and 25 μg of BmooPAi inhibited approximately 32% and 72% of ristocetin-induced platelet aggregation, respectively, whereas 50 μg completely inhibited platelet aggregation induced by ristocetin. On the other hand, BmooPAi was not able to inhibit the platelet aggregation induced by ADP and collagen, even at high concentrations (50 μg).

According to Coller and Gralnick [46], ristocetin is an antibiotic that induces vWF-GPIb binding *in vitro*, even at static conditions. This interaction plays a key role in the initial adhesion of platelets to the subendothelium, which

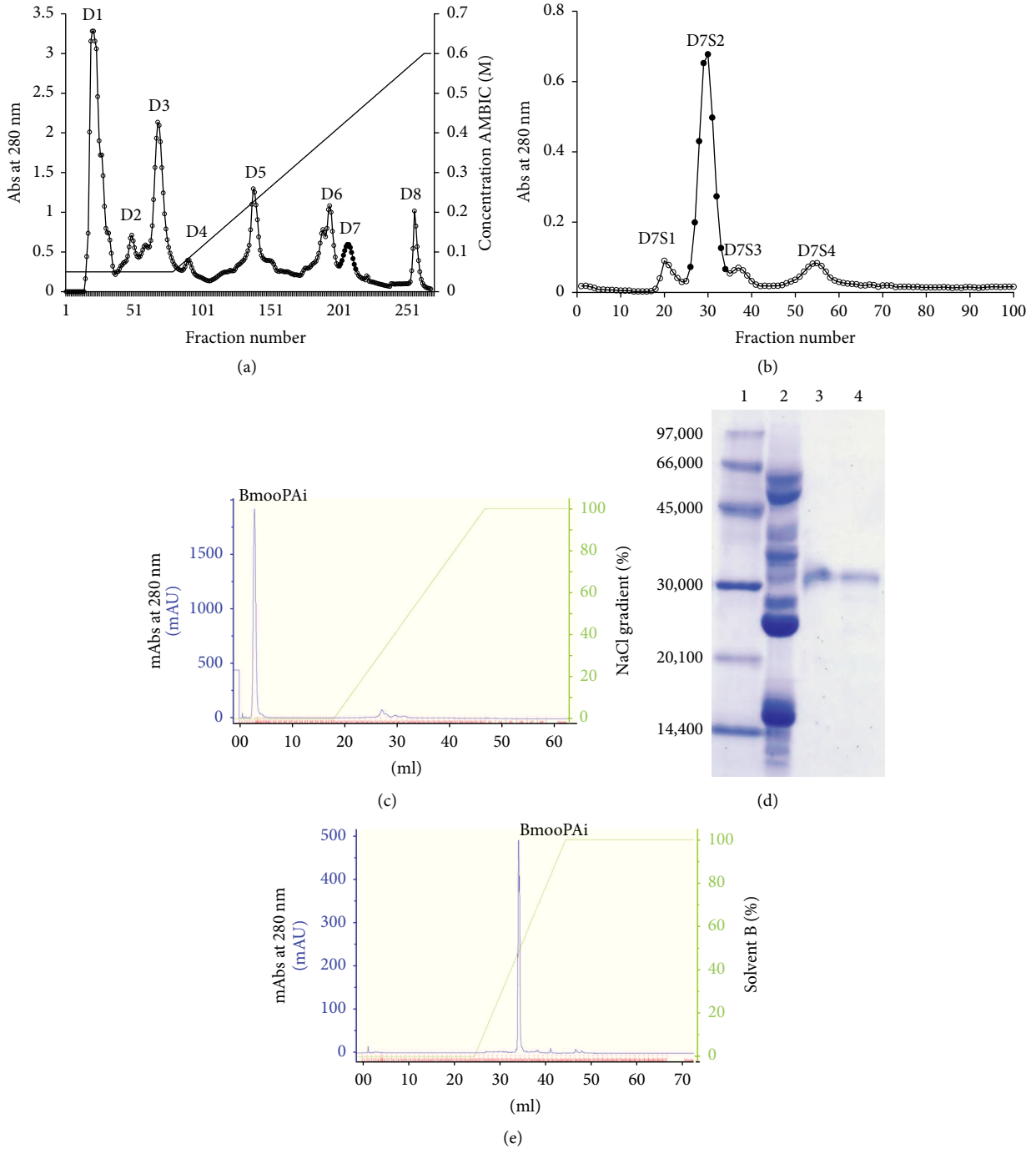

Figure 1: Purification of BmooPAi from *B. moojeni* snake venom. (a) Separation on DEAE-Sephacel ion-exchange chromatography: crude venom (200 mg) was applied to the column (2.5 × 20 cm) and elution was carried out at a flow rate of 20 mL/h with ammonium bicarbonate (Ambic) buffer gradients, pH 7.8, from 0.05 M to 0.6 M. Fractions of 3.0 mL/tube were collected and the absorbance was read at 280 nm. (b) Separation on Sephadex G-75 molecular exclusion chromatography: fraction D7 was applied to the column (1.0 × 100 