UNIVERSIDADE FEDERAL DO PARANÁ

ANA CAROLINA MARTINS WILLE

AVALIAÇÃO DA ATIVIDADE DE FOSFOLIPASE-D RECOMBINANTE DO VENENO DA ARANHA MARROM (*Loxosceles intermedia*) SOBRE A PROLIFERAÇÃO, INFLUXO DE CÁLCIO E METABOLISMO DE FOSFOLIPÍDIOS EM CÉLULAS TUMORAIS.

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Wille, Ana Carolina Martins

Avaliação da atividade de fosfolipase-D recombinante do veneno da aranha marrom (*Loxosceles intermedia*) sobre a proliferação, influxo de cálcio e metabolismo de fosfolipídios em células tumorais

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Departamento de Biologia Celular e Departamento de Fisiologia
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Curitiba, 17 de Fevereiro de 2014

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“A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original”

(Albert Einstein)

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RESUMO

As fosfolipases são uma família de enzimas encontradas em várias fontes biológicas incluindo os organismos procarióticos e eucarióticos. As aranhas do gênero *Loxosceles*, conhecidas popularmente como aranhas marrons, são eficazes em produzir em seus venenos uma grande proporção de fosfolipases-D. No veneno destas aranhas, estas enzimas estão relacionadas à dermonecrose, desregulação da resposta inflamatória, hemólise, nefrotoxicidade e agregação plaquetária. As fosfolipases-D catalizam a hidrólise de diferentes fosfolipídios gerando ácido fosfatídico, ou ácido lisofosfatídico, ou ceramida-1-fosfato, importantes mediadores nos eventos de sinalizações celulares. Alterações no padrão de expressão, atividade destas moléculas ou receptores para seus produtos tem sido relacionados com o desenvolvimento de muitos tumores. Desta forma, neste trabalho foi observado o efeito de uma fosfolipase-D exógena recombinante da glândula de veneno da aranha marrom *Loxosceles intermedia* (LiRecDT1) sobre as células das linhagens de melanoma murino B16-F10 e B16-F1. Por meio de experimentos de imunoblotting em duas dimensões com anticorpo contra a fosfolipase-D recombinante LiRecDT1 foi observada reatividade imunológica cruzada para pelo menos 25 spots no veneno bruto de *L. intermedia*, indicando alto nível de expressão para diferentes isoformas de fosfolipase-D. Experimentos de cinética de degradação de lipídios mostraram que esta toxina degrada de maneira tempo dependente tanto esfingomileina, gerando ceramida-1-fosfato e colina, quanto lisofosfatidilcolina, gerando ácido lisofosfatídico e colina e mostram menor atividade contra fosfatidilcolina. Através de experimentos de imunofluorescência com anticorpos contra LiRecDT1 e usando a toxina recombinante de fusão LiRecDT1-GFP foi observado a ligação direta de LiRecDT1 na membrana de células B16-F10. Também foi observado que a toxina recombinante LiRecDT1 tem acesso e degrada fosfolipídios das membranas das células B16-F10, observado em experimentos com extratos de membranas obtidos com detergente ou *Ghosts* de membrana pela geração de colina. Usando o Fluo-4 AM, um fluoróforo permeante sensível ao cálcio, foi possível observar que o tratamento das células B16-F10 e B16-F1 com a toxina LiRecDT1 induziu um aumento da concentração de cálcio no citoplasma. Em células B16-F10 também foram avaliadas a viabilidade e a morfologia após o tratamento com a fosfolipase-D recombinante e os resultados mostraram que não houve alterações sugerindo que a entrada de cálcio não foi decorrente a danos causados à membrana das células. Baseado no que é conhecido sobre a atividade de fosfolipases-D endógenas como indutores da proliferação celular e no fato de que LiRecDT1 se liga a superfície de células B16-F10 hidrolisando fosfolipídios e gerando lipídios bioativos, foi utilizada a toxina LiRecDT1 como uma fonte exógena de fosfolipase-D em células B16-F10. O tratamento destas células com a fosfolipase-D recombinante de aranha marrom foi efetivo no aumento da sua proliferação e de maneira tempo e concentração dependentes, especialmente na presença de esfingomielina sintética no meio. Estes resultados sugerem que a fosfolipase-D de aranha marrom pode ser usada como uma ferramenta bioativa para protocolos experimentais em biologia celular.

Palavras-chave: veneno de aranha marrom; fosfolipase-D; proliferação celular; metabolismo de lipídios; influxo de cálcio.

ABSTRACT

Phospholipases are a family of enzymes found in several biological sources including prokaryotic and higher organisms. The spiders of genus *Loxosceles*, popularly known as brown spiders, are effective in producing in their venom a great proportion of phospholipases-D. In the venom of brown spiders, these enzymes are related to dermonecrosis, dysregulated inflammatory responses, hemolysis, nephrotoxicity and platelet aggregation. The phospholipase-D catalyze the hydrolysis of different phospholipids generating phosphatidic acid or lysophosphatidic acid or ceramide-1-phosphate, important mediators in cellular signaling events. Changes in expression and activity of these molecules or receptors for their products have been related with several kinds of cancer. Thus, in this work was observed the effect of exogenous recombinant phospholipase-D (LiRecDT1) from brown spider (*Loxosceles intermedia*) venom gland on cells of murine melanoma B16-F10 and B16-F1. Through two-dimensional immunoblotting, with antibody against recombinant phospholipase-D (LiRecDT1) was observed immunological cross-reactivity for at least 25 spots in crude *L. intermedia* venom, indicating high expression levels for different isoforms of phospholipase-D. Phospholipid degrading kinetic experiments showed that this toxin mainly degrades synthetic sphingomyelin in a time-dependent manner, generating ceramide-1-phosphate plus choline, as well as *lyso*phosphatidylcholine, generating *lyso*phosphatidic acid plus choline, but exhibits little activity against phosphatidylcholine. Through immunofluorescence assays with antibodies against LiRecDT1 and using a recombinant GFP- LiRecDT1 fusion protein, was observed direct binding of LiRecDT1 to the membrane of B16-F10 cells. Addicionally, was shown that recombinant phospholipase-D LiRecDT1 degrades phospholipids in detergent extracts and from ghosts of B16-F10 cells, generating choline, indicating that the enzyme can access, modulates and has activity against membrane phospholipids. Using Fluo-4, a calcium-sensitive fluorophore, was observed that treatment of B16-F1 and B16-F10 cells with phospholipase-D, induced an increase of calcium concentration in the cytoplasm of cells. In B16-F10 cells, morphology and viability were also evaluated after treatment with the recombinant phospholipase-D and the results showed no changes suggesting that calcium influx was not caused by damage of cell membrane. Based on the known endogenous activity of phospholipase-D as an inducer of cell proliferation and on the fact that LiRecDT1 binds to B16-F10 cell surface, hydrolyzing phospholipids and generating bioactive lipids, we used LiRecDT1 toxin as an exogenous source of phospholipase-D in B16-F10 cells. Treatment of the cells with recombinant phospholipase-D (LiRecDT1) was effective in increasing their proliferation in a time- and concentration dependent manner, especially in the presence of synthetic sphingomyelin in the medium. The results suggested that phospholipase-D from brown spider can be used as a bioactive tool for experimental protocols in cell biology.

Keywords: Brown spider venom; phospholipase-D; cell proliferation; calcium influx,; lipid metabolism; melanoma cells.

LISTA DE ABREVIATURAS E SIGLAS

Å: Angstrom
AH: Ácido Hialurônico
ATCC: do inglês, “American type culture collection”
B16-F1: Linhagem celular de melanoma da pele de Mus musculus
B16-F10: Linhagem celular de melanoma da pele de Mus musculus
BCIP: 5-bromo-4cloro-3-indoil fosfato
BSA: Albumina de soro bovino
C1P: Ceramida-1-fosfato
cDNA: DNA complementar
CEEA: Comissão de ética e experimentação animal
DTT: Ditiotreitol
EDTA: Ácido etilenodiaminotetraacético
EGF: Fator de crescimento epidérmico
ELISA: Ensaio imunoenzimático, do inglês, “Enzime Linked Imuno Sorbent Assay”
FGF: Fator de crescimento de fibroblastos
GFP: Proteína verde fluorescente, do inglês “green fluorescente protein”
HRF: Fator liberador de histamina
ICK: Nó cistina inibidor
IgG: Imunoglobulina do Tipo G
ILGF: Fator de crescimento tipo insulina
IPTG: isopropil-β-D-tiogalatopiranosídeo
LALP: Metalo protease do tipo astacina.
LiRecDT: Toxina recombinante dermonecrótica de *Loxosceles intermedia*
LPA: Ácido lisofosfatídico
NBT: do inglês, “nitro blue tetrazolium”
Ni-NTA: Níquel-ácido nitrilotriacético
PBS: Solução salina tamponada com fosfato, do inglês, “phosphate buffer saline”
PDGF: Fator de crescimento derivado de plaquetas
PLD: Fosfolipase-D
PMSF: Fenilmetilsulfonil Fluoreto
SDS: dudecil sulfato de sódio, do inglês “sodium dudecyl sulfate”

SDS-PAGE: eletroforese em gel de poliacrilamida com SDS, do inglês, “sodium dudecyl sulfate-poliacrilamide gel eletrophoresis”)

SFB: Soro Fetal Bovino

TCTP: Proteína tumoral traducionalmente controlada, do inglês “translationally controlled tumor protein”

TIM: Triose fosfato isomerase, do inglês “triose phosphate isomerase”

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SUMÁRIO

1. INTRODUÇÃO

O Brasil é um país de proporções continentais com várias áreas climáticas que levam a grandes variações ecológicas, formando os diferentes biomas. A variedade de biomas reflete a enorme riqueza da flora e da fauna brasileiras. Diante da enorme biodiversidade encontrada no Brasil é comum a ocorrência de plantas, animais e microrganismos capazes de produzir moléculas de toxinas naturais com função de desencorajar ou matar seus predadores. Venenos e toxinas de origem natural podem exercer extraordinários efeitos farmacológicos e há muito representam uma fonte de fascínio para o homem, como por exemplo, a utilização pelos povos indígenas do “curare” (veneno de flecha), extraídos de plantas da América do Sul (Ministério do Meio Ambiente).

Atualmente toxinas de origem natural como as encontradas nos venenos de cobras, serpentes, aranhas e escorpiões têm sido isoladas, caracterizadas e estudadas com o objetivo de buscar aplicações biotecnológicas seja para exploração industrial ou para uso terapêutico porque interagem de modo específico com seus alvos moleculares gerando respostas biológicas específicas. Um exemplo é o Captopril, um remédio contra hipertensão amplamente descrito e utilizado, cujo princípio ativo é uma molécula sintética análoga a teprotida, uma toxina descoberta e isolada do veneno da serpente brasileira jararaca, *Bothrops jararaca*, pelo médico brasileiro Sérgio Henrique Ferreira na década de 60.

Cada vez mais a idéia de usar as toxinas do veneno como ferramentas para fins biológicos está ganhando aceitação mundial, bem como o uso de novas tecnologias para a produção de toxinas isoladas e em maiores quantidades do que as obtidas diretamente dos venenos. O avanço tecnológico tem levado a melhores técnicas de purificação de proteínas; diferentes modelos para a síntese de toxinas recombinantes; visualizações estruturais de domínios moleculares, sítios de ligação ou sítios catalíticos de moléculas de interesse, obtenção de inibidores sintéticos ou agonistas e finalmente, modelos celulares e animais para o teste dos produtos obtidos (SENFF-RIBEIRO *et al.,* 2008; CHAIM *et al.,* 2011a).

No Brasil, especialmente no estado do Paraná, entre os organismos vivos produtores de venenos ganham destaque as aranhas do gênero *Loxosceles* (Figura 1), seja pelos inúmeros acidentes que causam (Gráficos 1 e 2), seja pelos efeitos ocasionados pelas toxinas do veneno após a picada (Figura 5). Em geral, no veneno desta aranha existem toxinas protéicas ou peptídeos biologicamente ativos que podem ser úteis em protocolos experimentais para farmacologia, bioquímica, biologia celular e imunologia, bem como possíveis ferramentas biotecnológicas e industriais (SENFF-RIBEIRO *et al.,* 2008).

Entre as toxinas produzidas pelas aranhas do gênero *Loxosceles*, destacam-se as fosfolipases-D. De acordo com dados da literatura estas toxinas são as principais responsáveis pelos efeitos biológicos que ocorrem nos acidentados após a picada (da SILVA *et al*., 2004, APPEL *et al*., 2005, SWANSON e VETTER, 2006). Diante destes relatos e de outros com outras fosfolipases-D, como por exemplo a Autotaxina, uma enzima amplamente produzida em muitos tipos de tumores, especialmente nos melanomas, capaz de gerar ácido lisofosfatídico à partir da moléculas de lisofosfatidilcolina e estimular a sobrevivência, proliferação, migração e mobilização de cálcio nas células, se faz necessária uma melhor compreensão mecanística sobre essas moléculas (HWANG e*t al.,* 2012; MOOLENAAR and PERRAKIS, 2012).

A elucidação das atividades biológicas das fosfolipases-D loxoscélicas com base na sua organização molecular e seu modo de ação em diferentes modelos celulares, bem como receptores celulares e a comparação destes parâmetros com outra fosfolipase-D como a autotaxina, poderão transmitir informações sobre catálise e ações mecanísticas das toxinas, fornecendo dados úteis para a concepção de inibidores ou outras aplicações bioltecnológicas.

Diante disto, neste trabalho foi avaliada a atividade de uma fosfolipase-D recombinante obtida de *Loxosceles intermedia*, denominada LiRecDT1 sobre o metabolismo lipídico, influxo de cálcio e proliferação em linhagens de melanoma murino.

Figura 1: Aranha do gênero *Loxosceles.* Teia em forma de algodão desordenada e irregular. Modificada à partir de CABRERIZO *et al*., 2009a.

1.1 As fosfolipases

As fosfolipases são uma família de enzimas que hidrolizam ligações éster em fosfolipídios e são encontradas em muitos seres vivos incluindo procariotos e organismo superiores. Estas enzimas podem ser divididas em dois grupos: esterases alifáticas (A_1, A_2 e B) e fosfodiesterases (C e D) que liberam ácidos graxos e diacilglicerol ou ácido fosfatídico, respectivamente, quando fosfatidilcolina é hidrolisada (CHAVES-MOREIRA, 2011) (Figura 2).

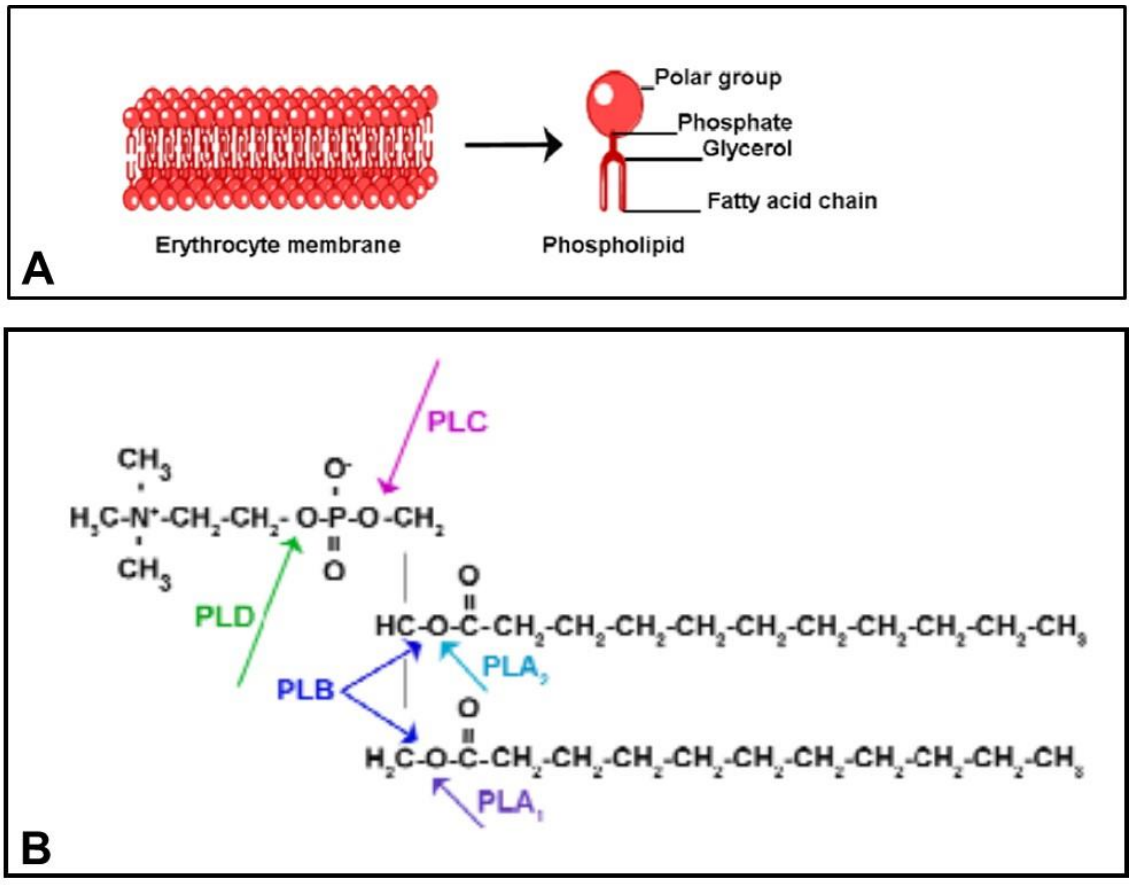


Figura 2. Fosfolipídios e sítios de clivagens das fosfolipases. (A) Representação esquemática dos fosfolipídios organizados em bicamadas nas membranas celulares e de um fosfolipídio isolado. **(B)** Locais de destino das clivagens promovidas pelas fosfolipases. Modificado a partir de CHAVES-MOREIRA *et al*., 2011.

Entre as diferentes classes de fosfolipases, as fosfolipases-D tem recebido atenção especial na literatura com base nas atividades biológicas dessas moléculas. Estas enzimas apresentam uma larga distribuição na natureza e têm sido descritas em diferentes organismos, tais como vírus, bactérias, plantas, leveduras, invertebrados e mamíferos (JENKINS e FROHMAN, 2005; RAGHU *et al.*, 2009). Nos venenos de aranhas do gênero *Loxosceles* foram caracterizadas diferentes classes de fosfolipases-D que correspondem a enzimas de massas moleculares muito próximas entre si e têm sido consideradas como isoformas de uma mesma enzima (TAVARES *et al.*, 2011). Tem sido postulado que as fosfolipases-D catalisam a hidrólise de glicerofosfolípidos ou esfingofosfolipídios, gerando ácido fosfatídico, ácido lisofosfatídico, ceramida-1-fosfato mais colina ou outras moléculas hidrofílicas, tais como serina, inositol, e etanolamina, estas moléculas ativam vias de sinalização em diferentes células, causando alterações patofisiológicas, tais como a resposta inflamatória, hemólise, doença renal aguda, agregação plaquetária e aumento da permeabilidade vascular.

2. REVISÃO BIBLIOGRÁFICA

2.1 As aranhas

O filo Arthropoda inclui os subfilos Trilobitomorpha, com todos os representantes já extintos, Crustacea, Hexapoda, Myriapoda e Chelicerata. No subfilo Chelicerata estão inclusas entre outras classes, a classe Arachnida. Dentro desta classe, a ordem Araneae é a que descreve as aranhas. (RUPPERT *et al*., 2004, GREMSKI, 2010). As aranhas são animais peçonhentos bastante comuns, possuem um par de glândulas produtoras de veneno que lançam seu conteúdo em um canal que atravessa as quelíceras, estruturas responsáveis pela inoculação do veneno durante a picada (RUPPERT *et al*., 2004).

Atualmente no mundo existem catalogadas 44032 espécies de aranhas, divididas em 3905 gêneros e 112 famílias (PLATINICK, 2013). Podem ser encontradas em quase todos os continentes com exceção da Antártida e do Ártico. Vivem nos mais diferentes ecossistemas como em buracos naturais no solo, em fendas de barrancos, em árvores e arbustos, sob troncos podres, em cupinzeiros, em bromélias, muitas vezes a grandes alturas do solo. Também podem ser encontradas em moradias humanas, em depósitos, garagens, construções (FISCHER e VASCONCELOS-NETO, 2005a).

Quase todas as espécies de aranhas são venenosas, porém algumas são incapazes de picar seres humanos pelo contato restrito ou inexistente ou por serem muito pequenas. As picadas de aranhas com morbidade significativa ocorrem devido a um número pequeno de espécies e mesmo assim o índice de óbitos é bastante baixo (WHITE *et al.*, 2003; WHITE, 2010; 2011, CHAVES-MOREIRA, 2011). Entre os gêneros de aranhas de importância médica mundialmente reconhecidos estão as dos gêneros: *Latrodectus* spp. (viúvas negras), *Loxosceles* spp. (TREVISAN-SILVA *et al.*, 2010, GREMSKI, 2010), seguido pelos gêneros *Atrax* e *Hadronyche* spp. ("funnel web spiders), localizadas na Austrália e *Phoneutria* spp. (aramadeiras), localizadas no Brasil (ISBISTER, 2004, GREMSKI, 2010).

2.2 As aranhas do gênero *Loxosceles*

As aranhas deste gênero pertencem à família *Sicariidae,* composta pelos gêneros *Sicarius* e *Loxosceles* e por 130 espécies (PLATINICK, 2013). O gênero *Loxosceles* apresenta aranhas com um colorido uniforme que varia do marrom claro (como a espécie *Loxosceles laeta*) até o marrom escuro (*Loxosceles gaucho*), por isso são popularmente denominadas de aranhas-marrons. Também podem ser conhecidas como aranhas violino por possuírem um desenho na parte superior do cefalotórax que lembra este instrumento (FUTRELL, 1992; da SILVA *et al*., 2004).

Podem ser encontradas em regiões de diferentes latitudes, e são adaptadas a distintas condições ambientais das zonas de clima tropical e temperado do globo terrestre (TAVARES *et al.*, 2011). A maioria destas aranhas estão presentes nas Américas, Índias Ocidentais e na África, algumas espécies têm sido descritas na Europa mediterrânica e China (BINFORD *et al*., 2008). Há evidências que demonstram que os gêneros *Loxosceles* e *Sicarius* se originaram na Gondwana Ocidental antes da separação dos continentes Africano e Sul-americano, de um ancestral comum sicariidae, (BINFORD *et al*., 2008). No Brasil atualmente são conhecidas 12 espécies de aranhas deste gênero (BERTANI *et al*., 2010; GONÇALVES de ANDRADE *et al*., 2012), conforme os dados contidos no mapa da Figura 3.

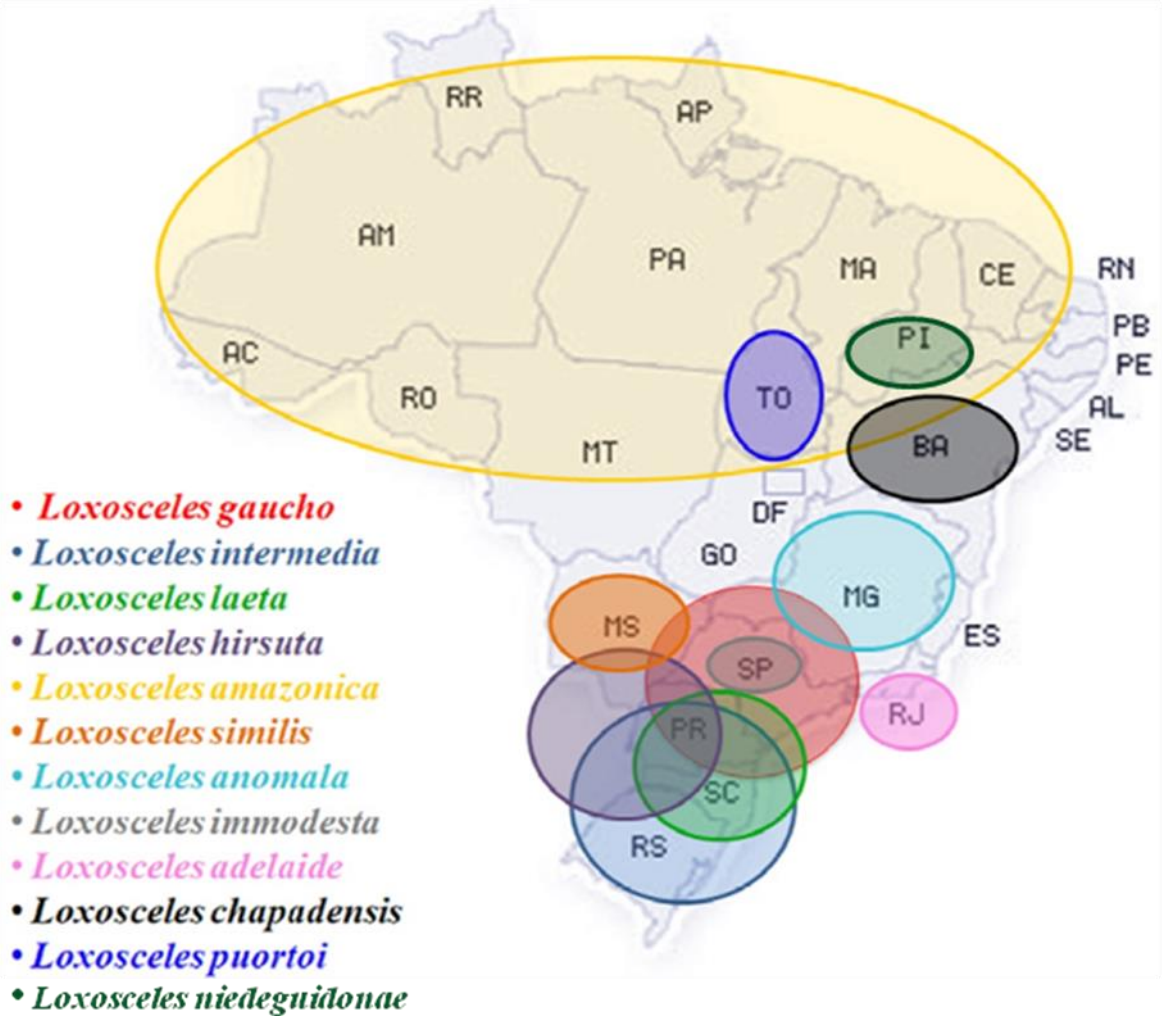


Figura 3: Distribuição geográfica das 12 espécies de aranhas do gênero *Loxosceles* encontradas no Brasil (Adaptado de CHAVES-MOREIRA, 2011).

As aranhas do gênero *Loxosceles* são pequenas, variando de 1 a 5 cm de comprimento, incluindo as pernas. Apresentam dimorfismo sexual, com as fêmeas geralmente maiores que os machos. Os machos têm palpos modificados na forma de pedipalpos com tarsos com uma estrutura adicional especializada para a transferência de esperma, o espermoporo (GILBERT, 1997, COSTA-AYUB *et al.,* 2007, MARGRAF *et al*., 2011). As aranhas do gênero *Loxosceles* possuem seis olhos dispostos em pares em semi-círculo sobre o cefalotórax, este posicionamento dos olhos tem sido descrito como o melhor meio para identificar as aranhas marrons (da SILVA *et al*., 2004; APPEL *et al*., 2005; VETTER, 2008; CHAIM *et al*., 2011a) (Figura 4). Elas constroem teias irregulares que lembram fios de algodão (da SILVA *et al*., 2004) (Figura 1).

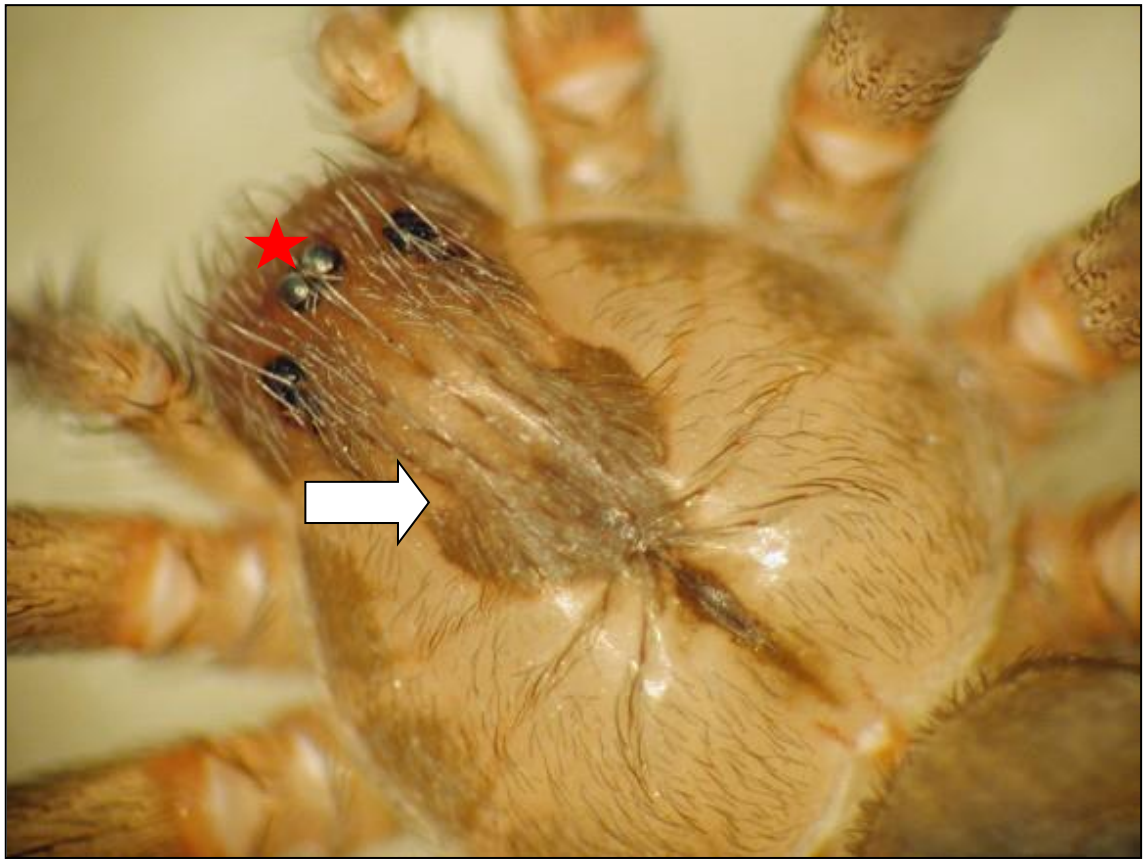


Figura 4: *Loxosceles reclusa.* Aspecto geral das aranhas do gênero *Loxosceles sp*. É possível observar uma região mais escura em forma de violino no cefalotórax (seta branca), o que lhe confere o nome popular de aranha violino e os olhos dispostos em semi-círculo (estrela vermelha). Imagem adaptada. Fonte: Image © Lisa Ames, University of Georgia (www.spiders.us/image/loxosceles-reclusa-3/Creative commons Attribution 3.0-Unported – CC BY 3.0)

As aranhas marrons podem sobreviver vários meses sem comida ou água e podem resistir a temperaturas que variam de 8 a 43 ° C (da SILVA *et al*., 2004). Segundo Fischer e Vasconcelos-Neto (2005b), a longevidade relatada pela *L. intermedia* é 1176 ± 478 dias para as fêmeas e 557 ± 87 dias para os machos.

Em geral, para a classicação das espécies deste gênero, os autores adotaram os grupos de espécies propostos por GERTSCH (1967), baseado principalmente nas características da genitália feminina e do órgão copulador masculino e propuseram quatro grupos de espécies para a América do Sul (BERTANI *et al*., 2010), de acordo com a Tabela 1 abaixo:

	Grupo amazonica	Grupo gaucho	Grupo laeta	Grupo spadicea
CARACTERÍSTICAS:	**Machos** com tarso palpal mais curto do que a tibia; **Fêmeas** com espermatecas com um grupo de pequenos lóbulos globulares no vértice	**Machos:** A tibia e o tarso palpal são equivalentes em comprimento (exceto *L. chapadensis*) **Fêmeas** tem uma placa esclerotizada transversalmente ligada as espermatecas.	**Machos:** A tíbia palpal é duas vezes maior do que tarso. **Fêmeas** as espermatecas variam, mas em geral elas são muito longas com receptáculos próximos e livres	**Machos:** têm um bulbo esférico e um embolo com carina na base. **Fêmeas:** espermateca com ductos epigênios pequenos bem-separados
ESPÉCIES:	***L. amazonica*** Gertsch, 1967 (Brasil)	***L.adelaida*** Gertsch, 1967 (Brasil)	***L.laeta*** (Nicolet, 1849) (Finlândia e Austrália);	***L.hirsuta*** Mello-Leitão, 1931 (Brasil, Paraguai, Argentina),
		L.gaucho Gertsch, 1967 (Brasil, Tunisia)	***L.accepta*** Gertsch, 1967,(Peru)	***L. intermedia*** Mello-Leitão, 1934 (Brasil, Argentina)
		L. similis Moenkhaus, (Brasil)	***L.alicea*** Gertsch, 1967, *(Peru)*	***L. spadicea*** Simon, 1907 (Peru, Bolívia, Argentina)
		L. variegata Simon, 1897 (Paraguai)	***L.bettyae*** Gertsch, 1967, *(Peru)*	
		L.chapadensis Bertani e Nagahama, 2010 (Brasil)	***L. conococha*** Gertsch, 1967, *(Peru)*	
			L.frizzelli Gertsch, 1967, *(Peru)*	
			L.harrietae Gertsch, 1967, *(Peru)*	
			L. herreri Gertsch, 1967, *(Peru)*	
			L.inca Gertsch, 1967, *(Peru)*	
			L.julia Gertsch, 1967, *(Peru)*	
			L.olmea Gertsch, 1967, *(Peru)*	
			L. piura Gertsch,1967,*(Peru)*	
			L.pucara Gertsch, 1967, *(Peru)*	
			L.surca Gertsch, 1967, *(Peru)*	
			L.gloria Gertsch, 1967(Peru e Equador),	
			L.taeniopalpis Simon, 1907 (Equador)	
			L.lutea Keyserling, 1877 (Colombia, Equador),	
			L.rulpes (Lucas, 1834) (Guatemala e Colombia),	
			L. lawrencei Caporiacco, 1955 (Venezuela, Trinidade, Curaçao)	
			L.panama Gertsch, 1958 (Panama),	
			L.coquimbo Gertsch, 1967 (Chile e Brasil)	
			L.puortoi Martins, Knysak & Bertani, 2002 (Brasil, Paraguai e Argentina)	

Tabela 1: Tabela de classificação das espécies do gênero *Loxosceles* da América do Sul baseado nas características da genitália da fêmeas e no órgão copulador dos machos de acordo com Gertsch (1967).

2.3 Epidemiologia

Cinco espécies do gênero *Loxosceles* (*L. rufescens , L. laeta , L. intermedia, L. gaucho e L. reclusa*) são responsáveis pela maioria dos casos de envenenamento em seres humanos, no entanto acidentes esporádicos causados por outras espécies do gênero Loxosceles (*L. deserta , L. arizonica , L. anomala*) têm sido descritas em várias regiões do globo (HOGAN *et al*., 2004 ; da SILVA *et al*., 2004; SWANSON e VETTER, 2006; BUCARETCHI *et al*., 2010; ISBISTER e FAN , 2011). Provavelmente o número de casos de loxoscelismo é substimado porque a maioria dos casos não são notificados (da SILVA *et al*., 2004; . HOGAN *et al*., 2004; DYACHENKO *et al*., 2006; ABDULKADER *et al*., 2008; MAKRIS *et al*., 2009; PIPPIRS *et al*., 2009; PERNET *et al*., 2010; BAJIN *et al*., 2011; LANE, *et al*., 2011; SÁNCHEZ-OLIVAS *et al*., 2011; HUGUET *et al*., 2012; RIBUFFO *et al*., 2012).

Diante dos danos causados as vítimas, as picadas de aranhas *Loxosceles* são considerados um problema de saúde pública em países como Brasil, Chile e Peru por causa de sua freqüência e morbidade associada (da SILVA *et al*., 2004; HOGAN *et al*., 2004; ZAMBRANO *et al*., 2005; SWANSON e VETTER, 2006; de SOUZA *et al*., 2008; MANRÍQUEZ e SILVA, 2009; VETTER, 2009; ISBISTER e FAN, 2011; MALAQUE *et al*., 2011).

No Brasil, no ano de 2012 foram notificados 7528 acidentes causados por aranhas do gênero *Loxosceles*, sendo 4130 registrados no estado do Paraná, destes 1967 ocorreram na cidade de Curitiba (Ministério da Saúde). Estes dados mostram que Curitiba e região metropolitana são áreas endêmicas para acidentes com aranhas marrons (Gráficos 1 e 2).

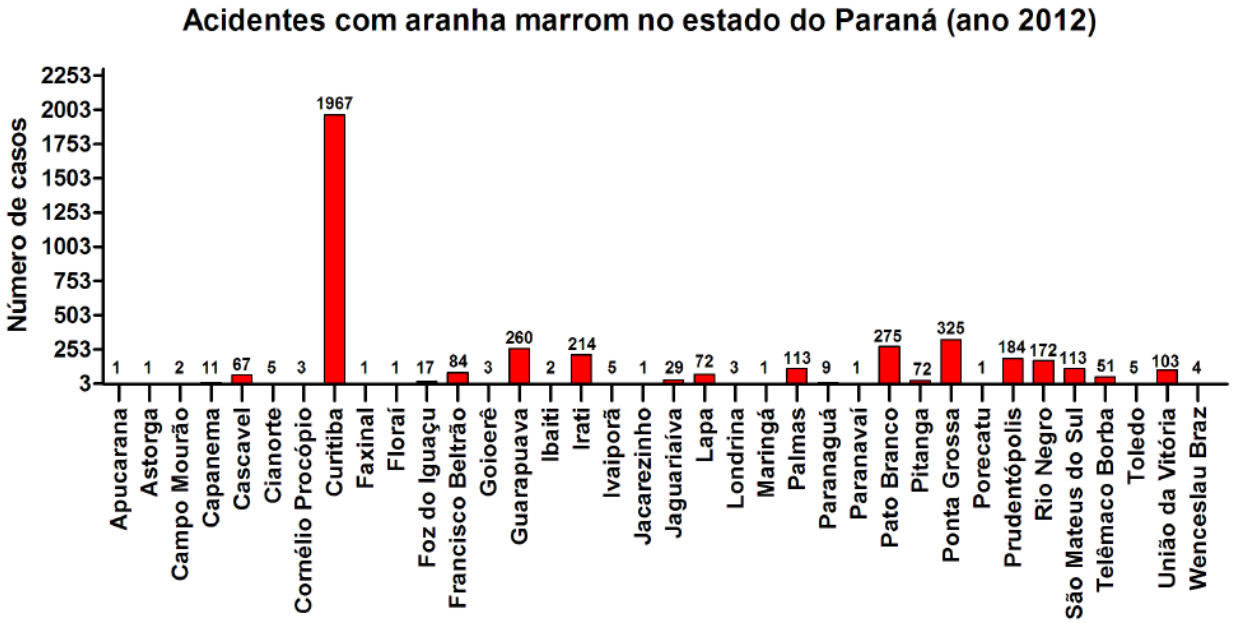


Gráfico 1: Número de acidentes com aranhas *Loxosceles sp.* notificados entre janeiro e dezembro de 2012 nas regionais de saúde do Estado do Paraná. Dados atualizados em 07/10/2013. Fonte: Ministério da Saúde/SVS – Sistema de Informação de Agravos de Notificação – Sinan Net.

Nas áreas urbanas dos estados do Paraná e Santa Catarina a espécie predominante é a *L. intermedia*; tal fato pode estar associado ao desequilíbrio ambiental e ausência de predadores (MARQUES-da-SILVA, 2002, MARQUES-da-SILVA e FISCHER 2005). As aranhas deslocadas de seu ambiente natural se abrigam em caixas, armários, brechas de paredes, gavetas e roupas. Como resultado, a maioria das picadas de aranha ocorrem quando as vítimas pressionam a aranha contra o seu corpo. Por essa razão, são frequentemente picadas no tronco, coxa e braço. As presas da aranha *Loxosceles* são pequenas, e é provável que o veneno seja injetado intra-epidermicamente. A picada destas aranhas ocorrem principalmente durante as estações mais quentes do ano (primavera e verão) (da SILVA *et al*., 2004; HOGAN *et al*., 2004; ISBISTER e FAN, 2011; VETTER, 2011; RADER *et al*., 2012) (Gráfico 2).

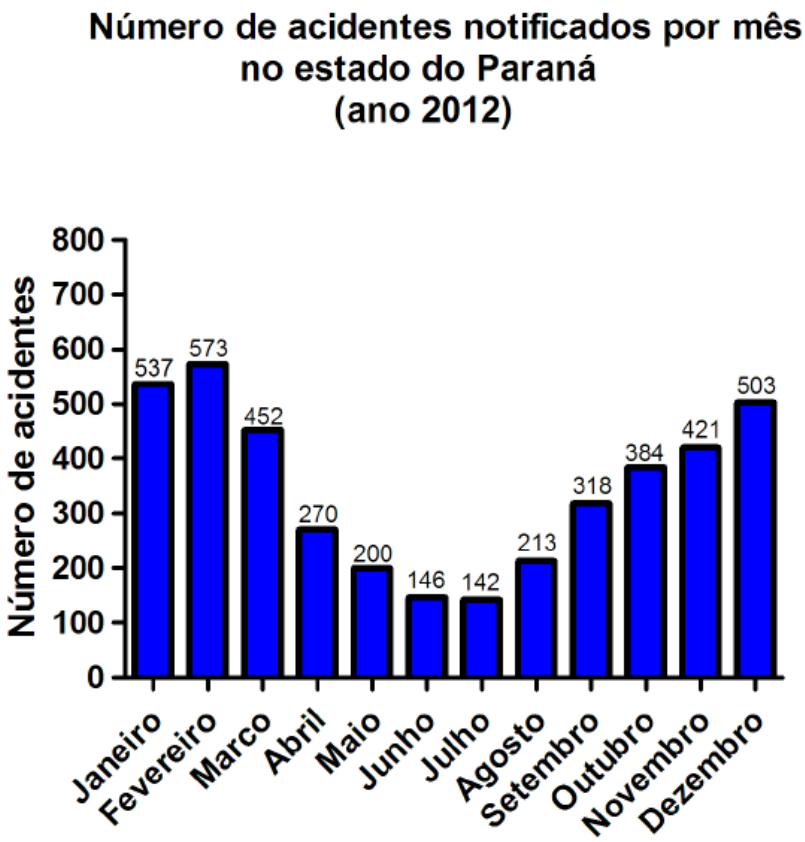


Gráfico 2: Número de acidentes com aranha marrom *Loxosceles sp.* notificados entre janeiro e dezembro de 2012 no Estado do Paraná. Dados atualizados em 07/10/2013. Fonte: Ministério da Saúde/SVS – Sistema de Informação de Agravos de Notificação – Sinan Net

Os eventos patológicos e clínicos desenvolvidos após as picadas são denominados de loxoscelismo (da SILVA *et al*., 2004; HOGAN *et al.*, 2004; SWANSON e VETTER, 2006; ISBISTER e FAN, 2011) e caracterizados por dermonecrose com espalhamento gravitacional próximo ao local da picada e em menor grau com toxicidade sistêmica, tais como alterações hematológicas como hemólise intravascular, trombocitopenia, coagulação intravascular disseminada e falência renal aguda (da SILVA *et al*., 2004).

Diante destes dados justifica-se a necessidade crescente de estudos que envolvam a biologia destas aranhas, as toxinas dos seus venenos, os efeitos biológicos destas toxinas, visto que poderão contribuir para a prevenção e compreensão dos mecanismos de ação das toxinas durante o envenenamento.

2.4 Loxoscelismo

O loxoscelismo pode gerar dois tipos de quadros clínicos distintos: o quadro cutâneo ou dermonecrótico (mais de 70% dos casos) e o quadro cutâneo-visceral ou sistêmico (0,7-27% dos casos) (BARBARO e CARDOSO, 2003 , HOGAN *et al*., 2004; ALBDULKADER *et al*., 2008, ISBISTER e FAN , 2011).

2.4.1 Loxoscelismo cutâneo

Inicialmente, as picadas por aranhas do gênero *Loxosceles* podem passar despercebidas pela vítimas, são geralmente indolores, causando em certos casos apenas um leve ardor no momento da picada. Os sinais e sintomas clínicos podem ser percebidos apenas entre 12 a 24 horas após a picada, quando já podem ser observados danos na pele. Após 2 a 8 horas, surge a dor local, tipo ardência ou “queimação”, podendo ocorrer também prurido, edema, mal-estar geral e febre. Ocorre uma reação inflamatória intensa no local da picada e após vários dias forma-se uma área de necrose com uma lesão circulada por halo vermelho, com uma zona pálida central, chamada de placa marmórea (FUTRELL, 1992) (Figura 5). Estas feridas necróticas podem levar várias semanas para cicatrizar (da SILVA *et al*., 2004; HOGAN *et al*., 2004 ; ISBISTER e FAN , 2011).