cm) and elution with 0.05 M ammonium bicarbonate was achieved at a flow rate of 20 mL/h. Fractions of 3.0 mL/tube were collected and the absorbance was read at 280 nm. (c) Separation by affinity chromatography on a HiTrap Heparin HP column using the ÄKTApurifier HPLC system: fraction D7S2 was applied to the column (5 × 1 mL), previously equilibrated with 20 mM Tris-HCl buffer (pH 7.0) containing 5 mM calcium chloride. The samples were eluted with an increasing concentration gradient of 20 mM Tris-HCl buffer (pH 7.0) containing 2.0 M sodium chloride, and the absorbance of the fractions was monitored at 280 nm. Fractions of 1.0 mL/tube were collected at a flow rate of 30 mL/h. (d) SDS-PAGE in 14% (w/v) polyacrylamide, Tris-glycine buffer, pH 8.3, and 20 mA. Lanes: 1, standard proteins; 2, reduced crude venom of *B. moojeni*; 3, reduced BmooPAi; 4, nonreduced BmooPAi. The gel was stained with Coomassie blue R-250. (e) Reverse-phase HPLC on a C2C18 column (4.6 × 100 mm) equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted with a linear concentration gradient from 0 to 100% of solution B (70% acetonitrile in 0.1% TFA).