Em certos casos, a lesão cutânea necrótica evolui em 2 a 6 semanas para formação de uma escara de difícil cicatrização, podendo originar sequelas deformantes. Microrganismos presentes nas quelíceras das aranhas, como por exemplo o *Clostridium perfringens*, um bacilo gram positivo anaeróbio, pode ser o agente responsável pelo agravamento da lesão, por gerar uma infecção secundária (MONTEIRO, *et al.*, 2002).

Experimentos *in vivo*, utilizando coelho como modelo mostraram que os venenos de *Loxosceles spp.* estão associados com o desenvolvimento de uma lesão dermonecrótica, com espalhamento gravitacional e equimoses. As análises da pele de coelhos expostos a venenos de *Loxosceles* mostraram as seguintes características: um edema inicial sob a derme, aumento da permeabilidade vascular, deposição intravascular de rede de fibrina, trombose dos vasos

sanguíneos da derme e degeneração das paredes dos vasos sanguíneos, assim como a infiltração e agregação de células inflamatórias. Às vezes, em períodos mais prolongados de exposição, podem ocorrer mionecrose das miofibrilas e infiltração de leucócitos no músculo esquelético. Finalmente podem ser observados, a destruição da integridade da epiderme, hemorragia e necrose de colágeno em torno do local e perto da epiderme (OSPEDAL *et al*., 2002; TAVARES *et al*., 2004; PRETEL *et al*., 2005; SILVESTRE *et al*., 2005; CHATZAKI *et al*., 2012).

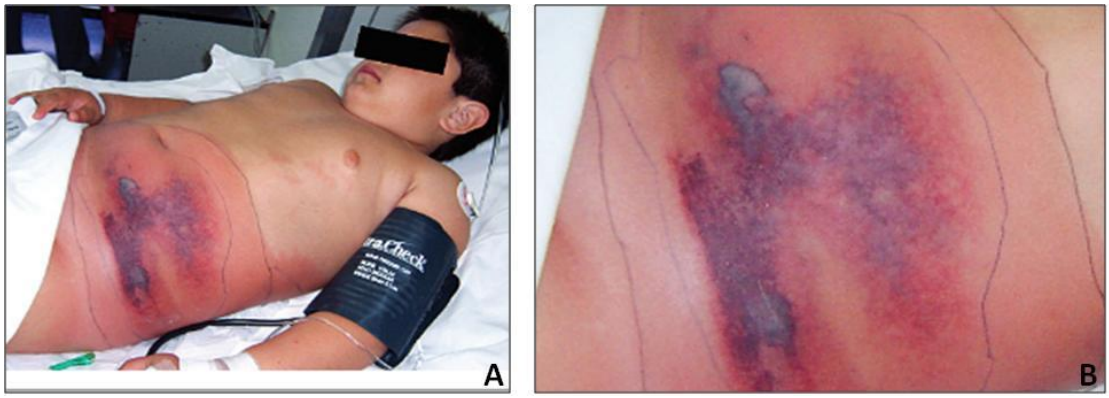


Figura 5: Lesão cutânea causada por cidente loxoscélico. (A) Paciente de 6 anos com lesão cutânea típica de acidente loxoscélico. **(B)** Placa marmórea após 36 horas do acidente. Adaptado de CABRERIZO *et al*., 2009a,b.

2.4.2 Loxoscelismo cutâneo-visceral ou sistêmico

Os casos mais graves de loxoscelismo são classificados como loxoscelismo cutâneo-visceral ou sistêmico e atingem de 0,7% à 27% dos casos, variando geograficamente ou de acordo com espécie de aranha envolvida (BARBARO e CARDOSO, 2003, HOGAN *et al*., 2004; ALBDULKADER *et al*., 2008 , ISBISTER e FAN, 2011). Alguns dados demonstram que loxoscelismo cutâneo-visceral tem maior incidência em países como o Chile (15,7%) e Peru (27,2%) , bem como no estado brasileiro de Santa Catarina (13,1%), onde *L. laeta* é mais encontrada (da SILVA *et al*., 2004; HOGAN *et al*., 2004). Os sintomas deste quadro são muito mais graves e incluem hematúria, hemoglobinúria, icterícia, febre, náuseas, leve coagulação intravascular disseminada e falência renal aguda, que é a maior responsável pelos casos de óbito decorrente do loxoscelismo (FUTRELL, 1992, da SILVA *et al*., 2004; HOGAN *et al*., 2004, ISBISTER e FAN, 2011). A análise

histológica de rins de camundongos submetidos a ação do veneno de *Loxosceles intermedia* mostrou a presença de eritrócitos no espaço de Bowman, colapso glomerular, citotoxicidade de células tubulares e deposição de material proteináceo no lúmen tubular (LUCIANO *et al*., 2004). Sintomas como astenia, episódios eméticos, rash cutâneo, cefaléia, convulsão e coma podem ser sugestivos de alterações sistêmicas (FUTRELL, 1992). Apesar de grandes estudos de caso relatarem loxoscelismo sistêmico em todos os grupos etários, a maioria dos casos ocorrem em crianças (SCHENONE *et al*., 2001; HOSTETLER *et al*., 2003; da SILVA *et al*., 2004; HOGAN *et al*., 2004; ELBAHLAWAN *et al*., 2005; HUBBARD e JAMES, 2011; ISBISTER e FAN , 2011; TASKESEN *et al*., 2011; ROSEN *et al*., 2012).

A letalidade do veneno de aranhas *Loxosceles* também foi descrita em camundongos. MOTA e BARBARO (1995) relataram que em camundongos injetados com venenos de *L. intermedia*, *L. gaucho* e *L. laeta*, as LD50s obtidas foram 0,48, 0,74 e 1,45 mg/kg, respectivamente. APPEL *et al*., (2008) obtiveram 100% de mortalidade nos camundongos testados, nas concentrações de 50 e 100µg/kg do veneno de *L. intermedia* após 16h. SILVESTRE *et al*., (2005) encontraram uma DL50 de 0,32 mg/kg de veneno de *L. similis*, e PRETEL *et al*., (2005) relataram uma DL50 de 0,696 mg/kg para o veneno de *L. adelaida*.

2.5 O veneno loxoscélico

O veneno das aranhas do gênero *Loxosceles* é um líquido incolor e cristalino produzido a partir de duas glândulas holócrinas situadas no cefalotórax da aranha e é eliminado através de um aparelho inoculador composto de um par de quelíceras (dos SANTOS *et al*., 2000, da SILVEIRA *et al*., 2002; da SILVA *et al*., 2004) (Figura 6). A análise histológica revelou que estas glândulas são constituídas por duas camadas de fibras de músculos estriados adjacentes, uma externa e o outra interna, em contacto com uma membrana basal subjacente que separa as células musculares do epitélio secretor (dos SANTOS *et al*., 2000).

Figura 6: Par de glândulas de veneno de *L. intermedia.* Glândulas produtoras de veneno de *L. intermedia* observadas em microscópio de dissecação ou estereomicroscópio (40x). Modificada a partir de CHAIM *et al*., 2011a.

As aranhas *Loxosceles* produzem pouco volume de veneno, geralmente poucos microlitros, contendo de 20 a 200µg de proteínas (BINFORD e WELLS, 2003; da SILVA *et al*., 2004; SENFF-RIBEIRO *et al*., 2008). A quantidade e o conteúdo do veneno produzido depende de diversos fatores como a espécie, o tamanho, o sexo, a idade e o estado nutricional das aranhas.

O veneno total das aranhas *Loxosceles* ainda está sob investigação, no entanto, vários estudos têm mostrado que a sua composição bioquímica consiste de uma mistura complexa de compostos biologicamente ativos, principalmente proteínas e peptídeos com ação tóxica e/ou enzimática. (VEIGA *et al*., 2000a, GREMSKI, *et al*., 2010). Já foram descritas enzimas como fosfatase alcalina, 5' ribonucleotídeo fosfohidrolase, peptídeos com atividade inseticida, hialuronidases, fosfolipases-D, serino-proteases e metaloproteases nos venenos das diferentes espécies de *Loxosceles sp* (FUTRELL, 1992; FEITOSA *et al*., 1998, VEIGA *et al*., 2000a, 2000b, YOUNG e PINCUS, 2001, VEIGA *et al*., 2001a, da SILVA *et al*., 2004, de CASTRO *et al*., 2004, BARBARO *et al*., 2005, da SILVEIRA *et al*., 2007a, 2007b, 2007c).

As proteínas dos venenos loxoscélicos apresentam perfil eletroforético similar, com predominância de proteínas de baixa massa molecular, sendo possível separar 4 grandes grupos de proteínas no perfil eletroforético dos venenos loxoscélicos: (a) um grupo de peptídeos, que se encontra no intervalo de 5- 10 kDa, (b) um grupo constituído principalmente por toxinas dermonecróticas com massa molecular encontradas no intervalo de 30-40 kDa, (c) um grupo, com amplo espectro de massa molecular, no qual se encontram as metaloproteases e, (d) um grupo de proteínas com alta massa molecular situado no intervalo de 60-95 kDa, do qual fazem parte as serino-proteases (da SILVEIRA, *et al.*, 2002; da SILVA *et al.*, 2004; SILVESTRE *et al.*, 2005, CHAVES-MOREIRA, 2011).

Análises de transcriptomas de glândulas de veneno de difentes espécies apresentaram diferentes perfis para as diferentes famílias de toxinas expressas. Fernandes-Pedrosa e colaboradores (2008) relataram para a glândula de veneno de *L. laeta* a predominância de transcritos que codificam para as fosfolipase D. Posteriormente, Gremski e colaboradores (2010) afirmaram que na glândula de veneno de *L. intermedia* existe a predominância de transcritos que codificam pequenos peptídeos, como os peptídeos ICKs (Gráfico 3).

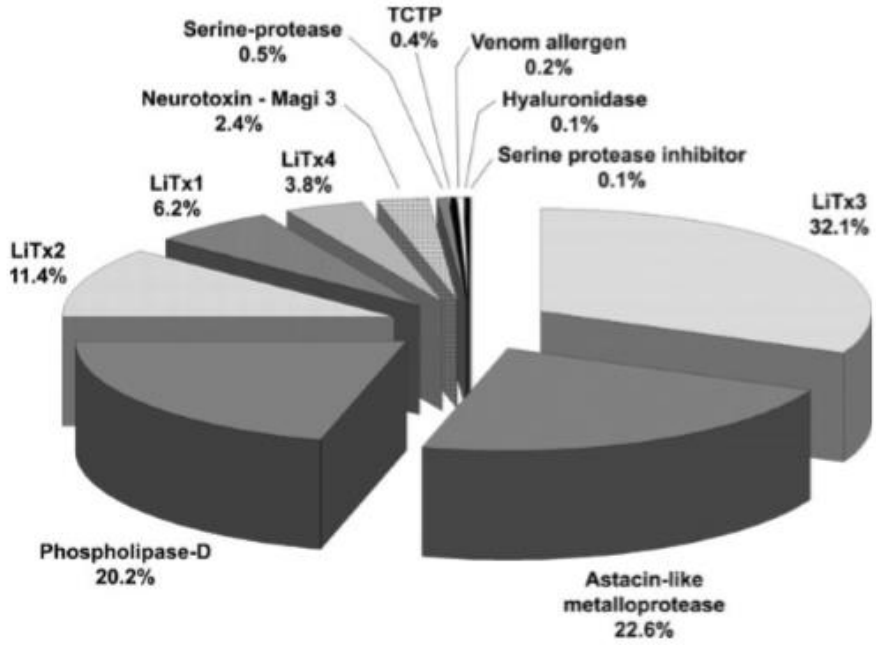


Gráfico 3: Proporções relativas de cada grupo de toxinas produzidas na glândula de veneno de *L. intermedia.* Modificada à partir de GREMSKI *et al*., 2010.

No entanto, tanto os transcriptomas das glândulas de veneno de *L. laeta* quanto de *L. intermedia* apresentaram alta expressão da fosfolipase D (PLD) e de metaloproteases. Sabendo que o principal papel do veneno dos aracnídeos é paralisar ou matar a presa e que, segundo ZOBEL-THROPP *et al*., (2012), as PLDs possuem potente atividade inseticida,, não é de estranhar que esta família de toxinas seja altamente expressa nas glândulas de veneno das aranhas marrons. Toxinas como as metaloproteases, têm sua alta expressão justificada porque podem estar envolvidas na digestão inicial de presa, nos eventos hemorrágicos do loxoscelismo ou na disseminação sistêmica de outras toxinas (da SILVEIRA *et al*., 2007b, TREVISAN-SILVA *et al*., 2010).

As fosfolipases-D (PLD) são as toxinas mais bem estudadas e bem caracterizadas dos venenos das aranhas *Loxosceles*. Esta família de toxinas têm sido denominada de Loxtox por alguns autores (de ANDRADE *et al*., 2005) ou família de toxinas dermonecróticas e correspodem a moléculas bastante conservadas. Estas toxinas, com massa molecular que varia de 30-35kDa, apresentam um peptídeo sinal e um pró-peptídeo. Catalisam a hidrólise de

glicerofosfolípidos ou esfingofosfolipídios, gerando ácido fosfatídico, ácido lisofosfatídico, ceramida-1-fosfato (C1P) mais colina ou outras moléculas hidrofílicas, tais como serina, inositol, e etanolamina (da SILVA *et al*., 2004, CHAIM *et al*, 2011b; WILLE *et al*., 2013). Estas toxinas têm sido relatadas como as principais responsáveis pelo desenvolvimento dos sinais clínicos e sintomas do loxoscelismo, promovendo agregação plaquetária, hemólise, letalidade, nefrotoxicidade e hiperpermeabilidade vascular (da SILVA *et al.*, 2004; CHAIM *et al.*, 2006; KALAPOTHAKIS *et al.*, 2007; KUSMA *et al*., 2008, SENFF-RIBEIRO *et al.*, 2008; CHAVES-MOREIRA *et al.,* 2009, PALUDO *et al.,* 2009). O grupo de pesquisa já possui clonadas sete isoformas de fosfolipase-D de *L. intermedia*, LiRecDT1 (CHAIM *et al.*, 2006), LiRecDT2 e LiRecDT3 (da SILVEIRA *et al.*, 2006), LiRecDT4 e LiRecDT5 (da SILVEIRA *et al.*, 2007a), LiRecDT6 (APPEL *et al.*, 2008) e LiRecDT7 (VUITIKA *et al.,* 2013). Estas toxinas foram clonadas a partir da análise de uma biblioteca de cDNA da glândula produtora de veneno de *L. intermedia* e identificadas por meio de sequenciamento aleatório dos clones obtidos nessa biblioteca (CHAVES-MOREIRA, 2011).

As metaloproteases encontradas nos venenos loxoscélicos fazem parte da família das astacinas, uma família de proteases pertencentes à superfamília metzincina, que são as metaloproteases dependentes de zinco (GOMIS-RUTH, 2003; STERCHI *et al*., 2008). As principais características dos membros dessa família (Merops M12A) são uma sequência consenso que formam o domínio catalítico HEXXHXXGXXHEXXRXDR - na qual três histidinas estão envolvidas na ligação do zinco (HEXXHXXGXXH), necessárias para a atividade catalítica e um “Met-turn (MXY)” na cadeia polipeptídica, promovido por um resíduo de metionina (STOCKER e BODE, 1995; STOCKER *et al.*, 1995; da SILVEIRA *et al.*, 2007b; SENFF-RIBEIRO *et al.*, 2008; TREVISAN-SILVA *et al.*, 2010).

Primeiramente, no veneno de *L. intermedia*, foram identificadas duas metaloproteases , Loxolisina A, de 20-28 kDa que degrada as cadeias Aα e Bβ de fibronectina e fibrinogênio, e Loxolisina B, uma protease 30-32 kDa com atividade gelatinolítica (FEITOSA *et al*., 1998). No veneno de *L. rufescens* também foram identificadas uma protease fibrinogenolítica de 23 kDa e uma protease gelatinolítica

de 27,5 kDa , que foram inibidas por 1,10- fenantrolina (YOUNG e PINCUS, 2001). Outras metaloproteases com atividade fibrinogenolítica também foram identificadas nos venenos de *L. reclusa* e *L. laeta* (ZANETTI *et al*., 2002). Outros componentes da matriz extracelular como entactina e proteoglicanos do tipo heparan sulfato também foram alvos para as metaloproteases loxoscélicas (VEIGA *et al*., 2000b, 2001b).

Uma seqüência que codifica uma metaloprotease do tipo astacina foi identificada pela primeira vez em uma biblioteca de cDNA de glândulas de veneno de *L. intermedia* (da SILVEIRA *et al*., 2007b). Esta sequência foi nomeada de LALP (Loxosceles protease astacin-like) que mostrou ser citotóxica em células subendoteliais de coelho e capaz de hidrolisar o fibrinogênio e fibronectina (da SILVEIRA *et al*., 2007b). Recentemente, duas novas isoformas de proteases do tipo astacinas foram identificadas no veneno de *L. intermedia* (nomeado LALP2 e LALP3), no veneno de *L. laeta* (LALP4) e de *L. gaucho* (LALP5). Estes resultados demonstram que as proteases do tipo astacinas são importantes constituintes dos venenos loxoscélicos (TREVISAN-SILVA *et al*., 2010).

No veneno de aranha-marrom (*L. intermedia*) também foram identificadas, duas serino-proteases com atividade gelatinolítica. São moléculas com alta massa molecular (85 e 95 kDa) e atividade ótima no pH entre 7.0 e 8.0 (VEIGA, *et al.*, 2000a, GREMSKI *et al.*, 2010). As serino-proteases, além da sua contribuição para a digestão da presa, podem ter uma importante função na destruição do tecido local e interferir na coagulação sanguínea e fibrinólise (VEIGA *et al*., 2000a, KINI 2005, DEVAJARA *et al*., 2008). O potencial de aplicabilidade terapêutica desta molécula é inquestionável, particularmente como droga antitrombótica com ação sobre os vasos sanguíneos (SENFF-RIBEIRO *et al*., 2008).

No transcriptoma da glândula de veneno de *L. intermedia* também foi possível identificar inibidores de serino-proteases como as serpinas (GREMSKI *et al*., 2010). As serpinas são proteínas com tipicamente 350-500 aminoácidos, distribuídas desde vírus até mamíferos. São considerados de grande interesse porque atuam como moduladores em uma variedade de funções fisiológicas como na coagulação sanguínea, fibrinólise, apoptose, desenvolvimento, inflamação e

ativação do sistema complemento (IRVING *et al*., 2000). O alinhamento da serpina identificada no transcriptoma da glândula de veneno de *L. intermedia*, mostra que a sequência tem identidade com serpinas de organismos bastante diversos, porém não apresenta similaridade com nenhuma outra serpina de venenos animais (GREMSKI *et al*., 2010).

Em 1973, Wright e colaboradores descreveram a atividade de enzimas do tipo hialuronidase no veneno de aranhas do gênero *Loxosceles*. Estas enzimas são hidrolases do tipo endo-β-N-acetil-D-hexosaminidase e exibem atividade contra ácido hialurônico (AH) e condroitin sulfato dos tipos A, B e C gerando resíduo terminal N-acetilglucosamina após a clivagem do AH. As hialuronidases podem ser encontradas em muitos venenos de animais, tais como lagartos, escorpiões, aranhas, abelhas, vespas, cobras e arraias e podem ser consideradas como "fatores de espalhamento" devido à sua capacidade de degradar os componentes da matriz extracelular para aumentar a difusão de outras toxinas a partir do local de inoculação (GIRISH e KEMPARAJU, 2005; KEMPARAJU e GIRISH, 2006; MAGALHÃES *et al*., 2008). Recentemente foi produzida a primeira hialuronidase recombinante a partir do veneno de aranhas *Loxosceles*. Esta toxina, com massa molecular em torno de 45 kDa, produzida a partir da glândula de veneno de *L. intermedia,* foi denominada de Hialuronidase de Dietrich e apresentou atividade contra ácido hialurônico e condroitin sulfato (FERRER *et al*., 2013).

Reações alérgicas causadas após as picadas por aranhas do gênero *Loxosceles* são raras (BIRCHER 2005, DONEPUDI *et al*., 2005; ROBB *et al*., 2007, MAKRIS *et al*., 2009, LANE *et al*., 2011). No entanto, pela análise de dois transcriptomas de glândulas de veneno de aranhas desse gênero foram encontradas sequências similares a toxinas alergênicas de outros venenos. No trasncriptoma de *L. laeta*, foram encontrados transcritos similares ao alérgeno III (sp|P35779|VA3_SOLRI) que representa 0.6% do total de sequências. Em 2010, Ferrer encontrou no veneno de *L. intermedia* um alérgeno composto por 145 aminoácidos com um peptídeo sinal de endereçamento para o retículo endoplasmático, típico de proteínas secretadas. Este alérgeno pertence à família *CAP* (proteínas ricas em cisteína) e que possui alta similaridade com o de espécies

de outros artrópodes como o parasita *Ixodes scapularis* e a aranha de jardim *(Lycosa singoriensis).* Esse fator alergênico pode ser capaz de desencadear reação alérgica e participar no desenvolvimento do *Rash* cutâneo observado nos acidentes com aranha marrom.

O transcriptoma da glândula de veneno de *L. intermedia* revelou a sequência de uma proteína identificada como membro da família TCTP (***T****ranslationally* ***C****ontrolled* ***T****umor* ***P****rotein)* esta proteína foi descrita por cientistas que estudam proteínas reguladas em nível de tradução (GREMSKI *et al*., 2010). Também foi demonstrado que esta proteína é um fator de liberação de histamina (HRF) (McDONALD *et al*., 1995) e fortilina (LI *et al*., 2001). Reações alérgicas, de hipersensibilidade ou dor observadas após o envenenamento podem estar associadas com eventos histaminérgicos, tais como um aumento na permeabilidade vascular e vasodilatação. As proteínas da superfamília TCTP já foram descritas nas secreções das glândulas de muitos artrópodes, tais como carrapatos ixodídeos e nas glândulas de veneno da aranha-lobo (*Lycosa godeffroyi*), a qual foi descrita como principal agente farmacológico secretado (MULENGA e AZAD, 2005; RATTMANN *et al*., 2008). A TCTP de *L. intermedia* foi clonada e expressa como uma proteína heteróloga num sistema de expressão de *E. coli*. A caracterização funcional da proteína recombinante, LiTCTP, mostrou que esta toxina causou edema e permeabilidade vascular aumentada (SADE *et al*., 2012).

Foram descritas nos venenos de animais muitos peptídeos ricos em cisteína, incluindo um motivo estrutural chamado "Nó de Cistina Inibidor" (Inhibitor Cystine Knot - ICK) (DALY e CRAIK, 2011). Estes peptídeos têm em comum três pontes dissulfeto e são compostos por três folhas β antiparalelas (CRAIK *et al*., 2001, ZHU *et al*., 2003, DALY e CRAIK, 2011). Duas das pontes dissulfeto formam um anel que é atravessado pela terceira ponte, o que forma o motivo estrutural ICK. As toxinas ICK podem ter diferentes atividades biológicas podendo atuar em canais voltagem-dependentes de sódio, potássio ou cálcio; canais mecanosensitivos; receptores nicotínicos de acetilcolina ou receptores rianodina (NICHOLSON, 2004; DUTERTRE e LEWIS, 2010). Nos venenos loxoscélicos, três peptídeos inseticidas foram inicialmente purificados, LiTx 1-3 (de CASTRO *et*

al., 2004). Fernandes-Pedrosa e colaboradores (2008) analisaram o transcriptoma de *L. laeta* e descobriram que 0,2% de todas as transcrições se pareciam com a neurotoxina ICK denominada Magi 3 de *Macrothele gigas*, um peptídeo com massa molecular de 5,2 kDa que mostrou efeito inibitório específico sobre canais de sódio de insetos (CORZO *et al*., 2003). A análise do transcriptoma da glândula de veneno de *L. intermedia* mostrou que 55,5% dos transcritos codificam toxinas que podem ser classificadas como peptídeos ICKs (GREMSKI *et al*., 2010) (Gráfico 3).

Além de proteínas, na composição dos venenos loxoscélicos são encontrados íons, sais minerais, aminoácidos livres, aminas, poliaminas neurotóxicas e neurotransmissores (ESCOUBAS *et al.*, 2000).

2.6 Fosfolipases-D (PLDs) loxoscélicas

Entre os organismos vivos que produzem fosfolipases-D, as aranhas do gênero *Loxosceles* são notáveis em produzir uma mistura de isoformas destas moléculas em seus venenos (da SILVA *et al*., 2004, KALAPOTHAKIS *et al*., 2007). As fosfolipases-D dos venenos loxoscélicos, também conhecidas como toxinas dermonecróticas, são as principais responsáveis pelo desenvolvimento da lesão dermonecrótica nos acidentados, principal evento do loxoscelismo (da SILVA *et al*., 2004, APPEL *et al*., 2005, SWANSON e VETTER, 2006).

Essas enzimas catalisam a hidrólise de fosfolipídios como esfingomielina na ligação fosfodiéster terminal para liberar colina e produzir ceramida 1-fosfato (C1P) e também fosfatidilcolina de uma maneira dependente de íon Mg^{+2} (da SILVA *et al*., 2004; van MEETEREN *et al*., 2004; CHAIM *et al*., 2011b; HORTA *et al*., 2013; WILLE *et al*., 2013).

Gremski e colaboradores (2010) revelaram que no transcriptoma da glândula de veneno de *L. intermedia* 9% dos transcritos analisados correspondem a fosfolipases-D. Estes dados estão de acordo com os descritos por Machado e colaboradores (2005), que identificaram pelo menos 11 isoformas de PLD no veneno de *L. gaucho*, denominadas Loxonecroginas, ou dados relatados por WILLE *et al*., (2013), que por eletroforese bidimensional seguido por imunoblotting encontraram no veneno de *L. intermedia* pelo menos 25 spots imunologicamente relacionados com as toxinas PLDs.

Estas toxinas são proteínas com massa molecular de 30 a 35kDa, que contém de 284 a 285 aminoácidos e incluem um peptídeo sinal seguido por um pró-peptídeo. Apresentam uma única cadeia polipeptídica que dobra-se para formar um barril distorcido onde a superfície interna do barril é revestida com oito folhas β paralelas (denominadas A-H) e por oito α-hélices (denominadas de hélices 1-8) que formam a superfície externa do barril (MURAKAMI *et al*., 2005). Este motivo estrutural foi observado pela primeira vez na estrutura da Triose Fosfato Isomerase (TIM) e é referido como um barril TIM ou como um barril (α/β)8. Estas enzimas apresentam um loop catalítico (azul), um loop variável (verde), um loop flexível (vermelho) e outros loops curtos que cercam o sítio ativo (Figura 7A). O loop catalítico contém um resíduo catalítico importante, a His47, que forma um gancho, devido à presença de uma ponte dissulfeto (Cys51-Cys57).

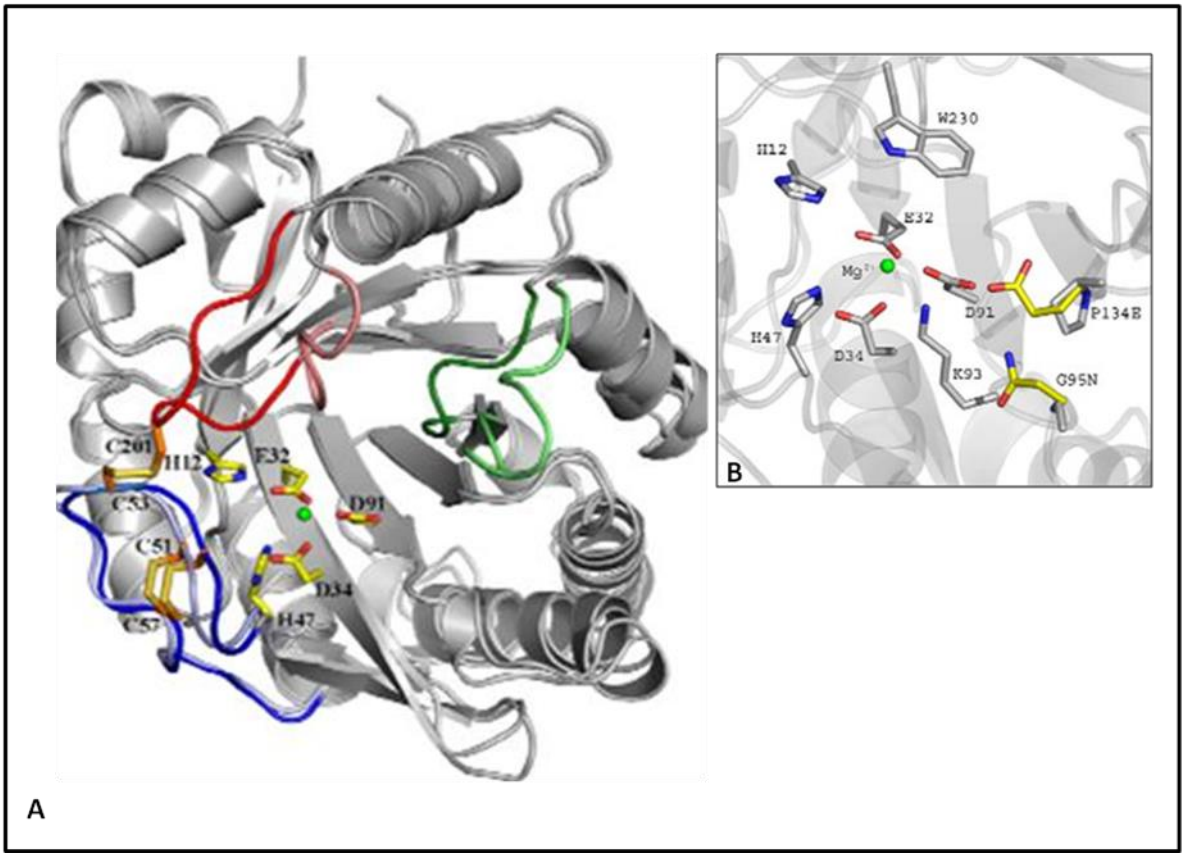


Figura 7: Estrutura de fosfolipases-D de aranhas do gênero *Loxosceles*. (A) Alinhamento estrutural entre a LiRecDT1 de *Loxosceles intermedia* (classe II) e a PLDI de *Loxosceles laeta*. Os loops catalíticos estão coloridos em azul, loops flexíveis em vermelho, loops variáveis em verde (Modificado à partir de GIUSEPPE *et al*., 2011). **(B)** Diferenças entre os aminoácidos do sítio ativo da PLD classe IIa (átomos em branco) e classe IIb (átomos em amarelo). Observar a presença do átomo de Mg^{2+} no sítio catalítico. Modificado a partir de MURAKAMI *et al*., 2006, CHAVES-MOREIRA, 2011.

As sequências de aminoácidos das fosfolipases-D são altamente conservadas (55 a 99%), especialmente nos resíduos próximos a fenda catalítica, no entanto, essas enzimas podem ser agrupadas em duas classes com base na sua sequência, estruturas e dados bioqímicos (MURAKAMI *et al*., 2006, de GIUSEPPE *et al*., 2011). A classe I representada pela PLDI de *L. laeta* é caracterizada pela presença de apenas uma ponte dissulfeto (Cys-51-Cys57) e um circuito hidrofóbico variável (loop variável). Já a classe II compreende PLDs que contêm uma ponte dissulfeto adicional (Cys53-Cys201) intra-cadeia adicional ligando o loop flexível ao loop catalítico. A classe II pode ser subdividida em duas classes dependendo da sua capacidade de hidrolisar esfingomielina, classe IIa (maior atividade catalítica) e IIb (menos ativa ou inativa) (MURAKAMI *et al*., 2005, de ANDRADE *et al*., 2006, MURAKAMI *et al*., 2006) (Figuras 7A e 7B).

O íon Mg^{2+} é essencial para a atividade catalítica dessas moléculas e seu sítio de ligação é completamente conservado em todas as fosfolipases-D do veneno de aranha marrom. Este íon é octaedricamente coordenado (com uma significativa distância média Mg^{2+}-O de 1,98 Å), equatorialmente por oxigênios carboxilados nas cadeias laterais de Glu^{32} e Asp^{34} e por duas moléculas de água fortemente ligadas e apicalmente por átomos de oxigênio carboxilados de cadeias laterais de Asp^{91} e por uma molécula de água que é também ligada ao átomo de hidrogênio do $Glu^{32}O^{\varepsilon 1}$. A estrutura da enzima é determinada pela presença da ligação de um íon sulfato, o qual é considerado por ocupar a metade da posição fosfato do substrato e é coordenado por três moléculas de água, duas das quais também coordenam o íon Mg^{2+}. O anel indol de Trp^{230} é parcialmente desordenado e provavelmente desempenha um papel na estabilização do grupo de cabeça de colina do substrato (MURAKAMI *et al*., 2005).

Com base na estrutura do cristal da fosfolipase D, foi sugerido um mecanismo catalítico dividido em dois passos nos quais His12 e His47 desempenham importantes funções. No primeiro passo a His12 atua como um nucleófilo que inicia o ataque sobre o substrato no ponto de quebra da ligação, o qual é seguido pela formação de um intermediário penta-coordenado que posteriormente é desestabilizado pela doação de um átomo de hidrogênio pela His47, levando à formação de uma molécula de colina. Em um Segundo passo da reação a His47 retira um próton da molécula de água o que inicia o ataque nucleófilo sobre o estado intermediário covalente da histidina resultando na formação de um segundo produto ceramida 1-fosfato, e o retorno para o estado inicial. O íon Mg^{2+} é importante para o reconhecimento e ligação do substrato e para uma estabilização adicional do estado intermediário do mecanismo catalítico de dois passos (MURAKAMI *et al*., 2006) (Figura 8).

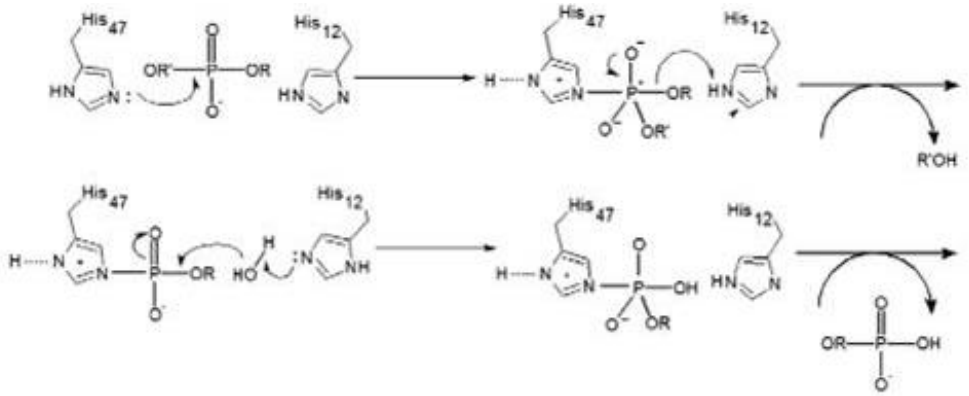


Figura 8: Mecanismo catalítico da fosfolipase-D. Hidrólise de esfingomielina envolvendo duas histidinas (His 12 e His47) encontradas no sítio catalítico da enzima. Adaptado de MURAKAMI *et al*., 2006.

Muitas isoformas de PLD do veneno de espécies de *Loxosceles* foram bem caracterizadas e clonadas (RAMOS-CERRILLO *et al*., 2004, BARBARO *et al*., 2005, MAGALHÃES *et al*., 2013). No veneno de *L. intermedia*, muitas isoformas de PLD têm sido descritas e destas nove isoformas já foram expressas como proteínas recombinantes. São capazes de reproduzir a maioria dos efeitos tóxicos observados no loxoscelismo e as propriedades antigênicas do veneno (KALAPOTHAKIS *et al*., 2002, FERNANDES-PEDROSA *et al*., 2002**,** CHAIM *et al*., 2006; da SILVEIRA *et al*., 2006, da SILVEIRA *et al*., 2007a, APPEL *et al*., 2008, VUITIKA *et al*., 2013). Tanto as formas nativas quanto as recombinantes tem sido relatadas no desenvolvimento de lesões dermonecróticas, aumento da permeabilidade vascular, aumento da resposta inflamatória no local de inoculação e em nível sistêmico, agregação plaquetária, hemólise, nefrotoxicidade e letalidade (CUNHA *et al*., 2003, APPEL *et al*., 2005, SWANSON e VETTER, 2006, SENFF-RIBEIRO *et al*., 2008; TAMBOURGI *et al*., 2010, CHAIM *et al*., 2011b).

2.7 O papel das fosfolipases-D no desenvolvimento do câncer

Atualmente é bastante conhecida na literatura a molécula de autotaxina (ATX), uma enzima ectonucleotídica do tipo fosfodiesterase pirofosfatase (E-NPP) com atividade lisofosfolipase-D, isolada pela primeira vez de células de melanoma humano e originalmente identificada como “fator de motilidade autócrino” de células tumorais (STRACKE *et al.,* 1992). Atualmente sua atividade está relacionada a vários processos fisiológicos e patofisiológicos incluindo desenvolvimento vascular e neural, transporte de linfócitos, fibrose e progressão tumoral (HOUBEN E MOOLENAAR, 2011; MOOLENAAR & PERRAKIS, 2012).

A autotaxina produz como mediador lipídico o ácido lisofosfatídico (LPA) à partir da degradação da lisofosfatidilcolina (LPC) (GIGANTI *et al*., 2008; MOOLENAAR and PERRAKIS, 2012). O LPA por sua vez atua como regulador do crescimento de muitos tipos celulares e têm sido relacionado ao crescimento e a metástase em muitos tipos de tumores (WANG *et al*. 2010). O LPA se liga a receptores específicos, denominados receptores LPA_{1-8} de superfície celular, acoplados a proteína G, os quais sinalizam para diferentes respostas biológicas (HOUBEN E MOOLENAAR, 2011; TIGYI *et al.,* 2013). A lista de respostas biológicas para LPA é diversa, dependendo do tipo de receptor LPA o resultado da estimulação resulta em: proliferação, sobrevivência, migração, remodelação do citoesqueleto e mobilização de cálcio (HOUBEN E MOOLENAAR, 2011; MOOLENAAR & PERRAKIS, 2012) (Figura 9).

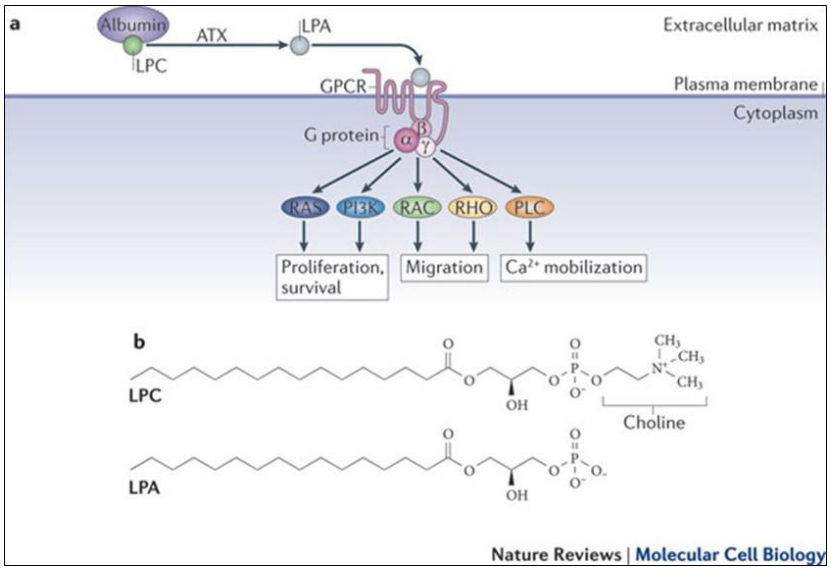


Figura 9: Hidrolise de lisofosfatidilcolina (LPC) por autotaxina (ATX) leva a produção de ácido lisofosfatídico bioativo (LPA) e ativação de diferentes cascatas de sinalização celular. (A) O LPA atua em receptores específicos (GPCRs), ligados a proteína G, distintamente distribuídos e sobrepostos entre os tecidos. Esses receptores quando ativados levam a diferentes respostas de sinalização celular que incluem: via mitogênica Ras Kinase regulada por sinal extracellular; via de sobrevivência fosfoinositol 3-Kinase (PI3K)-AKT; RHO e RAC mediadores de remodelação dos citoesqueleto e migração cellular e ativação de fosfolipase-C levando a mobilização de cálcio. **(B)** Estruturas de LPA e LPC. ATX remove o grupo cabeça colina de LPC para produzir LPA (MOOLENAAR & PERRAKIS, 2012).

Em muitos tipos de câncer a expressão de ATX ou de receptores LPA se encontram aumentados, este aumento está relacionado com a malignidade, metástase e resistência a drogas (TIGYI *et al.,* 2013). Linhagens celulares de melanoma secretam autotaxina e promovem a migração e a proliferação celular (HWANG *et al.,* 2012). Em células de cólon de camundongos a maior expressão de LPA_2 foi relacionada a carcinogênese (HOUBEN E MOOLENAAR, 2011). Em outros casos, a diminuição ou perda de função dos receptores é que foram associadas ao câncer, como no caso de câncer de bexiga, em que a perda da função LPA_6 está relacionada ao aparecimento do tumor, sugerindo que LPA_6 possa ser um suppressor tumoral (HOUBEN E MOOLENAAR, 2011). Nas células de melanoma em linhagens B16 o receptor LPA_5 é predominantemente expresso e a diminuição destes receptores por siRNA resulta numa diminuição da invasão tumoral (TIGYI *et al.,* 2013).

Com base na atividade catalítica da autotaxina, uma Lisofosfolipase-D, em gerar LPA e desta forma promover o crescimento e invasão tumoral, neste trabalho foi avaliada a atividade catalítica de uma outra fosfolipase-D, uma enzima recombinante obtida à partir da glândula de veneno da aranha marrom denominada LiRecDT1, frente a substratos lipídicos sintéticos (esfingomielina, lisofosfatidilcolina e fosfatidilcolina) e da membrana de células da linhagem de melanoma B16-F10. Os resultados mostraram que a toxina recombinante LiRecDT1 assim como a autotaxina têm atividade sobre lisofosfatidilcolina sendo capaz de gerar LPA e colina, além de degradar esfingomielina e produzir ceramida-1-fosfato e colina.

Os resultados descritos acima abriram a possibilidade da fosfolipase-D recombinante LiRecDT1 em atuar como indutores de efeitos biológicos relacionados ao desenvolvimento do câncer. Desta forma, neste trabalho também foram estudados a capacidade destas moléculas em ligar-se na superfície das células da linhagem tumoral B16-F10, promover o influxo de cálcio, desencadear aumento da proliferação além de seus efeitos sobre a viabilidade e morfologia. O influxo de cálcio também foi avaliado em células da linhagem de melanoma B16-F1, com menor potencial metastático que as células da linhagem B16-F10.

Pesquisas que elucidam o eixo Fosfolipases-D/LPA/receptores de LPA têm sido um alvo emergente que visam o desenvolvimento para terapias contra o câncer e a busca por inibidores para estas moléculas têm sido constantes. O estudo comparativo com outras fosfolipases-D com atividade Lisofosfolipase-D, como a toxina recombinante LiRecDT1 de aranha marrom, potencialmente mais estável que a autotaxina, podem ser de grande utilidade para a elucidação dos efeitos biológicos desencadeados por essas moléculas. A produção de isoformas de fosfolipases-D mais estáveis e com melhor atividade catalítica podem contribuir grandemente para muitas áreas de toxinologia e para a compreensão completa das características das PLDs assim como se abre a possibilidade do uso desta enzima como uma nova bioferramenta nos estudos sobre fosfolipídios, proliferação celular e metabolismo de cálcio, câncer além de muitas aplicações

biotecnológicas e mecanismos moleculares relacionados. Além disso, isoformas de fosfolipases-D têm sido evidenciadas como possíveis modelos para o desenvolvimento de drogas ou outras aplicações biotecnológicas (SENFF-RIBEIRO *et al*., 2008, TAMBOURGI *et al*., 2010, CHAIM *et al*., 2011a).

3. OBJETIVOS:

3.1 OBJETIVO GERAL:

- ✓ Verificar e avaliar a atividade da fosfolipase-D recombinante do veneno da aranha marrom (LiRecDT1) sobre a proliferação, influxo de cálcio e metabolismo de fosfolipídios em linhagens de melanoma murino;

3.2 OBJETIVOS ESPECÍFICOS:

- ✓ Expressar e purificar a fosfolipase-D recombinante (LiRecDT1) de *Loxosceles intermedia*;

- ✓ Detectar isoformas de fosfoslipases-D do veneno bruto de *Loxosceles intermedia* por imunoblotting a partir de eletroforese bidimensional (SDS-PAGE 2D).