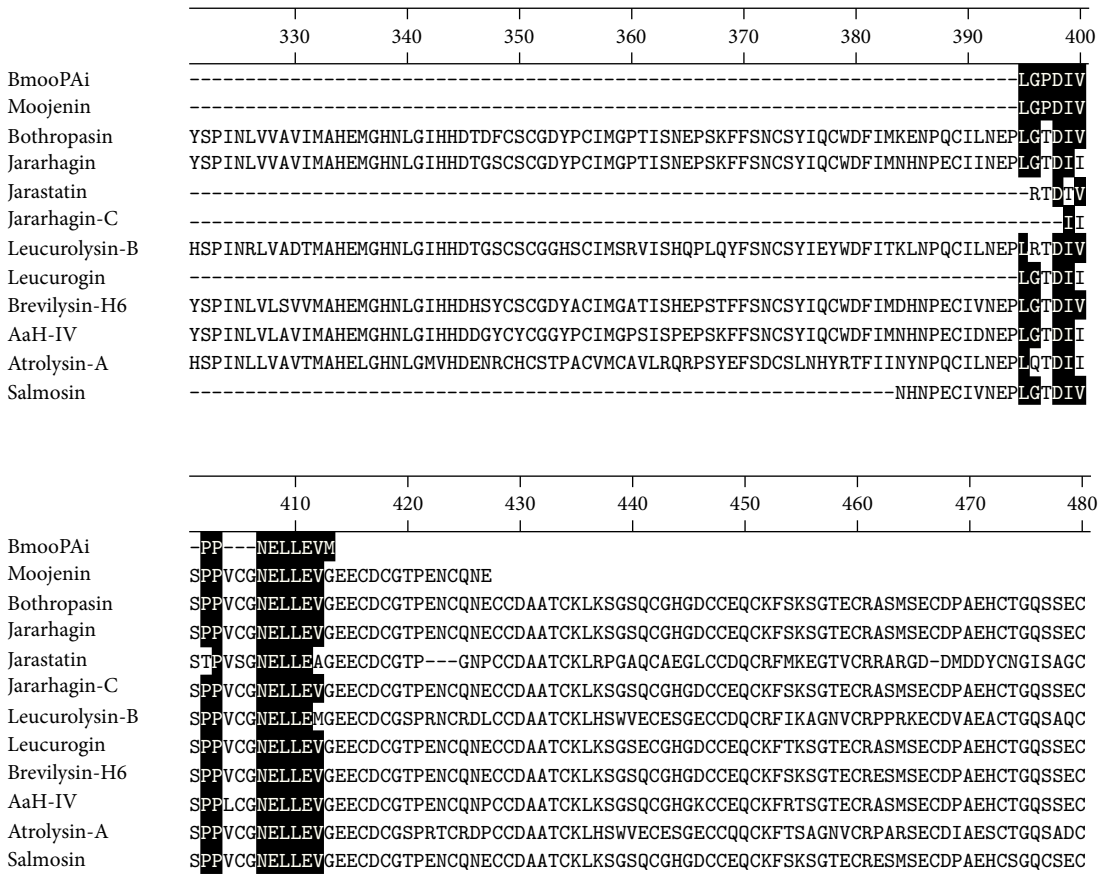

Figure 2: Sequence alignment of BmooPAi and other metalloproteinases/dis-cys proteins: Moojenin (GI: P0DKR0.1), Bothropasin (GI: 209870468), Jararhagin (GI: P30431.1), Jarastatin (GI: Q0NZX5.1), Jararhagin-C (GI: P30431.1), Leucurolysin-B (GI: P86092.1), Leucurogin (GI: P0DJ87.1), Brevilysin-H6 (GI: P0C7B0.2), AaH-IV (GI: 255917952), Atrolysin-A (GI: Q92043.1), and Salmosin (GI: O93518.1). The nonconserved residues are shown in black frames. The alignment and figure were generated by the MegAlign program from Lasergene (DNAStar Inc., Madison, WI, USA).

in turn induces a complex cascade of signals which results in platelet aggregation [47]. Since the circulating native form of vWF does not bind to platelet GPIb under static conditions, the antibiotic ristocetin allows the reproduction of the platelet aggregation events *in vitro* [48]. The importance of vWF-GPIb binding has been demonstrated for the clinical subsidiary diagnosis of hemostatic disorders, such as Bernard-Soulier syndrome [49] and von Willebrand disease [50].

A number of haemostatically active proteins that inhibit ristocetin (or botrocetin, a snake venom protein that also promotes *in vitro* vWF-GPIb binding)-induced platelet aggregation have been isolated and characterized from the venom of several snake species [3, 15]. Echicetin [51], Agkicetin [52], Jararaca GPIb-BP [53], Tokaracetin [54], Crotalin [55], Jararhagin [56], Kaouthiagin [57], Acurhagin [58], Kistomin [59], and Mocarhagin [60] are some examples.

Echicetin, Agkicetin, Jararaca GPIb-BP, and Tokaracetin toxins belong to the C-type lectin family and are able to bind directly to GPIb, blocking the binding of vWF to GPIb [61]. Jararhagin, Kaouthiagin, Acurhagin, Kistomin, Mocarhagin, and Crotalin belong to the SVMP family and are able to inhibit ristocetin-induced platelet aggregation due to their catalytic effect on GPIb and/or vWF [15]. BmooPAi also interferes with the vWF-GPIb interaction, but since it has no catalytic effect, we suggest that BmooPAi acts as an antagonist of the GPIb receptor, as described in other works. It is probable that the presence of the dis-cys domain directs the toxin to the platelet receptor. Furthermore, as BmooPAi did not inhibit ADP- or collagen-induced platelet aggregation, we can rule out the possibility of activity toward $\alpha_2\beta_1$, GPVI, P2Y1, and P2Y12 receptors. The initial findings from this work in progress provide impetus to further investigate the mechanism of action of BmooPAi on the platelet receptor.

4. Conclusions

In conclusion, we describe the procedures for isolation of BmooPAi (32 kDa), a toxin comprised of the dis-cys domain, originating from autolysis/proteolysis of PIII SVMPs from *B. moojeni* venom. BmooPAi may be of medical interest because it is a platelet aggregation inhibitor that interferes with the vWF-GPIb interaction. This toxin can be used to better understand the mechanisms concerning haemostasis

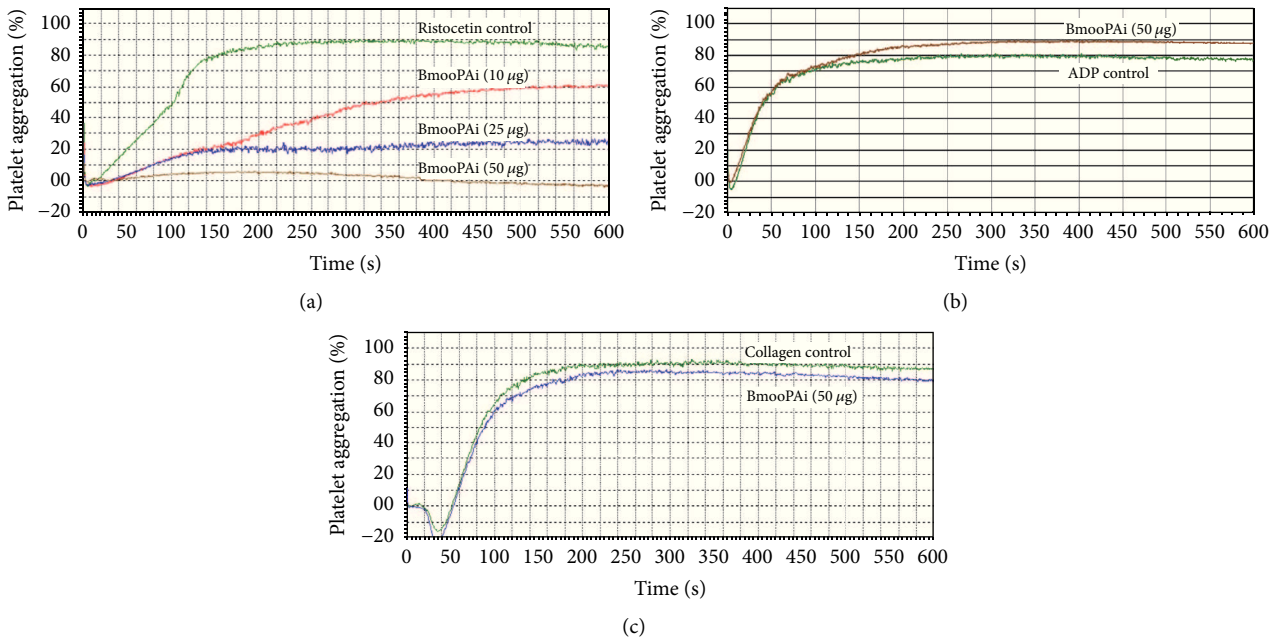

FIGURE 3: Effect of BmooPAi from *B. moojeni* venom on (a) ristocetin-, (b) ADP-, and (c) collagen-induced platelet aggregation. Aggregation was triggered with the agonists immediately after adding the indicated doses of BmooPAi to citrated human PRP at 37°C. Platelet aggregation was recorded for 10 min in an AggRAM platelet aggregation system with four-channel laser optics (Helena Laboratories, EUA). Results are expressed as an increase in light transmission, where PPP represents the maximum response (100%). Control experiments were performed in the absence of BmooPAi.

and could potentially be used as a tool for the development of novel antithrombotic agents.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

This work was supported by Fundação de Amparo á Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Ministério de Ciências e Tecnologia (MCT) of Brazil.

References

[1] X.-Y. Du, D. S. Sim, W.-H. Lee, and Y. Zhang, "Blood cells as targets of snake toxins," *Blood Cells, Molecules, and Diseases*, vol. 36, no. 3, pp. 414–421, 2006.