- ✓ Detectar a atividade enzimática da toxina loxoscélica recombinante LiRecDT1 em substratos lipídicos sintéticos e fosfolipídios da membrana citoplasmática (*ghost* e extrato) de células B16F10;

- ✓ Verificar a capacidade da toxina LiRecDT1 em ligar-se na membrana de células da linhagem tumoral B16F10, através de ensaios de fluorescência (fluorescência direta com a toxina de fusão LiRecDT1-GFP ou imunofluorescência com anticorpos que reconhecem LiRecDT1);

- ✓ Verificar e comparar a ocorrência de influxo de cálcio entre células das linhagens tumorais B16-F10 e B16-F1 (com menor potencial metastático) e entre as toxinas recombinantes de *L. intermedia* LiRecDT1 e LiRecDT1 H12A (com menor atividade catalítica comparada a LiRecDT1) após o tratamento com toxina loxoscélica LiRecDT1;

- ✓ Estudar o efeito da fosfolipase-D loxoscélica LiRecDT1 recombinante sobre a viabilidade e a morfologia das células da linhagem de melanoma murino B16F10;

- ✓ Verificar o efeito da toxina recombinante LiRecDT1 sobre a proliferação das células B16-F10 em diferentes concentrações e tempos de tratamento e na presença de substratos lipídicos sintéticos (esfingomielina);

3. MATERIAL E MÉTODOS:

4.1 Material

4.1.1 Reagentes

Para as diversas soluções preparadas, especialmente as utilizadas com as linhagens celulares foram utilizados sais da Merck (Darmstadt, Germany). Os marcadores de massa molecular para O SDS-PAGE, o BSA, o ágar-ágar, os adjuvantes utilizados nas imunizações, o Ponceau-S, a albumina de Soro Bovino (BSA), o Hepes, a D-Glucose e os anticorpos secundários utilizados nos ensaios de “Western Blotting” foram adquiridos da Sigma (St. Louis, MI, EUA). O extrato de levedura utilizado foi provenientes da HiMedia (Mumbai, Índia). O BCIP, o NBT foram adquiridos da Promega (Madison, WI, EUA). Os reagentes SDS, corante Azul de Coomassie, Tris e Glicina foram adquiridos da GibcoBRL (Grand Island, NY, EUA). A resina Ni-NTA agarose, o Amplex Red, kit de atividade enzimática, o CyQuant, Kit para ensaio de proliferação e o Fluo4-AM foram adquiridos da Invitrogen (Carlsbad, CA, EUA). O DAPI e o Alexa Fluor 594, que reconhece anticorpos de coelho foram adquiridos da Molecular Probes (Eugene, EUA). Antibióticos utilizados para a expressão de proteínas foram adquiridos da USB (Cleaveland,OH, EUA). O Soro Fetal Bovino e os meios de cultura RPMI e DMEM foram adquiridos da Cultilab (Campinas, Brasil). Os fosfolipídios foram adquiridos da Avanti Polar Lipids (Alabaster, AL, USA). As placas (24 e 96 poços – Polysorp e Maxysorp) foram compradas da Nalge Nunc International Corporation (Naperville, IL, EUA). Garrafas para cultivo de células foram adquiridas da Nalge Nunc International Corporation (Naperville, IL, EUA) e da Jet Biofil (Guangzhou Jet Bio-Filtration Products Co., Ltd – Guangzhou, China).

4.1.2 Animais

As aranhas utilizadas para obtenção do veneno foram mantidas em condições apropriadas, com água à vontade e com restrição alimentar (alimentadas somente durante os 15 dias antecedentes à coleta do veneno para evitar contaminação com egesta). A utilização de organismos geneticamente modificados (cepas de *Eescherichia coli* BL21(DE3) pLysS) foi autorizada pela Comissão Técnica Nacional de Biossegurança (CTNBIO) (parecer técnico N° 542/2006, DOU N°79, Seção 1, página 9, 26/abril/2006 – em anexo)

4.2 Métodos

4.2.1 Esterilização de materiais

Toda vidraria utilizada nos procedimentos de cultivo celular, expressão e purificação de proteínas foram esterilizados em autoclave vertical (Phoenix, São Paulo, Brasil) à 120ºC, durante 40 minutos, à 1 atm.

4.2.2 Extração do veneno loxoscélico por eletrochoque

O veneno de *L. intermedia* foi obtido de aranhas adultas mantidas em condições apropriadas e submetidas a eletrochoque de 15 volts no cefalotórax (FEITOSA *et al*., 1998). Após a coleta, os venenos foram diluídos em PBS e mantidos à temperatura de -80ºC até o momento do uso.

4.2.3 Dosagem de proteínas

A dosagem da concentração de proteínas presentes no veneno de *L. intermedia* bem como das toxinas recombinantes foi realizada pelo método de Azul de Coomassie, como descrito por BRADFORD, 1976. A absorbância foi obtida em 610nm em leitor ELISA (Meridian, ELx 800, Auto Reader Diagnostics inc, EUA).

4.2.4 Eletroforese de proteínas em gel de poliacrilamida

As eletroforeses foram realizadas em gel de poliacrilamida (SDS-PAGE) 12,5% em condições redutoras e não redutoras como descrito por LAEMMLI e colaboradores (1970). Para observação das proteínas, os géis foram corados com azul de Coomassie 0,02% em metanol 50%, ácido acético 10% em água deionizada. Os géis foram mantidos nesta solução por vinte minutos em temperatura ambiente, sob agitação constante e descorados com sucessivas trocas de metanol 50% com ácido acético 10% em água destilada (FAIRBANKS *et al.*, 1971). Finalmente, utilizou-se marcadores de massa molecular para avaliar a altura das proteínas purificadas. Os marcadores utilizados foram miosina (200kDa), β-galactosidase (116,25 kDa), fosforilase B (97,4kDa), soroalbumina (66,2 kDa), ovoalbumina (45 kDa), anidrase carbônica (31 kDa), inibidor de tripsina (21,5kDa), lisozima (14,4 kDa), e aprotinina (6,5 kDa) adquiridos da BioRad (Hercules, CA, EUA).

4.2.5 Cultivo celular

Células B16-F10 e B16-F1 adquiridas da ATCC (American Type Culture Collection, Rockville, MD), foram cultivadas em meio RPMI contendo 40 µg/mL de sulfato de gentamicina, suplementado com 10% soro fetal bovino (SFB). As culturas foram mantidas a 37°C numa atmosfera umidificada com 5% de CO_2. As células foram soltas das garrafas de cultivo pelo tratamento com uma solução 10mM de ácido etilenodiaminotetracético (EDTA) em PBS livre de metais bivalentes durante 10 minutos. Decorrido este tempo, foram coletadas, centrifugadas, contadas e ressuspensas em meio de cultura suplementado com soro fetal bovino e deixadas para aderir e crescer até atingirem confluência.

4.2.6 Expressão e Purificação das toxinas recombinantes LiRecDT1, LiRecDT1 H12A e LiRecDT1-GFP

A expressão da proteína recombinante LiRecDT1 (número de acesso ao Genbank ABA62021) foi realizada de acordo com CHAIM *et al.,* (2006). A LiRecDT1 foi expressa em *E. coli* BL21 (DE3) pLysS (Invitrogen) como proteína de fusão, com etiqueta de 6 Histidinas no N-terminal e 13 amino ácidos ligados incluindo um sítio de ligação de trombina entre a etiqueta de 6 histidinas e a proteína madura.

A toxina mutada LiRecDT1 H12A foi obtida a partir da toxina dermonecrótica LiRecDT1 de *L. intermedia* com uso da técnica de "Megaprimer" adaptada de SAMBROOK e RUSSELL (2001) (KUSMA *et al,* 2008). A LiRecDT1 H12A possui uma mutação no primeiro resíduo de histidina (His12), que desempenha uma importante função no sítio catalítico, sendo este aminoácido substituído por uma alanina. A expressão da LiRecDT1 H12A foi realizada da mesma forma que para LiRecDT1 (descrita acima).

A proteína recombinante LiRecDT1-GFP foi obtida por subclonagem com a construção prévia da LiRecDT1 (CHAIM *et al.,* 2006). Nessa seqüência foi adicionada a seqüência da "green fluorescence protein" (EGFP) em pET-14b, usando-se a estratégia de *Blunt-Cut-Cut* em *Nde*I (pET-14b) e dois sítios *Bam*H I (entre LiRecDT1, EGFP e o Vetor). O Resultado foi a construção de uma proteína de fusão com uma cauda "6x His-Tag" na região N-terminal, seguida da seqüência da LiRecDT1 madura e da EGFP na região C-terminal.

Após a indução da expressão das toxinas recombinantes, as bactérias foram sedimentadas por centrifugação e rompidas por criofratura com o auxilio de Lizosima (1mg/mL), adquirida da Sigma (St. Louis, MO, USA) em tampão de ligação (fosfato de sódio 50mM, pH 8,0; NaCl 500mM; imidazol 10mM). Os materiais lisados foram centrifugados em 20.000g por 20 minutos. Posteriormente as suspensões foram levadas a uma coluna de Ni-NTA agarose para a purificação das proteínas recombinantes. As colunas foram lavadas exaustivamente em tampão de lavagem (fosfato de sódio 50mM, pH 8,0; NaCl 500mM; imidazol 20mM) até a densidade óptica (D.O.) de 0.01 em 280nm e eluídas em tampão de eluição (fosfato de sódio 20mM, pH 8,0; NaCl 500mM; imidazol 250mM). Foram obtidas frações de 1ml de cada que posteriormente foram analisados por SDS/PAGE 12% em condições redutoras. Após a purificação as toxinas recombinantes foram dosadas pelo método de Bradford, aliquotadas e mantidas à -20°C até o momento do uso (CHAIM *et al*., 2006).

4.2.7 Detecção de fosfolipases-D presentes no veneno de *L. intermedia* por imunoblotting usando anticorpos que reconhecem a toxina loxoscélica recombinante LiRecDT1 a partir de eletroforese bidimensional (2-DE)

Amostras de veneno foram preparadas a partir de 100µg de veneno de *L. intermedia* em tampão de amostra 2-DE (250µL de volume final) aplicado em 2 tiras de gel de 13cm com faixa linear de pH (3-10) (GE Healthcare Bio-Sciences Corp.) e incubadas por 12h para rehidratação das tiras. A focalização isoelétrica ocorreu na unidade IPGphor (GE) em várias etapas: 500V constante por 1h; de 500V à 1.000V por 1h; de 1.000 à 8.000V em 2h e 30min; 8.000V constante por 22min (tempo total de corrida 5h). Depois da focalização as proteínas foram submetidas à redução e alcalinização. As fitas foram lavadas primeiramente em solução de redução (6M uréia, 75mM de Tris, pH 8,8, 29,3% glicerol, 2% SDS, 0,002% de azul de bromofenol e 125mM de DTT) e posteriormente em solução de alquilação (6M uréia, 75mM de Tris, pH 8,8, 29,3% glicerol, 2% SDS, 0,002% de azul de bromofenol e 125mM de iodoacetamida).

As proteínas separadas por eletroforese 2-DE foram transferidas para membranas de nitrocelulose em 1h na voltagem constante de 100V, bloqueadas com tampão PBS/Molico 5% (p/v). Posteriormente, as membranas de nitrocelulose foram incubadas em temperatura ambiente e sob agitação constante com soro pré-imune ou hiperimune de coelho que reconhece a toxina loxoscélica recombinante LiRecDT1 (1:1000) diluídos em PBS/Molico 5% (p/v). As proteínas foram detectadas usando anticorpos secundários de cabra anti IgG de coelho conjugado com fosfatase alcalina (1:8000) (Sigma) diluídos em PBS/Molico 5% (p/v), lavadas em tampão ótimo para fosfatase alcalina e revelados com o substrato e desenvolvedor de cor BCIP/NBT.

4.2.8 Hidrólise dos fosfolipídios esfingomielina, lisofosfatidilcolina e fosfatidilcolina pela toxina loxoscélica recombinante LiRecDT1

Este ensaio foi realizado com a finalidade de verificar a atividade enzimática e a especificidade da proteína recombinante LiRecDT1 frente a diferentes substratos lipídicos. Os tempos de incubação foram de 5, 15 e 30 minutos, 1, 3, 6, 12 e 24 horas, e os substratos foram esfingomielina, lisofosfatidilcolina e fosfatidilcolina, importantes constituintes das membranas biológicas das células. Como controle negativo foi utilizada esfingomielina não incubada com a fosfolipase-D.

Neste ensaio, a atividade hidrolítica da toxina recombinante LiRecDT1 foi determinada pela medição indireta da colina liberada do substrato lipídico com auxílio do reagente 10-acetil-3,7-dihidroxifenoxazine Amplex Red® (Molecular Probes, Eugene, EUA), um reagente fluorogênico sensível para H_20_2. Primeiramente, a toxina hidrolisa a esfingomielina à ceramida-1-fosfato e colina ou a lisofosfatidilcolina a ácido lisofosfatídico e colina. A colina por sua vez, é oxidada pela enzima colina oxidase à betaina e H_20_2. Finalmente, a H_20_2 na presença da peroxidase reage com o Amplex Red estequiometricamente (1:1) gerando um produto altamente fluorescente, o Resorufina. Os ensaios foram realizados em triplicata, sendo as amostras incubadas a 37ºC em cinética temporal. Após isso, a fluorescência foi determinada em espectrofluorímetro Tecan Infinite® M200 (Tecan, Männedorf, Switzerland) usando comprimento de onda de excitação em 540nm e emissão em 570nm.

4.2.9 Avaliação da ligação da toxina LiRecDT1 na superfície de células da linhagem tumoral B16F10

Com o objetivo de verificar se a toxina LiRecDT1 era capaz de ligar-se as membranas das células B16-F10 foram feitos ensaios de fluorescência com a toxina recombinante LiRecDT1 conjugada com a proteína fluorescente GFP. As células plaqueadas em placas de 24 poços sobre lamínulas de 13mm na densidade de $0,5X10^3$ foram mantidas em cultura por um período de 5 dias. Posteriormente as células foram expostas ao tratamento com a quimera LiRecDT1-GPF na concentração de 10µg/mL ou com a solução de competição - LiRecDT1-GPF (10µg/mL) associada à toxina recombinante LiRecDT1 (100µg/mL) – ambos os tratamentos foram realizados por um período de 5 horas. Para os controles negativos, as células foram mantidas em meio de cultura na presença de GFP. Após o tratamento as células foram fixadas em paraformaldeído 4% em PBS por 30 minutos; os radicais aldeídicos foram bloqueados com solução de glicina 0,1M por 20 minutos seguido de 10 lavagens em PBS. Após a fixação, as células foram marcadas com DAPI (Molecular Probes, Eugene, EUA) para a visualização dos núcleos, montadas sobre uma lâmina e observadas em Microscópio de Fluorescência Observer Z1 (Carl Zeiss).

Também foram realizados ensaios de imunofluorescência para a verificação da ligação da toxina loxoscélica recombinante LiRecDT1 sobre as células B16F10. As células B16F10 foram plaqueadas e mantidas em cultura nas mesmas condições descritas acima. Posteriormente foram expostas ao tratamento com a toxina loxoscélica recombinante LiRecDT1 na concentração de 10µg/mL ou com a solução de competição - LiRecDT1(100µg/mL) misturada a anticorpos policlonais produzidos em coelho que reconhecem a toxina LiRecDT1 (1:1000) – ambos os tratamentos foram realizados por um período de 5 horas. Para os controles negativos as células não receberam qualquer tratamento e foram mantidas apenas em meio de cultura. Posteriormente as células foram fixadas em paraformaldeído 4% em PBS por 30 minutos; os radicais aldeídicos foram bloqueados com solução de glicina 0,1M por 20 minutos e lavadas 10 vezes em PBS. A detecção da toxina loxoscélica recombinante foi realizada pela incubação por uma hora com soro hiperimune, produzido em coelho, que reconhece a toxina recombinante LiRecDT1, seguido pela

incubação com anticorpo secundário Alexa Fluor 594 (Molecular Probes) que reconhece anticorpos de coelho. As células foram também marcadas com DAPI (Molecular Probes) para a visualização dos núcleos. Posteriormente, as lamínulas foram montadas sobre lâmina e observadas em Microscópio Invertido de Fluorescência Zeiss Axio Observer Z1 (Carl Zeiss, Germany).

4.2.10 Detecção da liberação de colina da membrana citoplasmática (*ghost*) e do extrato do *ghost* de células B16F10

Células B16F10 ($5X10^8$) foram lisadas, lavadas e centrifugadas (4X12.000*g*, 10min. 4ºC) em tampão hiposmótico, (NaH_2PO_4 5mM, PMSF 2mM, pH 8,0); o sobrenadante foi aspirado e os *ghosts* obtidos. Posteriormente, os *ghosts* foram ressuspensos em tampão de extração (Tris-HCl 50mM, NaCl 150mM, Triton X-100 0,5%), homogeneizados por 10 minutos a 4ºC e centrifugados em 20.000g por 20 minutos a 4ºC e os sobrenadantes foram coletados para uso como substratos (extrato). Tanto os *ghosts* quanto extratos foram utilizados como substratos para a toxina LiRecDT1. Os ensaios foram realizados nas mesmas condições descritas no (item 4.2.8) sendo a atividade enzimática medida pela liberação de colina que reage com Amplex Red (Molecular Probes, Eugene, EUA). Como controle negativo da reação foi utilizado *ghost* não incubado com a fosfolipase-D. A determinação da concentração protéica foi realizada utilizando-se o método de Bradford, como descrito anteriormente.

4.2.11 Influxo de cálcio gerado nas células da linhagem B16-F10 e B16-F1 após o tratamento com toxina loxoscélica recombinante LiRecDT1 ou LiRecDT1 H12A

Células das linhagens B16F10 e B16-F1 após serem soltas das garrafas foram lavadas em meio de cultura, centrifugadas e ressuspensas duas vezes em Solução de Ringer (122,5mM NaCl; 5,4mM KCl; 0,8mM $MgCl_2$; 10mM de Hepes, 11mM de glucose; 1mM de NaH_2PO_4, pH 7,4). Posteriormente as células foram incubadas por com Fluo-4 (5µM) em ácido pluorônico F-127 (0,01%) por 30minutos à 37ºC. Este indicador tem uma alta afinidade de ligação com Ca^{2+} (K_d = 345 nM) e emite um alta intensidade de fluorescência em resposta ao cálcio (>100). Subseqüentemente, as células foram novamente lavadas duas vezes com Solução de Ringer para desesterificação por 30 minutos, à temperatura ambiente. As células foram incubadas com 25µg/ml da toxina LiRecDT1 ou LiRecDT1 H12A, diluídas em Solução de Ringer contendo 2,5mM de $CaCl_2$, por 0, 5, 15, 30, 60 e 90 minutos. Como controle negativo as células foram incubadas apenas com Solução de Ringer com cálcio. Após a reação as células foram transferidas para uma placa de 96 poços, na densidade de $1X10^6$ células por poço em um volume total de 200µL. A fluorescência foi lida em Florímetro Tecan Infinite® M200 usando para excitação o comprimento de onda de 485nm e a emissão foi lida em 535nm.

Células B16F10 plaqueadas sobre lamínulas em placas de 24 poços na densidade de 2 X 10^4 células por poço foram observadas em Microscópio de Fluorescência Observer Z1 (Carl Zeiss, Germany). O Fluo-4 AM foi excitado em 488nm e emissão foi de 505nm. As imagens foram obtidas usando lente objetiva de 63X com óleo e a intensidade da fluorescência foi obtida em câmera monocromática.

4.2.12 Ação da toxina recombinante loxoscélica LiRecDT1 sobre a viabilidade e morfologia de células da linhagem de melanoma B16-F10

Células da linhagem B16F10 foram plaqueadas em placas de 24 poços (TPP, Trasadingen, Suíça), na densidade de 4 X 10^4 células por poço e deixadas aderir e crescer durante 24 horas. Posteriormente foram incubadas com a toxina recombinante LiRecDT1 nas concentrações de 100, 200 e 300µg/mL por 24, 48 e 72 horas em hexaplicata. Após a incubação com a toxina, a viabilidade celular foi determinada pelo método de exclusão Azul de Tripan (Merck, Darmstadt, Alemanha), como descrito por FRESHNEY (2000). As mesmas condições experimentais foram aplicadas ao grupo controle, exceto que o meio continha PBS em vez de toxina. A viabilidade celular do grupo controle (sem toxina) foi estabelecida como 100%.

As células tratadas nas mesmas condições descritas acima foram fotografadas após 24, 48 e 72 horas usando um microscópio invertido (Leica-DMIL, Wetzlar, Germany) e as mudanças na morfologia celular foram avaliadas.

4.2.13 Efeito da fosfolipase-D recombinante de aranha marrom LiRecDT1 sobre a proliferação de células B16-F10

Devido a autaxina, uma fosfolipase-D exógena ter sido demonstrada como um potente indutor da proliferação celular, foi estudado o efeito da fosfolipase-D recombinante da aranha marrom sobre a proliferação de células B16-F10. Estas células (5 × 10^3 células/poço) foram tratadas com a fosfolipase-D recombinante LiRecDT1 (10 e 25 µg/mL) por 48 horas. Também foram feitos ensaios em que as células tratadas na concentração de 10g/mL da toxina LiRecDT1 foram avaliadas por 24, 48 e 72 horas.

Outros ensaios foram feitos em meio de cultura contendo esfingomielina exógena (5 e 10 mM), visto que a esfingomielina é um substrado para a fosfolipase-D recombinante LiRecDT1 (como demonstrado anteriormente no item 4.2.8) em que as células foram tratadas com 10µg/mL da toxina recombinante LiRecDT1 por 48 horas.

Em todos os ensaios a avaliação da proliferação celular foi realizada com o Kit de proliferação CyQuant (Molecular Probes) e a leitura da fluorescência foi obtida em um Florímetro Tecan Infinite® M200, usando para excitação o comprimento de onda de 485nm e a emissão foi lida em 535nm.

5. RESULTADOS

5.1 Gel expressão e purificação da proteína loxoscélica recombinante LiRecDT1

A expressão da proteína recombinante LiRecDT1 (número de acesso ao Genbank ABA62021) foi realizada de acordo com CHAIM *et al.,* (2006). Após a indução da expressão com IPTG as bactérias *E. coli* BL21 (DE3) pLysS (Invitrogen) foram lisadas em lisozima, centifugadas e o sobrenadante foi levado para purificação em coluna de Ni-NTA agarose. Na figura 10 abaixo é possível verificar no gel a presença da proteína expressa em diferentes etapas de expressão e purificação.

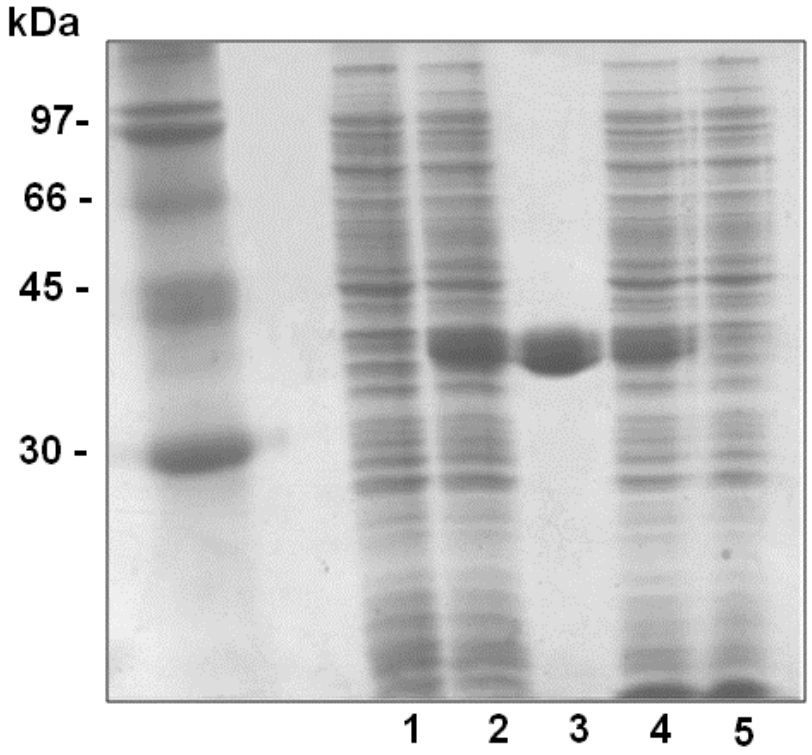


Figura 10. Purificação da proteína recombinante LiRecDT1 em resina Ni2+-NTA-agarose. À esquerda estão indicadas as massas moleculares; 1 – cultura bacteriana antes da expressão; 2 - cultura bacteriana 3,5 horas após adição de IPTG; 3 – proteína recombinante eluída da resina. 4 - lisado bacteriano contendo a fração de proteínas solúveis; 5 – *void* da cromatografia após incubação do lisado com resina Ni-NTA-agarose; SDS-PAGE 12,5% em condições redutoras.

No gel de purificação da proteína acima, é possível observar que existe pequena expressão basal da proteína pela bactéria no tempo 0h. O plasmídio pLysS reprime a expressão na ausência de IPTG, evitando que uma proteína tóxica para a bactéria seja expressa sem a presença do indutor. A ligação à resina de níquel é satisfatória, pois não é observada uma banda considerável da proteína no *void* (material que não se ligou à resina).

5.2 Fosfolipases-D presentes no veneno de *L. intermedia* foram detectadas a partir de eletroforese bidimensional (2-DE) seguido por imunoblotting com anticorpos policlonais que reconhecem a toxina loxoscélica recombinante LiRecDT1

Amostras de veneno de *L. intermedia* submetidas a focalização isoelétrica e posteriormente separadas por eletroforese 2-DE foram transferidas para membrana de nitrocelulose para realização de imunoblotting. As membranas foram primeiramente incubadas com anticorpos pré e hiperimune de coelho que reconhece a toxina recombinante LiRecDT1 e a revelação ocorreu com a incubação de anticorpos secundários IgG de cabra conjugado com fosfatase alcalina que reconhecem IgG de coelho (Figura 11).

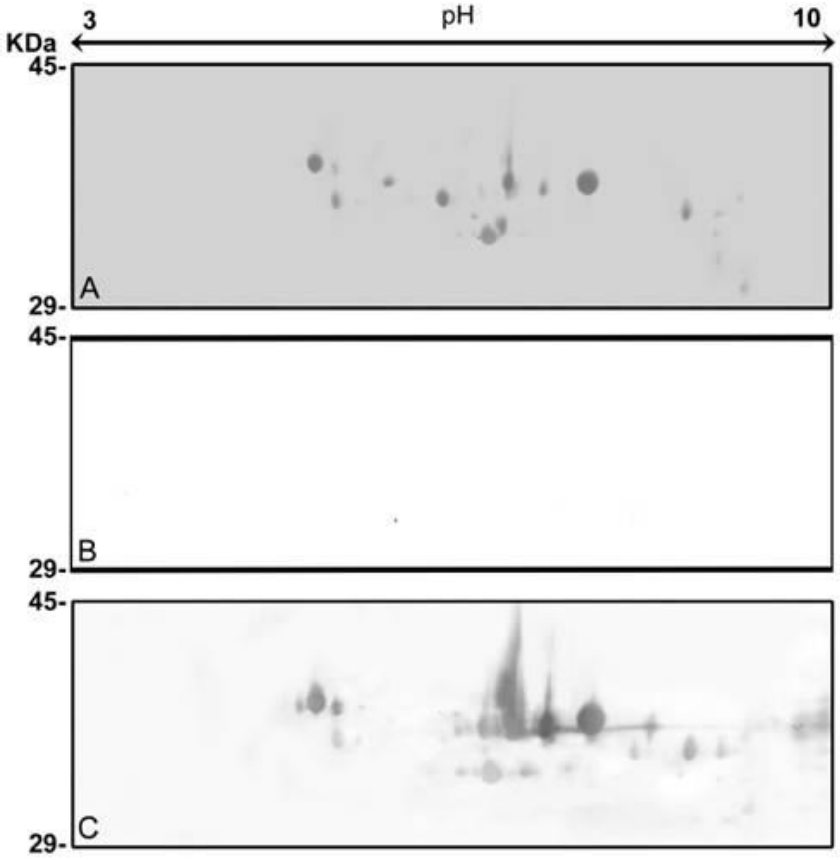


Figura 11: Perfil de imunomarcação das fosfolipases-D presentes no veneno de *L. intermedia*. Imunoblotting realizado a partir de gel 2-DE usando tiras de gel com gradiente de pH 3-10 e SDS-PAGE 12,5%. **(A)** Proteínas do veneno de *Loxosceles intermedia* coradas com Ponceau-S, 0,5%. **(B)** Imunoblotting realizado com anticorpos pré-imunes de coelho **(C)** Fosfolipases-D imunodetectadas por anticorpos que reconhecem a toxina loxoscélica recombinante LiRecDT1, seguido de reação com anticorpos que reconhecem imunoglobulinas de coelho conjugados com fosfatase alcalina. As fosfolipases-D foram observadas entre os marcadores moleculares de 29-45 KD.

O immunoblotting da eletroforese bidimensional revelou vários pontos de reconhecimento na membrana de nitrocelulose, indicando a presença de muitas isoformas de fosfolipases-D no veneno de *L. intermedia* e a especificidade dos anticorpos por essas toxinas. Os resultados obtidos apontam a existência de uma família de toxinas na espécie, antigenicamente e molecularmente relacionadas (visualizadas nos pontos escuros - spots) (Figura 11) que reforçam a idéia da importância biológica desta toxina para a biologia das aranhas *Loxosceles intermedia* e estão de acordo com o descrito por GREMSKI *et al.* (2010), que observou que no transcriptoma da glândula de veneno de *L. intermedia* as fosfolipases-D correspondem a cerca de 20% do total de RNAs transcritos para toxinas.

5.3 Os fosfolipídios esfingomielina, lisofosfatidilcolina e fosfatidilcolina foram hidrolisados pela toxina loxoscélica recombinante LiRecDT1

Neste ensaio foi avaliada a atividade enzimática da toxina loxoscélica recombinante LiRecDT1 frente a diferentes substratos lipídicos (como esfingomielina, lisofosfatidilcolina e fosfatidilcolina) em diferentes tempos de incubação. Para isso foi observada a liberação de colina, um produto gerado após a quebra dos diferentes substratos, analisados através do Kit Amplex Red (Molecular Probes, Eugene, EUA). Os resultados podem ser visualizados no Gráfico 4 abaixo.

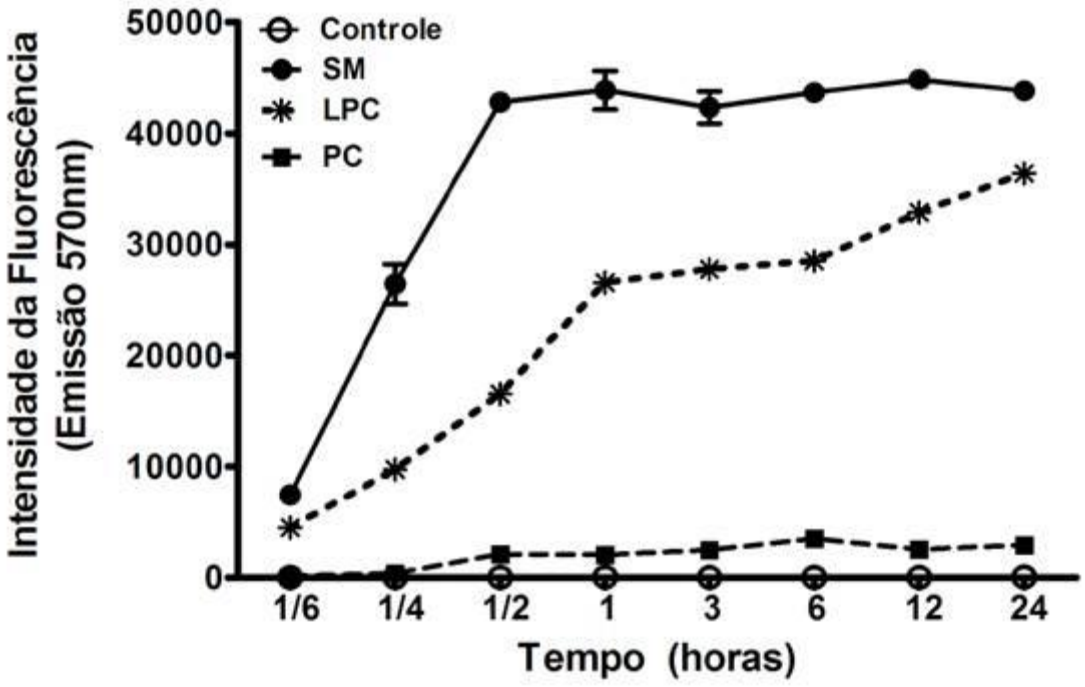
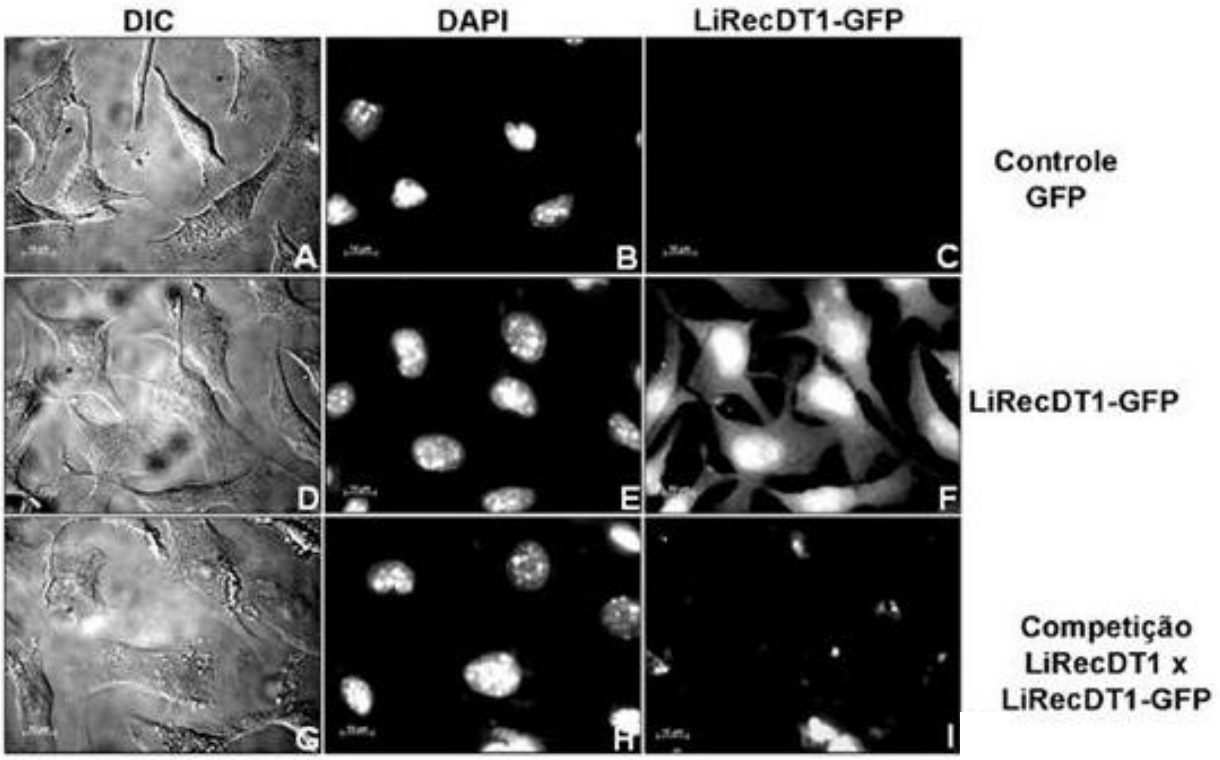


Gráfico 4: Atividade Hidrolítica de LiRecDT1. Quantificação da colina gerada indiretamente pela atividade enzimática da toxina recombinante LiRecDT1 sobre os substratos esfingomielina, lisofosfatidilcolina e fosfatidilcolina em diferentes tempos de incubação. A cinética enzimática foi demonstrada através da incubação dos substratos esfingomielina, lisofosfatidilcolina e fosfatidilcolina com a toxina recombinante LiRecDT1 por 1/6, 1/4, 1/6, 1, 3, 6, 12 e 24 horas. Como controle negativo foi utilizada esfingomielina não incubada com a fosfolipase-D. (SM: esfingomielina tratada com LiRecDT1; LPC: lisofosfatidilcolina tratada com LiRecDT1; PC: fosfatidilcolina tratada com LiRecDT1. Resultados obtidos de experimentos realizados em triplicata.

Como demonstrado no gráfico 4 a toxina recombinante LiRecDT1 purificada hidrolisou tanto esfingomielina quanto lisofosfatidilcolina, mas apresentou pouca atividade sobre a fosfatidilcolina nos diferentes tempos testados. O perfil de atividade da LiRecDT1 demonstra que esta enzima apresenta maior atividade sobre esfingomielina.

5.4 A toxina recombinante LiRecDT1 foi capaz de ligar-se as membranas das células B16-F10

Com a finalidade de observar se a toxina recombinante era capaz de ligar-se as membranas das células B16-F10 foram feitos ensaios de fluorescência com a toxina recombinante LiRecDT1 conjugada com a proteína fluorescente verde (Green fluorescent protein – GFP) e de imunofluorescência indireta. Nos ensaios de imunofluorescência as células B16-F10 após o tratamento com a toxina recombinante LiRecDT1 foram incubadas com anticorpos policlonais hiperimunes de coelho que reconhecem a toxina, seguido pela incubação com anticorpo secundário Alexa-Fluor 594 (Molecular Probes) que reconhecem anticorpos de coelho. Em ambas as situações os núcleos foram marcados com DAPI.



F**igura 12: Ligação da toxina quimérica recombinante LiRecDT1-GFP em células da linhagem B16F10. (A, B e C)** mostram células não tratadas com LiRecDT1 (controle). **(D, E e F)** células tratadas com a toxina quimérica LiRecDT1-GFP. **(G, H e I)** células tratadas com a solução de competição entre a toxina quimérica LiRecDT1-GFP e a toxina loxoscélica recombinante LiRecDT1 em excesso. **(A, D e G)** células observadas por DIC. **(B, E e H)** marcação dos núcleos com DAPI. **(C, F e I)** marcação da superfície das células com a toxina quimérica LiRecDT1-GFP. Aumento de 68X.

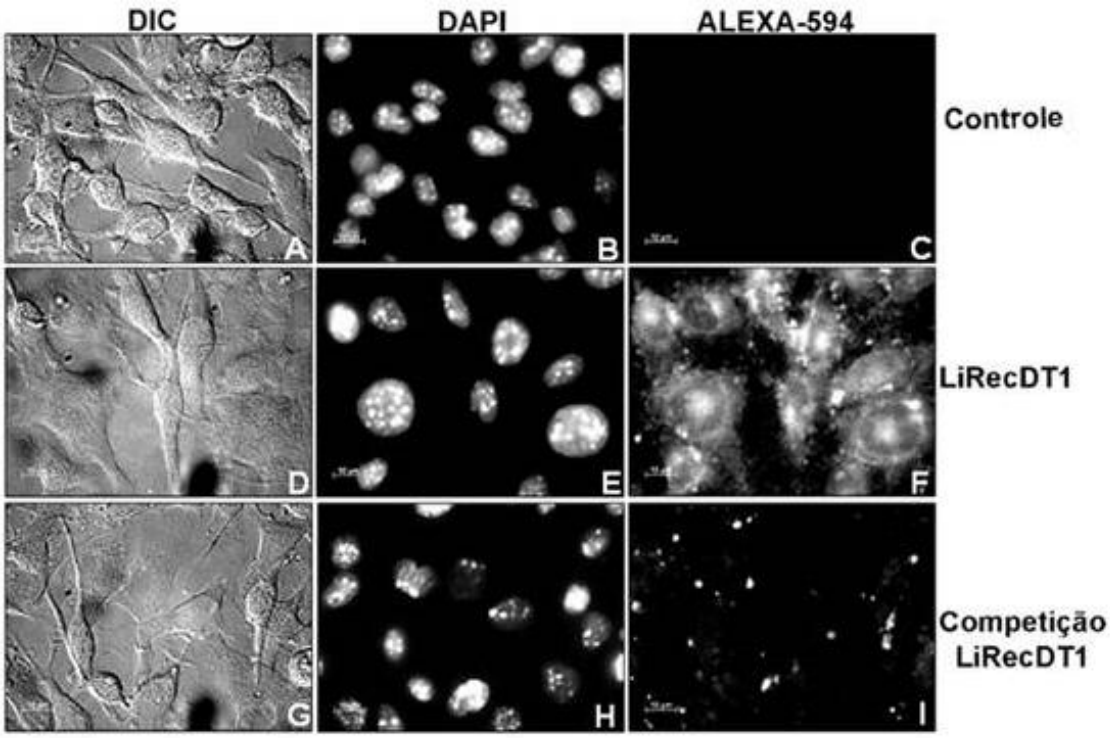


Figura 13: Análise da ligação da toxina loxoscélica recombinante LiRecDT1 em células da linhagem B16F10 por microscopia de fluorescência convencional. (A, B e C) mostram células não expostas à toxina e incubadas com o anticorpo secundário fluorescente. **(D, E e F)** células tratadas com a toxina LiRecDT1 e incubadas com anticorpos que reconhecem a toxina loxoscélica recombinante LiRecDT1 e com o conjugado fluorescente. **(G, H e I)** células tratadas com a solução de competição - LiRecDT1 associada a anticorpos policlonais produzidos em coelho que reconhecem a toxina LiRecDT1 e incubadas com o anticorpo conjugado fluorescente . **(A, D e G)** células observadas por DIC. **(B, E e H)** marcação dos núcleos com DAPI. **(C, F e I)** células imunomarcamarcadas. Aumento de 68X.

Os resultados expressos nas figuras 12 e 13 demonstraram a existência de sítios de ligação da toxina loxoscélica recombinante LiRecDT1 sobre a membrana de células B16F10 e sugerem a hipótese de atividade desta molécula sobre os constituintes da membrana desta célula. Mudanças nestas células induzidas por fosfolipases-D podem ocorrer como resultado da ligação com a membrana citoplasmática.

5.5 A fosfolipase-D recombinante de aranha marrom LiRecDT1 foi capaz de hidrolisar fosfolipídios das membranas citoplasmáticas (*ghosts*) e do extrato do *ghost* de células B16F10

Com o objetivo de avaliar a atividade enzimática da toxina recombinante LiRecDT1 sobre os fosfolipídios da membranas das células B16-F10 foram obtidos os *ghosts* e os extratos de *ghosts* o qual foram utilizados como substratos. A colina, produto gerado pela degradação do substrato foi revelada com o Kit Amplex Red (Molecular Probes, Eugene, EUA) e mensurada em espectrofluorímetro Tecan Infinite® M200 usando comprimento de onda de excitação em 540nm e emissão em 570nm Os resultados estão sendo mostrados no gráfico 5 abaixo.

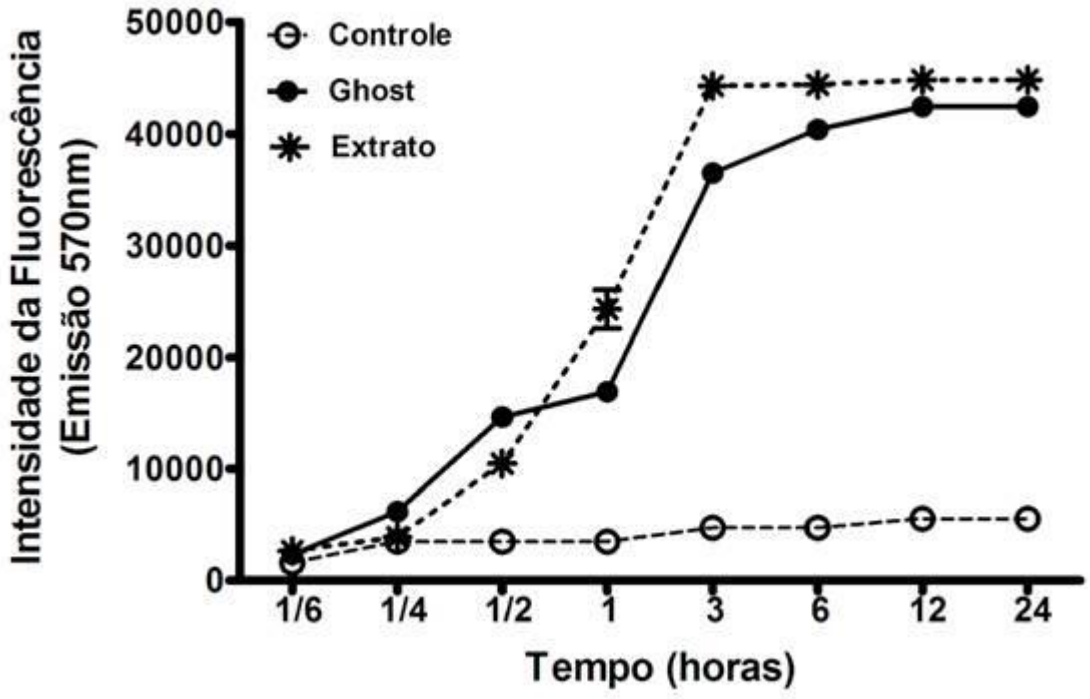


Gráfico 5: Atividade Hidrolítica de LiRecDT1. Quantificação da colina gerada indiretamente pela atividade enzimática da toxina recombinante LiRecDT1 sobre o *ghost* e o extrato de células B16F10 em diferentes tempos de incubação. A cinética enzimática foi demonstrada pela incubação do *ghost* e do extrato de células B16F10 como substratos para a toxina recombinante LiRecDT1 por 1/6, 1/4, 1/6, 1, 3, 6, 12 e 24 horas. Como controle negativo foi utilizado *ghost* não incubado com a fosfolipase-D. Resultados foram obtidos de experimentos realizados em triplicata.