[2] R. M. Kini and H. J. Evans, "Effects of snake venom proteins on blood platelets," *Toxicon*, vol. 28, no. 12, pp. 1387–1422, 1990.

[3] F. S. Markland, "Snake venoms and the hemostatic system," *Toxicon*, vol. 36, no. 12, pp. 1749–1800, 1998.

[4] T. Sajevic, A. Leonardi, and I. Križaj, "Haemostatically active proteins in snake venoms," *Toxicon*, vol. 57, no. 5, pp. 627–645, 2011.

[5] J. W. Fox and S. M. T. Serrano, "Timeline of key events in snake venom metalloproteinase research," *Journal of Proteomics*, vol. 72, no. 2, pp. 200–209, 2009.

[6] J. M. Gutiérrez and A. Rucavado, "Snake venom metalloproteinases: their role in the pathogenesis of local tissue damage," *Biochimie*, vol. 82, no. 9-10, pp. 841–850, 2000.

[7] F. S. Markland and S. Swenson, "Snake venom metalloproteinases," *Toxicon*, vol. 62, pp. 3–18, 2013.

[8] N. Marsh and V. Williams, "Practical applications of snake venom toxins in haemostasis," *Toxicon*, vol. 45, no. 8, pp. 1171–1181, 2005.

[9] J. J. Calvete, P. Juárez, and L. Sanz, "Snake venomics. Strategy and applications," *Journal of Mass Spectrometry*, vol. 42, no. 11, pp. 1405–1414, 2007.

[10] A. M. Moura-da-Silva, D. Butera, and I. Tanjoni, "Importance of snake venom metalloproteinases in cell biology: Effects on platelets, inflammatory and endothelial cells," *Current Pharmaceutical Design*, vol. 13, no. 28, pp. 2893–2905, 2007.

[11] S. Swenson and F. S. Markland Jr., "Snake venom fibrin(ogen)olytic enzymes," *Toxicon*, vol. 45, no. 8, pp. 1021–1039, 2005.

[12] J. W. Fox and S. M. T. Serrano, "Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases," *Toxicon*, vol. 45, no. 8, pp. 969–985, 2005.

[13] J. W. Fox and S. M. T. Serrano, "Insights into and speculations about snake venom metalloproteinase (SVMP) synthesis, folding and disulfide bond formation and their contribution to venom complexity," *FEBS Journal*, vol. 275, no. 12, pp. 3016–3030, 2008.

[14] S. Braud, C. Bon, and A. Wisner, "Snake venom proteins acting on hemostasis," *Biochimie*, vol. 82, no. 9-10, pp. 851–859, 2000.

[15] A. S. Kamiguti, "Platelets as targets of snake venom metalloproteinases," *Toxicon*, vol. 45, no. 8, pp. 1041–1049, 2005.

[16] C. Y. Koh and R. M. Kini, "From snake venom toxins to therapeutics—cardiovascular examples," *Toxicon*, vol. 59, no. 4, pp. 497–506, 2012.

[17] T. Matsui, Y. Fujimura, and K. Titani, "Snake venom proteases affecting hemostasis and thrombosis," *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, vol. 1477, no. 1-2, pp. 146–156, 2000.

[18] O. H. P. Ramos and H. S. Selistre-De-Araujo, "Snake venom metalloproteases - Structure and function of catalytic and disintegrin domains," *Comp Biochem Physiol C Toxicol Pharmacol*, vol. 142, no. 3-4, pp. 328–346, 2006.

[19] J. J. Calvete, C. Marcinkiewicz, D. Monleón et al., "Snake venom disintegrins: Evolution of structure and function," *Toxicon*, vol. 45, no. 8, pp. 1063–1074, 2005.

[20] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[21] V. M. Rodrigues, A. M. Soares, R. Guerra-Sá, V. Rodrigues, M. R. M. Fontes, and J. R. Giglio, "Structural and functional characterization of neuwiedase, a nonhemorrhagic fibrin(ogen)olytic metalloprotease from Bothrops neuwiedi snake venom," *Archives of Biochemistry and Biophysics*, vol. 381, no. 2, pp. 213–224, 2000.