Como demonstrado nos gráficos 4 e 5, os resultados obtidos por este método fluorimétrico apontam que a toxina recombinante LiRecDT1 causa a degradação tanto de fosfolipídios sintéticos (esfingomielina e lisofosfatidilcolina) quanto dos fosfolipídios organizados na membrana (*ghost* e extrato) das células B16F10 em todos os tempos analisados, especialmente após ½ hora. Os resultados estão de acordo com o demonstrado por CHAVES-MOREIRA *et al.,* (2011) em experimentos usando eritrócitos e mostram que a enzima tem acessibilidade e é capaz de hidrolisar os fosfolipídios organizados na membrana das células B16F10.

5.6 A toxina recombinante LiRecDT1 é capaz de gerar influxo de cálcio em células das linhagens B16-F10 e B16-F1

Primeiramente foram realizados ensaios com as células da linhagem B16-F10 com o objetivo de verificar se a toxina recombinante LiRecDT1 era capaz de promover a entrada de íons cálcio para o interior das células. Com o mesmo objetivo, posteriormente foram realizados ensaios com células da linhagem B16-F1 e estudos comparando o influxo de cálcio nas duas linhagens de células, visto que, por serem células com diferentes potenciais metastáticos, poderiam apresentar diferentes respostas ao cálcio, seja pelo número de canais ou pelas quantidades de outras proteínas/enzimas intracelulares relacionadas a este evento. Tais características particulares de cada tipo celular poderiam ser os diferenciais relacionados com o seu grau de malignidade.

Com a finalidade de verificar se o influxo de cálcio estaria relacionado com a atividade enzimática da proteína recombinante LiRecDT1, também foram feitos ensaios com a toxina recombinante mutada LiRecDT1 H12A que contém uma importante mutação no sítio catalítico que tornam a atividade desta enzima diminuida. Estes ensaios foram realizados nas duas linhagens celulares analisadas.

Células B16F10 também foram plaqueadas sobre lamínulas em placas de 24 poços na densidade de 2 X 10^4 células por poço, observadas em Microscópio de Fluorescência Observer Z1 (Carl Zeiss). As imagens foram obtidas usando lente objetiva de 63X com óleo e a intensidade da fluorescência foi obtida em câmera monocromática.

Em todos os ensaios as células foram primeiramente incubadas com Fluo 4AM (um fluoróforo celular permeante sensível ao cálcio) e posteriormente tratadas com as toxinas, a fluorescência foi obtida em Florímetro Tecan Infinite® M200 usando para excitação o comprimento de onda de 485nm e a emissão foi lida em 535nm. Os resultados dos ensaios envolvendo o influxo de cálcio nas células B16-F10 e B16-F1 após o tratamento com a fosfolipase-D recombinante estão expostos nos gráficos 6 a 9 e na Figura 14.

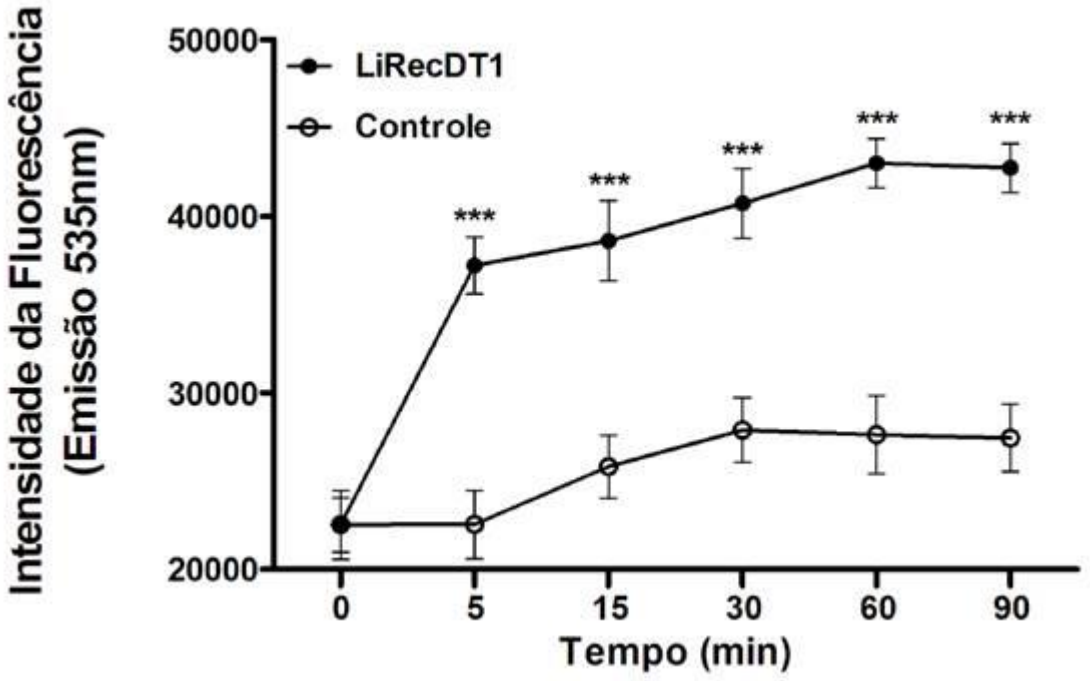


Gráfico 6: O tratamento das células B16-F10 com a fosfolipase-D recombinante provoca influxo de cálcio. As células B16-F10 foram incubadas com LiRecDT1 (25 µg/mL) na presença de Fluo-4 em um tampão contendo cálcio e a fluorescência de Fluo-4 foi medida após vários períodos de tempo. Como controle negativo, as células B16-F10 foram incubadas nas mesmas condições laboratoriais, exceto pela ausência de tratamento com a fosfolipase-D. Os valores apresentados são a média de cinco experimentos ± desvio padrão e a significância foi definida como *** $p<0,001$. O desenvolvimento de fluorescência (de 5 à 90 minutos) foi obtido no espectrofluorímetro (Tecan Infinite® M200) usando um comprimento de onda de excitação de 485nm e a emissão foi obtida em 535 nm. O eixo Y indica a intensidade da fluorescência e o eixo X o tempo de tratamento com a fosfolipase-D. Houve um aumento da absorção de cálcio após a exposição das células à LiRecDT1.

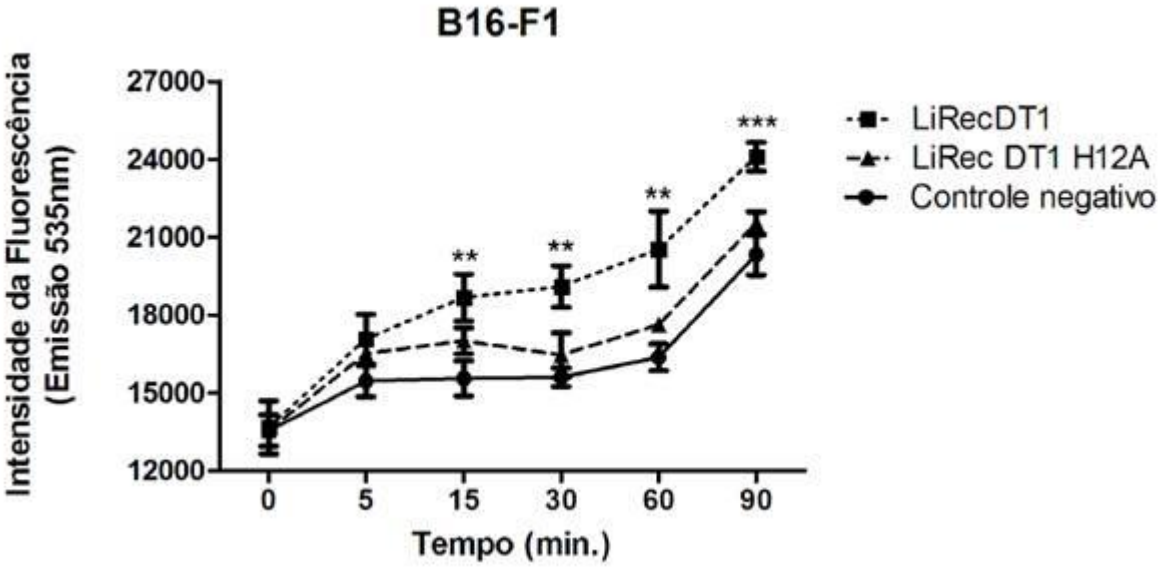


Gráfico 7: Influxo de cálcio gerado nas células B16-F1 após o tratamento com a fosfolipase-D recombinante LiRecDT1. As células B16-F1 foram incubadas com LiRecDT1 e LiRecDT1 H12A, ambas nas concentrações de 25µg/mL na presença de Fluo-4 em um tampão contendo cálcio e a fluorescência do Fluo-4 foi medida após vários períodos de tempo. Como controle negativo, as células B16-F10 foram incubadas nas mesmas condições laboratoriais, exceto pela ausência de tratamento com a fosfolipase-D. Os valores apresentados são a média de três experimentos ± desvio padrão e a significância foi definida como ** $p<0,01$, *** $p<0,001$. O desenvolvimento de fluorescência (de 0 à 90 minutos) foi obtido no espectrofluorímetro (Tecan Infinite® M200) usando um comprimento de onda de excitação de 485nm e a emissão foi obtida em 535 nm. O eixo Y indica a intensidade da fluorescência e o eixo X o tempo de tratamento com a fosfolipase-D. Houve um aumento da absorção de cálcio após a exposição das células à LiRecDT1.

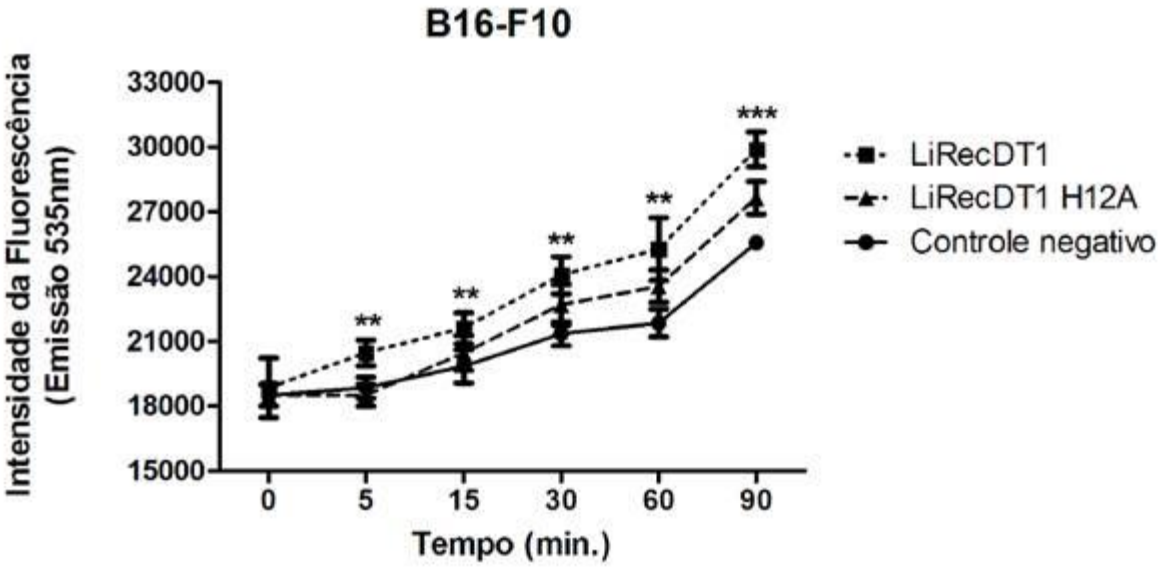


Gráfico 8: Influxo de cálcio gerado nas células B16-F10 após o tratamento com a fosfolipase-D recombinante LiRecDT1. As células B16-F10 foram incubadas com LiRecDT1 e LiRecDT1 H12A, ambas nas concentrações de 25µg/mL na presença de Fluo-4 em um tampão contendo cálcio e a fluorescência do Fluo-4 foi medida após vários períodos de tempo. Como controle negativo, as células B16-F10 foram incubadas nas mesmas condições laboratoriais, exceto pela ausência de tratamento com a fosfolipase-D. Os valores apresentados são a média de três experimentos ± desvio padrão e a significância foi definida como ** $p<0,01$, *** $p<0,001$. O desenvolvimento de fluorescência (de 0 à 90 minutos) foi obtido no espectrofluorímetro (Tecan Infinite® M200) usando um comprimento de onda de excitação de 485nm e a emissão foi obtida em 535 nm. O eixo Y indica a intensidade da fluorescência e o eixo X o tempo de tratamento com a fosfolipase-D. Houve um aumento da absorção de cálcio após a exposição das células à LiRecDT1.

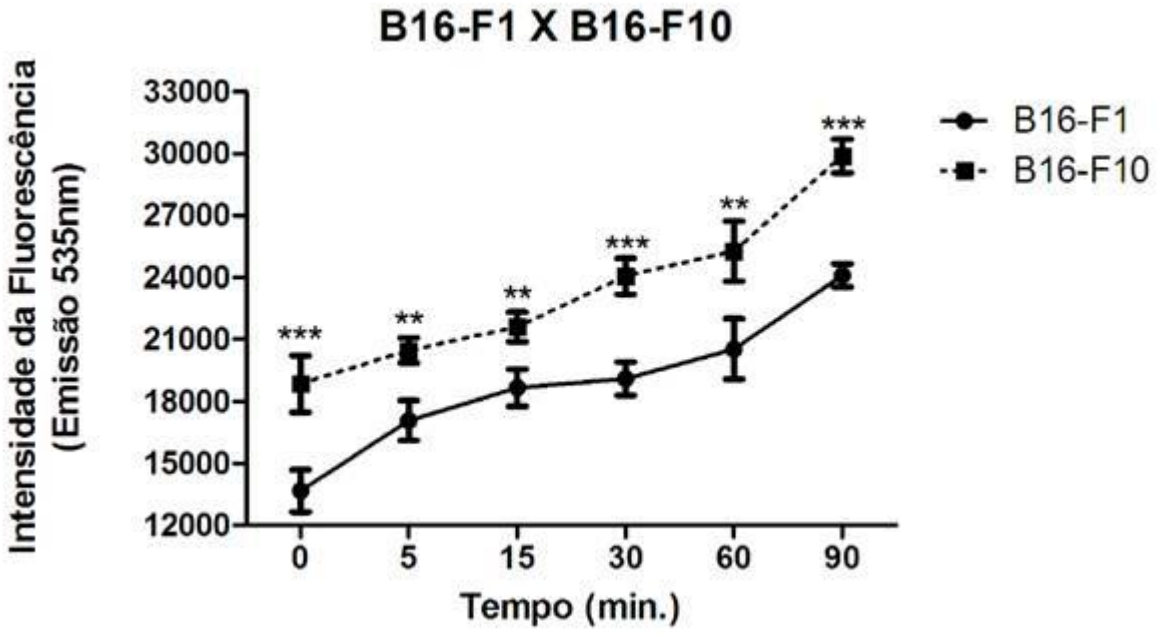


Gráfico 9: Estudo comparativo sobre o influxo de cálcio gerado nas células das linhagens B16-F10 e B16-F1 após o tratamento com a fosfolipase-D recombinante de aranha marrom LiRecDT1. As células foram incubadas com LiRecDT1 nas concentração de 25µg/mL na presença de Fluo-4 em um tampão contendo cálcio e a fluorescência do Fluo-4 foi medida após vários períodos de tempo. Os valores apresentados são a média de três experimentos ± desvio padrão e a significância foi definida como ** $p<0,01$, *** $p<0,001$. O desenvolvimento de fluorescência (de 0 à 90 minutos) foi obtido no espectrofluorímetro (Tecan Infinite® M200) usando um comprimento de onda de excitação de 485nm e a emissão foi obtida em 535 nm. O eixo Y indica a intensidade da fluorescência e o eixo X o tempo de tratamento com a fosfolipase-D. Houve um aumento significativo da absorção de cálcio após a exposição das células à LiRecDT1.

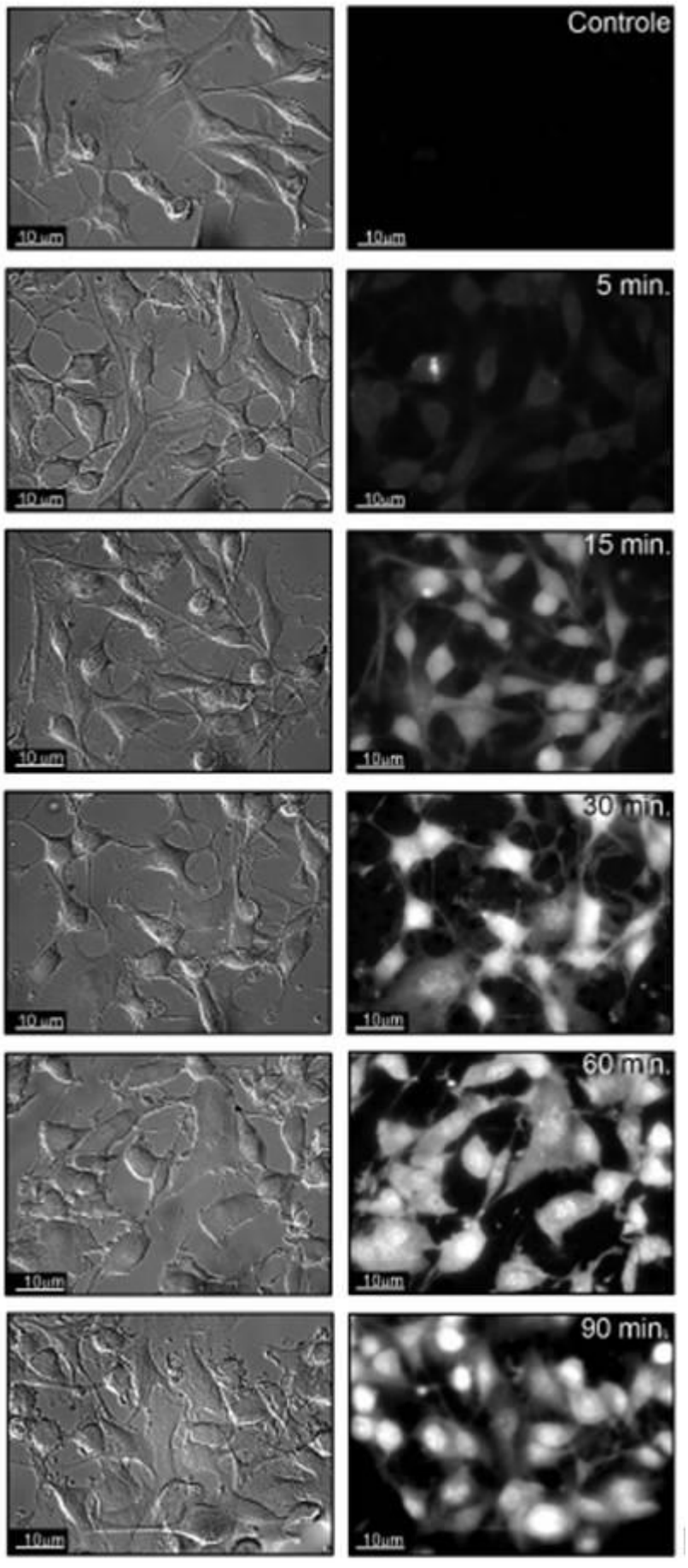


Figura 14. Em células B16-F10 na presença de Fluo-4, o influxo de cálcio gerado após o tratamento com LiRecDT1 pode ser observado por imagem de fluorescência. Células B16-F10 expostas à 25 µg/mL da fosfolipase-D recombinante (LiRecDT1) em diferentes tempos (5, 15, 30, 60 e 90 minutos), foram observadas por meio de microscopia de transmissão (microscópio invertido Zeiss Axio Observer.Z1). Foram obtidas imagens com DIC e da fluorescência das células contendo Fluo-4. O Fluo-4 AM foi excitado à 488nm e a emissão foi detectada utilizando um filtro LP 505nm (filtro verde). Imagens individuais foram obtidas usando uma lente objetiva de 63X com óleo de imersão em microscópio invertido com contraste diferencial de interferência (DIC) e a intensidade da fluorescência foi analisada com uma câmera monocromática (AxioCam HRm, Zeiss). O controle constituiu de células B16-F10 cultivadas sem fosfolipase-D, mas contendo Fluor-4. O tratamento com a fosfolipase-D induziu um aumento de cálcio no interior das células de maneira tempo-dependente. Barras de escala são mostradas no canto inferior esquerdo das figuras.

Foi observado por espectrofluorimetria que após o tratamento das células B16-F10 e B16-F1 com a fosfolipase-D recombinante LiRecDT1 ocorreu um aumento da geração de influxo de cálcio intracelular. Como mostrado nos gráficos 6, 7 e 8, ocorreu rápido influxo de cálcio nas células B16-F10 e B16-F1 de modo tempo dependente. Tanto as células B16-F10 quanto as células B16-F1 tratadas com a toxina mutada LiRecDT1 H12A monstraram um discreto aumento na entrada de cálcio e de maneira pouco significativa quando comparadas ao controle. Tal fato, mostra que o influxo de cálcio desencadeado por esta toxina é dependente de sua atividade catalítica, uma vez que na toxina mutada LiRecDT1 H12A uma importante mutação no sítio catalítico é capaz de reduzir a atividade enzimática.

Células B16-F10 e B16-F1 em igual número ($1X10^8$ células/mL) incubadas com Fluo-4 AM e tratadas com a mesma concentração da toxina recombinante LiRecDT1 (25µg/mL) foram capazes de revelar que o influxo de cálcio pode ser menor em células B16-F1 (Gráfico 9). Uma possível hipótese para este fato pode estar relacionada a diferentes quantidades de canais de cálcio das duas linhagens celulares ou a diferentes quantidades de outras proteínas/enzimas intracelulares que atuam neste evento. Estes diferenciais podem estar relacionados ao potencial metastático destas células, visto que já é conhecido que células B16-F10 têm maior índice metastático do que células B16-F1.

A geração de influxo de cálcio de modo tempo dependente em células B16-F10 também foi observada com uso de microscopia de fluorescência em Microscópio de Fluorescência Observer Z1 (Carl Zeiss) (Figura 14).

5.7 Mesmo em altas concentrações e em períodos de tempo prolongados a toxina recombinante LiRecDT1 não foi capaz de alterar a viabilidade e a morfologia de células B16-F10

Para descartar a possibilidade de que o influxo de cálcio nas células B16-F10 fosse uma consequência do efeito deletério das toxinas na membrana plasmática, causando uma alteração na integridade da membrana e influxo artificial de cálcio, a viabilidade das células em diferentes concentrações da toxina (100, 200 e 300µg/mL) e em diferentes períodos de tempo (24, 48 e 72h) foi determinada por meio do método de exclusão do corante Azul de Tripan.