[22] T. Nikai, N. Mori, M. Kishida, H. Sugihara, and A. T. Tu, "Isolation and biochemical characterization of hemorrhagic toxin f from the venom of Crotalus atrox (Western Diamondback Rattlesnake)," *Archives of Biochemistry and Biophysics*, vol. 231, no. 2, pp. 309–319, 1984.

[23] J. Gené, A. Roy, G. Rojas, J. Gutiérrez, and L. Cerdas, "Comparative study on coagulant, defibrinating, fibrinolytic and fibrinogenolytic activities of Costa Rican crotaline snake venoms and their neutralization by a polyvalent antivenom," *Toxicon*, vol. 27, no. 8, pp. 841–848, 1989.

[24] L. O. Randall and J. J. Selitto, "A method for measurement of analgesic activity on inflamed tissue," *Archives Internationales de Pharmacodynamie et de Thérapie*, vol. 111, no. 4, pp. 409–419, 1957.

[25] M. R. De Queiroz, C. C. N. Mamede, K. C. Fonseca et al., "Rapid purification of a new P-I class metalloproteinase from Bothrops moojeni venom with antiplatelet activity," *BioMed Research International*, vol. 2014, Article ID 352420, 2014.

[26] R. K. Andrews and M. C. Berndt, "Snake venom modulators of platelet adhesion receptors and their ligands," *Toxicon*, vol. 38, no. 6, pp. 775–791, 2000.

[27] M. R. de Queiroz, B. B. de Sousa, D. F. da Cunha Pereira et al., "The role of platelets in hemostasis and the effects of snake venom toxins on platelet function," *Toxicon*, vol. 133, pp. 33–47, 2017.

[28] S. M. T. Serrano, J. Kim, D. Wang et al., "The cysteine-rich domain of snake venom metalloproteinases is a ligand for von Willebrand factor A domains: Role in substrate targeting," *The Journal of Biological Chemistry*, vol. 281, no. 52, pp. 39746–39756, 2006.

[29] S. M. T. Serrano, D. Wang, J. D. Shannon, A. F. M. Pinto, R. K. Polanowska-Grabowska, and J. W. Fox, "Interaction of the cysteine-rich domain of snake venom metalloproteinases with the A1 domain of von Willebrand factor promotes site-specific proteolysis of von Willebrand factor and inhibition of von Willebrand factor-mediated platelet aggregation," *FEBS Journal*, vol. 274, no. 14, pp. 3611–3621, 2007.

[30] F. S. Torres, B. Rates, M. T. Gomes et al., " Bmoo FIBMP-I: A New Fibrinogenolytic Metalloproteinase from ," *ISRN Toxicology*, vol. 2012, pp. 1–10, 2012.

[31] C. P. Bernardes, N. A. Santos-Filho, T. R. Costa et al., "Isolation and structural characterization of a new fibrin(ogen)olytic metalloproteinase from Bothrops moojeni snake venom," *Toxicon*, vol. 51, no. 4, pp. 574–584, 2008.

[32] T. Escalante, A. Rucavado, J. W. Fox, and J. M. Gutiérrez, "Key events in microvascular damage induced by snake venom hemorrhagic metalloproteinases," *Journal of Proteomics*, vol. 74, no. 9, pp. 1781–1794, 2011.

[33] L.-G. Jia, X.-M. Wang, J. D. Shannon, J. B. Bjarnason, and J. W. Fox, "Function of disintegrin-like/cysteine-rich domains of atrolysin A. Inhibition of platelet aggregation by recombinant protein and peptide antagonists," *The Journal of Biological Chemistry*, vol. 272, no. 20, pp. 13094–13102, 1997.

[34] M. T. Assakura, C. A. Silva, R. Mentele, A. C. M. Camargo, and S. M. T. Serrano, "Molecular cloning and expression of structural domains of bothropasin, a P-III metalloproteinase from the venom of Bothrops jararaca," *Toxicon*, vol. 41, no. 2, pp. 217–227, 2003.

[35] F. R. Mandelbaum, A. P. Reichel, and M. T. Assakura, "Isolation and characterization of a proteolytic enzyme from the venom of the snake Bothrops jararaca (Jararaca)," *Toxicon*, vol. 20, no. 6, pp. 955–972, 1982.

[36] S. Fujimura, K. Oshikawa, S. Terada, and E. Kimoto, "Primary structure and autoproteolysis of brevilysin H6 from the venom of Gloydius halys brevicaudus," *The Journal of Biochemistry*, vol. 128, no. 2, pp. 167–173, 2000.

[37] E. F. Sanchez, L. M. Gabriel, S. Gontijo et al., "Structural and functional characterization of a P-III metalloproteinase , leucurolysin -B, from *Bothrops leucurus* venom," *Archives of Biochemistry and Biophysics*, vol. 468, no. 2, pp. 193–204, 2007.

[38] Z. Zhu, W. Gong, X. Zhu, M. Teng, and L. Niu, "Purification, characterization and conformational analysis of a haemorrhagin from the venom of Agkistrodon acutus," *Toxicon*, vol. 35, no. 2, pp. 283–292, 1997.

[39] M. J. I. Paine, H. P. Desmond, R. D. G. Theakston, and J. M. Crampton, "Purification, cloning, and molecular characterization of a high molecular weight hemorrhagic metalloprotease, jararhagin, from *Bothrops jararaca* venom. Insights into the disintegrin gene family," *The Journal of Biological Chemistry*, vol. 267, no. 32, pp. 22869–22876, 1992.

[40] Y. Usami, Y. Fujimura, S. Miura et al., "A 28-kDa Protein with Disintegrin-like Structure (Jararhagin-C) Purified from Bothrops jararaca Venom Inhibits Collagen- and ADP-Induced Platelet Aggregation," *Biochemical and Biophysical Research Communications*, vol. 201, no. 1, pp. 331–339, 1994.

[41] J. Zang, Z. Zhu, Y. Yu et al., "Purification, partial characterization and crystallization of acucetin, a protein containing both disintegrin-like and cysteine-rich domains released by autoproteolysis of a P-III-type metalloproteinase AaH-IV from Agkistrodon acutus venom," *Acta Crystallographica - Section D Biological Crystallography*, vol. 59, no. 12, pp. 2310–2312, 2003.

[42] H. S. Selistre-de-Araujo, M. R. Cominetti, C. H. B. Terruggi et al., "Alternagin-C, a disintegrin-like protein from the venom of Bothrops alternatus, modulates $\alpha 2\beta 1$ integrin-mediated cell adhesion, migration and proliferation," *Brazilian Journal of Medical and Biological Research*, vol. 38, no. 10, pp. 1505–1511, 2005.

[43] D. A. Higuchi, M. C. Almeida, C. C. Barros et al., "Leucurogin, a new recombinant disintegrin cloned from Bothrops leucurus

(white-tailed-jararaca) with potent activity upon platelet aggregation and tumor growth," *Toxicon*, vol. 58, no. 1, pp. 123–129, 2011.

[44] I. Limam, A. Bazaa, N. Srairi-Abid et al., "Leberagin-C, A disintegrin-like/cysteine-rich protein from Macrovipera lebetina transmediterranea venom, inhibits alphavbeta3 integrin-mediated cell adhesion," *Matrix Biology*, vol. 29, no. 2, pp. 117–126, 2010.

[45] R. K. Andrews, A. S. Kamiguti, O. Berlanga, M. Leduc, R. D. G. Theakston, and S. P. Watson, "The use of snake venom toxins as tools to study platelet receptors for collagen and von Willebrand factor," *Pathophysiology of Haemostasis and Thrombosis*, vol. 31, no. 3-6, pp. 155–172, 2001.

[46] B. S. Coller and H. R. Gralnick, "Studies on the mechanism of ristocetin induced platelet agglutination. Effects of structural modification of ristocetin and vancomycin," *The Journal of Clinical Investigation*, vol. 60, no. 2, pp. 302–312, 1977.

[47] K. Jurk and B. E. Kehrel, "Platelets: Physiology and biochemistry," *Seminars in Thrombosis and Hemostasis*, vol. 31, no. 4, pp. 381–392, 2005.

[48] A. Navdaev, H. Subramanian, A. Petunin, K. J. Clemetson, S. Gambaryan, and U. Walter, "Echicetin coated polystyrene beads: A novel tool to investigate GPIb-specific platelet activation and aggregation," *PLoS ONE*, vol. 9, no. 4, Article ID e93569, 2014.

[49] A. T. Nurden, D. Didry, and J. P. Rosa, "Molecular defects of platelets in Bernard-Soulier syndrome," *Blood Cells*, vol. 9, no. 2, pp. 333–358, 1983.

[50] Z. M. Ruggeri, "Developing basic and clinical research on von Willebrand factor and von Willebrand disease," *Thrombosis and Haemostasis*, vol. 84, no. 2, pp. 147–149, 2000.

[51] M. Peng, W. Lu, L. Beviglia, S. Niewiarowski, and E. P. Kirby, "Echicetin: A snake venom protein that inhibits binding of von Willebrand factor and alboaggregins to platelet glycoprotein Ib," *Blood*, vol. 81, no. 9, pp. 2321–2328, 1993.

[52] Y. L. Chen and I. H. Tsai, "Functional and Sequence Characterization of Agkicetin, a New Glycoprotein IB Antagonist Isolated from Agkistrodon acutus Venom," *Biochemical and Biophysical Research Communications*, vol. 210, no. 2, pp. 472–477, 1995.

[53] Y. Fujimura, Y. Ikeda, S. Miura et al., "Isolation and characterization of jararaca GPIb-BP, a snake venom antagonist specific to platelet glycoprotein Ib," *Thrombosis and Haemostasis*, vol. 74, no. 2, pp. 743–750, 1995.

[54] T. Kawasaki, Y. Taniuchi, N. Hisanichi et al., "Tokaracetin, a new platelet antagonist that binds to platelet glycoprotein Ib and inhibits von Willebrand factor-dependent shear-induced platelet aggregation," *Biochemical Journal*, vol. 308, no. 3, pp. 947–953, 1995.

[55] B. S. Coller, "Anti-GPIIb-IIIa drugs: current strategies and future directions," *Thrombosis and Haemostasis*, vol. 86, no. 1, pp. 427–443, 2001.

[56] A. S. Kamiguti, C. R. M. Hay, R. D. G. Theakston, and M. Zuzel, "Insights into the mechanism of haemorrhage caused by snake venom metalloproteinases," *Toxicon*, vol. 34, no. 6, pp. 627–642, 1996.

[57] J. Hamako, T. Matsui, S. Nishida et al., "Purification and characterization of kaouthiagin, a von Willebrand factor-binding and -cleaving metalloproteinase from Naja kaouthia cobra venom," *Thrombosis and Haemostasis*, vol. 80, no. 3, pp. 499–505, 1998.

[58] W.-J. Wang and T.-F. Huang, "Purification and characterization of a novel metalloproteinase, acurhagin, from Agkistrodon acutus venom," *Thrombosis and Haemostasis*, vol. 87, no. 4, pp. 641–650, 2002.

[59] T.-F. Huang, M.-C. Chang, and C.-M. Teng, "Antiplatelet protease, kistomin, selectively cleaves human platelet glycoprotein Ib," *BBA - General Subjects*, vol. 1158, no. 3, pp. 293–299, 1993.

[60] C. M. Ward, R. K. Andrews, A. I. Smith, and M. C. Berndt, "Mocarhagin, a novel cobra venom metalloproteinase, cleaves the platelet von Willebrand factor receptor glycoprotein Ibα. Identification of the sulfated tyrosine/anionic sequence Tyr-276-Glu-282 of glycoprotein Ibα as a binding site for von Willebrand factor and α-thrombin," *Biochemistry*, vol. 35, no. 15, pp. 4929–4938, 1996.

[61] G. Xu, H. Ulrichts, S. Vauterin et al., "How does agkicetin-C bind on platelet glycoprotein Ibα and achieve its platelet effects?" *Toxicon*, vol. 45, no. 5, pp. 561–570, 2005.