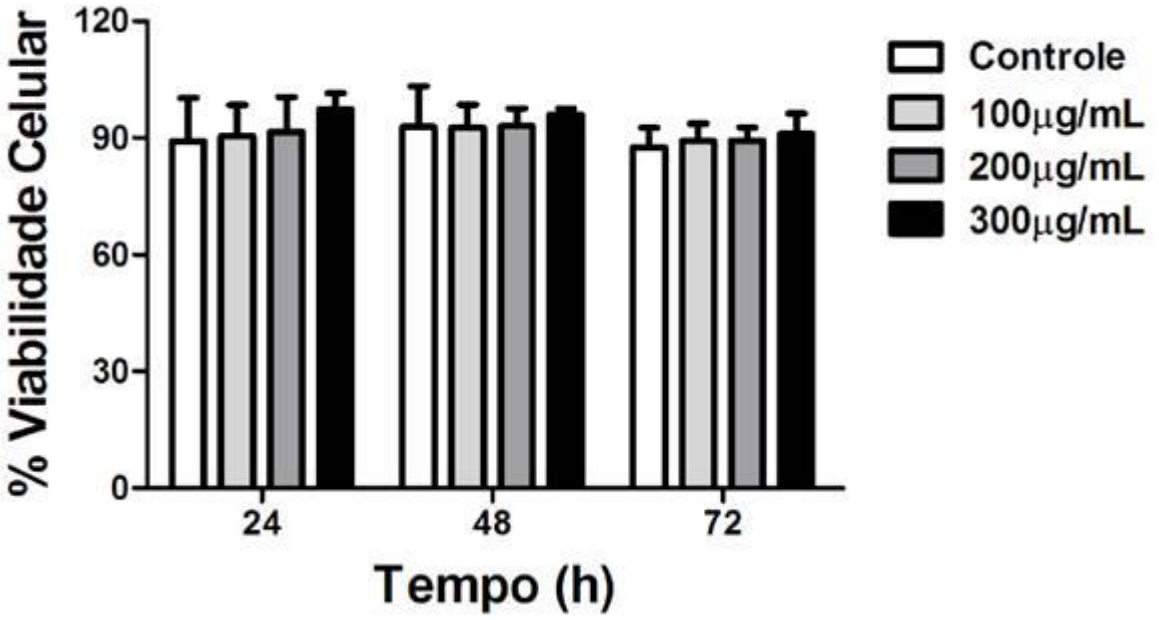


Gráfico 10: Ensaio de citotoxicidade. Efeito de LiRecDT1 sobre a viabilidade de células B16-F10. O efeito citotóxico de LiRecDT1 em células B16-F10 foi analisado pelo método de exclusão com o corante Azul de Tripan. Os efeitos citotóxicos de LiRecDT1 foram determinados após 24, 48 e 72h nas concentrações indicadas da fosfolipase-D purificada. Os experimentos foram feitos em hexaplicata e os valores obtidos mostram a média ± desvio padrão. Mesmo na maior concentração (300 µg/mL), a exposição à fosfolipase-D não causou citotoxicidade nas condições testadas.

Apesar da toxina loxoscélica recombinante LiRecDT1 se ligar e metabolizar fosfolípidos da membrana (dados mostrados nas figuras 12 e 13, gráfico 5), esta fosfolipase-D recombinante aplicada em concentrações 10X maior do que aquela que induziu um influxo de cálcio, (300µg/mL por 72h), concentração suficiente para matar ratos e coelhos e até mesmo causar problemas graves em seres humanos (da SILVA *et al*., 2004; KUSMA *et al*., 2008), não alterou a viabilidade das células B16-F10 (Gráfico 10).

Ainda com o objetivo de verificar se o influxo de cálcio gerado nas células B16-F10 após o tratamento com a toxina recombinante LiRecDT1 não ocorria devido a danos na membrana plasmática, também foram realizadas micrografias das células tratadas em diferentes concentrações da toxina e em diferentes períodos de tempo (Figura 15).

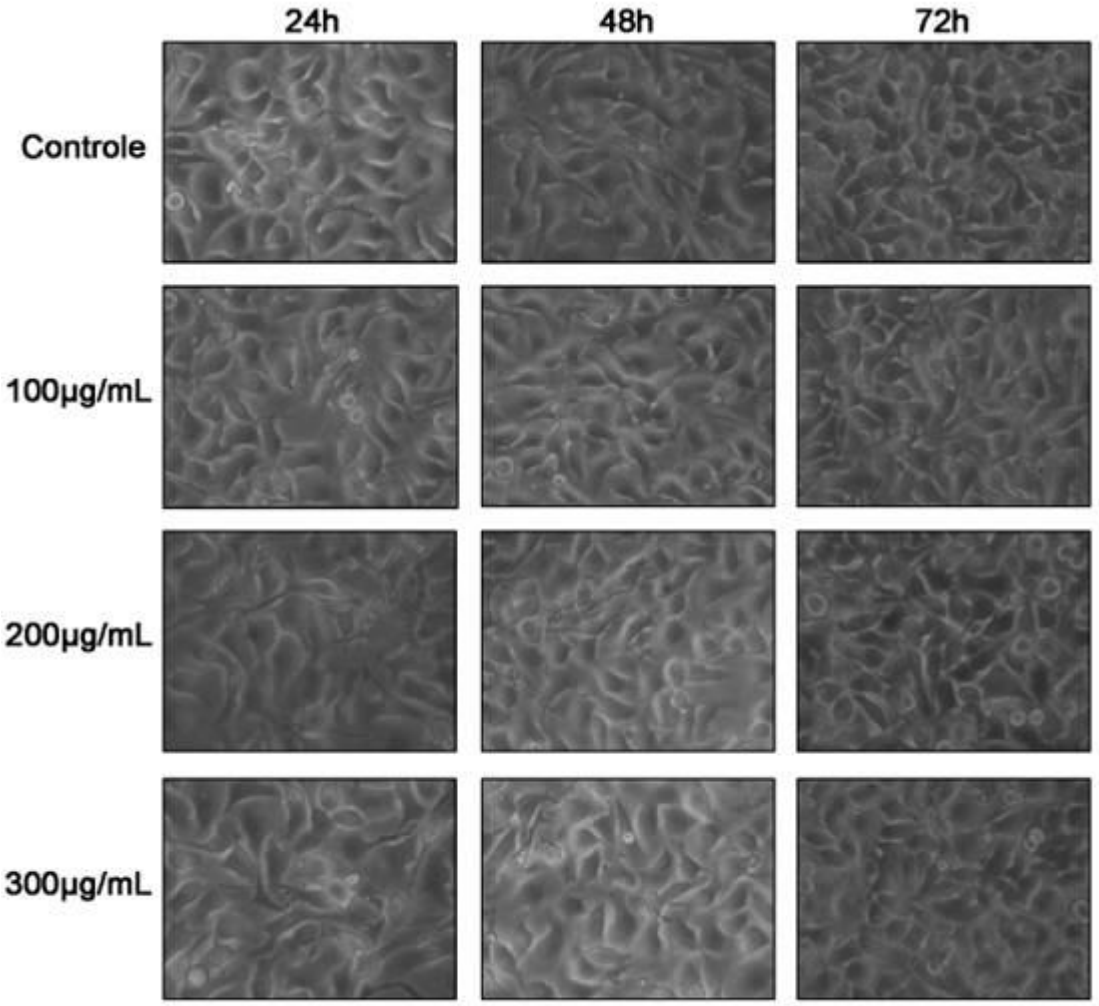


Figura 15. Ensaio de citotoxicidade. Efeito de LiRecDT1 sobre a morfologia das células B16-F10. Células B16-F10 expostas à LiRecDT1 foram observadas com microscópio invertido (Leica-DMIL). As análises foram feitas após 24, 48 e 72h de exposição à LiRecDT1. A concentração da toxina purificada em meio de cultura variou de 100µg/mL à 300µg/mL. As células controle foram analisadas na ausência da toxina. Os tratamentos aplicados às células não provocaram sinais de citotoxicidade, tais como vacuolização do citoplasma, alterações no espalhamento e na adesão celular e alterações na morfologia nas condições de cultura analisadas.

Como é possível observar na figura 15 acima, as células não sofreram qualquer tipo de modificação morfológica, como vacuolização citoplasmática, arredondamento, desadesão do substrato, agregação ou lise celular. Estas observações sugerem ausência de efeitos deletérios desta fosfolipase-D sobre as células B16-F10. Desta forma, é possível concluir que o influxo de íons de cálcio para o interior das células após o tratamento com a fosfolipase-D recombinante da aranha marrom não foi uma consequência do extravasamento pela membrana da célula.

5.8 A fosfolipase-D recombinante de aranha marrom LiRecDT1 estimula a proliferação de células B16-F10

Com a finalidade de observar os efeitos da toxina recombinante LiRecDT1 sobre a proliferação de células B16-F10 foram realizados ensaios com as células submetidas a diferentes concentrações da toxina (10 e 25 µg/mL por 48 horas – gráfico 11), em diferentes períodos de tempo (10µg/mL por 24, 48 e 72horas – gráfico 12) e na presença de esfingomielina (5 e 10mM de esfingomielina, com 10µg/mL da toxina por 48 horas – gráfico 13). A proliferação foi realizada com o Kit de proliferação CyQuant (Molecular Probes) e a leitura da fluorescência foi obtida em um Florímetro Tecan Infinite® M200, usando para excitação o comprimento de onda de 485nm e a emissão foi lida em 535nm.

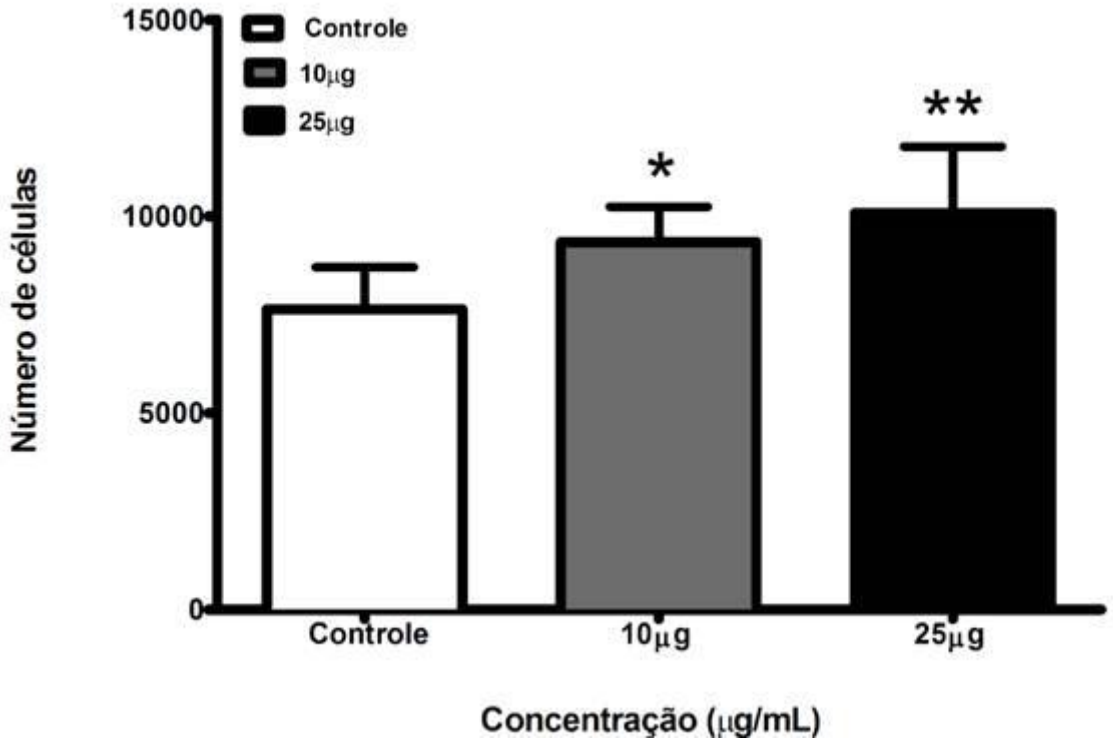


Gráfico 11: A fosfolipase-D recombinante de aranha marrom estimula a proliferação de células B16-F10. As células B16-F10 foram cultivadas em meio RPMI suplementado com 10% de soro fetal bovino. Vinte e quatro horas antes do ensaio de proliferação as células foram cultivadas nas mesmas condições descritas acima, exceto pela ausência de soro no meio de cultura, para sincronizar o ciclo celular. As células B16-F10 foram plaqueadas em placas de 96 poços na diluição de $5X10^3$ células/poço e incubadas com fosfolipase-D recombinante nas concentrações de 10 e 25µg/mL por 48 horas em pentaplicata. No grupo controle foi adicionado a quantidade apropriada do veículo (PBS), em vez de LiRecDT1. Depois da incubação com a fosfolipase-D, a quantificação da proliferação celular foi feita usando o Kit de proliferação CyQuant. A fluorescência foi obtida no espectrofluorímetro (Tecan Infinite® M200) usando um comprimento de onda de excitação de 480nm e a emissão foi obtida em 520 nm. O eixo Y indica o número de células, e o eixo X a concentração da fosfolipase-D. Os experimentos foram feitos em pentaplicata e os valores obtidos mostram a média ± desvio padrão, a significância foi definida como * $p<0,05$ e ** $p<0,01$.

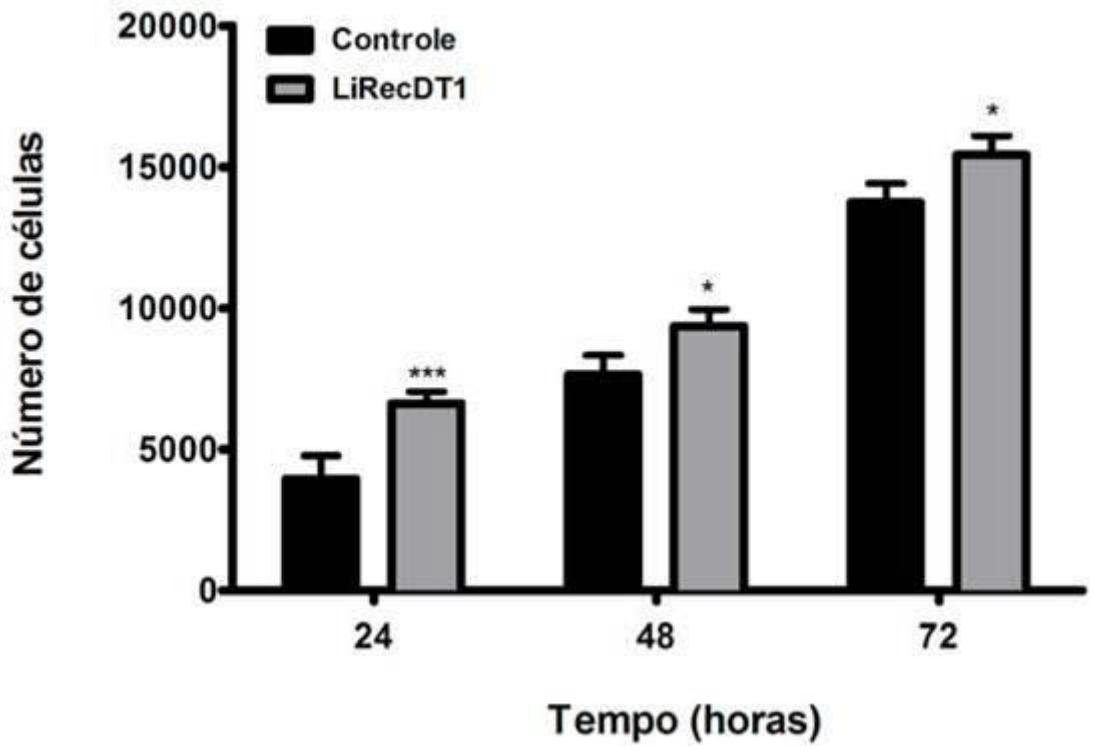


Gráfico 12: Efeito da fosfolipase-D de aranha marrom sobre a proliferação de células B16-F10 após 24, 48 e 72 horas de tratamento. Células B16-F10 plaqueadas em placas de 96 poços na diluição de $5X10^3$ células/poço foram cultivadas e mantidas por 24 horas em meio RPMI suplementado com 10% de soro fetal bovino. Posteriormente, o meio de cultura contendo soro foi substituido por meio sem soro e mantido por 24 horas para sincronizar o ciclo celular. Foi realizado um ensaio de proliferação em que as células B16-F10 foram tratadas por 24, 48 e 72h com 10µg/mL de LiRecDT1. A quantificação da proliferação celular foi feita usando o Kit de proliferação CyQuant. A fluorescência foi obtida no espectrofluorímetro (Tecan Infinite® M200) usando um comprimento de onda de excitação de 480nm e a emissão foi obtida em 520 nm. O eixo Y indica o número de células, e o eixo X o tempo de tratamento com a fosfolipase-D. Os experimentos foram feitos em pentaplicata e os valores obtidos mostram a média ± desvio padrão, a significância foi definida como * $p<0,05$ e *** $p<0,001$.

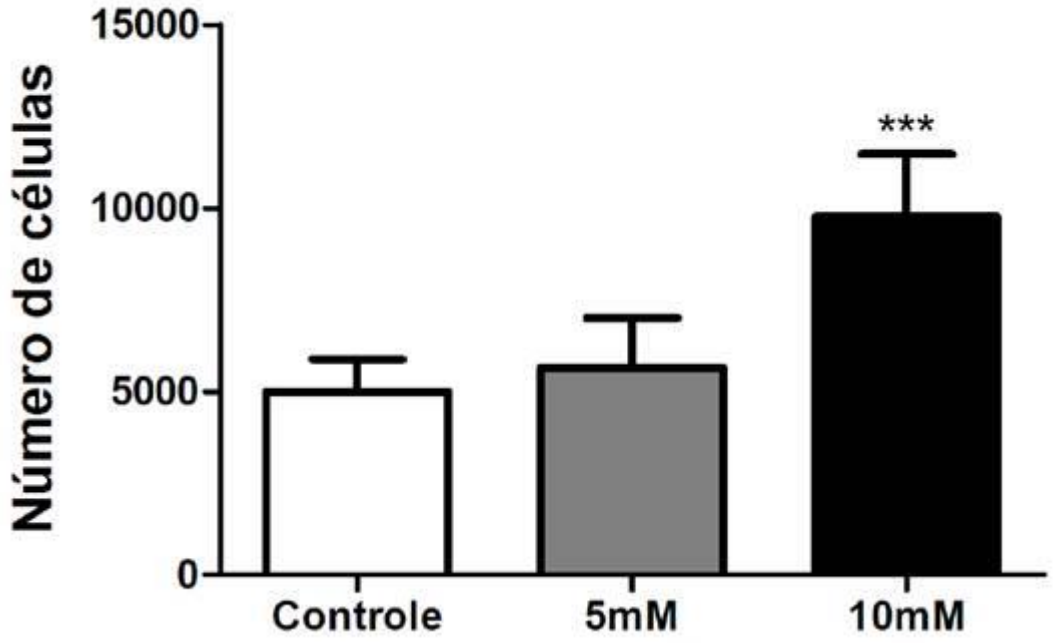


Gráfico 13. O efeito da fosfolipase-D recombinante de aranha marrom sobre proliferação de células B16-F10 é superior quando as células são incubadas com esfingomielina exógena. Células B16-F10 plaqueadas em placas de 96 poços na diluição de $5X10^3$ células/poço foram cultivadas e mantidas por 24 horas em meio RPMI suplementado com 10% de soro fetal bovino. Após 24 horas, o meio de cultura contendo soro foi substituido por meio sem soro para sincronizar o ciclo celular. Para a realização deste ensaio de proliferação as células B16-F10 foram mantidas por 48h em meio de cultura contendo esfingomielina sintética (5 e 10mM). A quantificação da proliferação celular foi feita usando o Kit de proliferação CyQuant. A fluorescência foi obtida no espectrofluorímetro (Tecan Infinite® M200) usando um comprimento de onda de excitação de 480nm e a emissão foi obtida em 520 nm. O eixo Y indica o número de células, e o eixo X o tempo de tratamento com a fosfolipase-D. Os experimentos foram feitos em pentaplicata e os valores obtidos mostram a média ± desvio padrão, a significância foi definida como ***p<0,001.

Os resultados apresentados acima confirmam a capacidade da fosfolipase-D de aranha marrom em estimular a proliferação celular de uma maneira dependente da concentração e do tempo (gráficos 11 e 12). Além disso, o aumento na taxa de proliferação das células B16-F10 após a exposição à LiRecDT1 foi superior quando as células foram incubadas com esfingomielina exógena (o qual é, como anteriormente relatado, um bom substrato para a fosfolipase-D recombinante) (gráfico 13). Uma explicação hipotética para este evento é que a esfingomielina exógena aumenta a concentração e acessibilidade aos substratos de enzimas, gerando lípidos bioativos após o tratamento com a toxina recombinante de aranha marrom (tais como ceramida-1-fosfato ou lípidos interconversíveis, tais como, ceramida, esfingosina e esfingosina-1-fosfato), comparado aos substratos lipídicos localizados na bicamada lipídica das membranas celulares. Estes resultados indicam que a fosfolipase-D recombinante de aranha marrom LiRecDT1 pode atuar como um fator exógeno estimulante da proliferação.

6. DISCUSSÃO DOS RESULTADOS

As fosfolipases-D foram descritas como importantes reguladoras de vários processos fisiológicos (EXTON, 2002). Estas enzimas catalisam a hidrólise de diversos fosfolípidos, gerando moléculas bioativas que desempenham um importante papel em diferentes cascatas de sinalização intracelular. Em mamíferos, três isoformas de fosfolipases-D endógenas foram relatadas: PLD-1 (que se encontram principalmente no aparelho de Golgi, vesículas secretoras e endossomos), PLD-2 (que se encontram principalmente na membrana plasmática, e em pequenas percentagens em caveolas e endossomos), e a isoforma mitoPLD (associada as mitocôndrias), recentemente descrita (JENKINS e FROHMAN, 2005; CHOI *et al*., 2006, DONALDSON, 2009). Ao nível celular, estas moléculas atuam no tráfico vesicular intracelular, regulando vesículas secretoras derivadas da rede trans-golgi, reciclagem de membrana, endocitose e fusão de mitocôndrias (JENKINS e FROHMAN, 2005, CHOI *et al.*, 2006, DONALDSON, 2009). Também têm sido mostrado que as fosfolipases-D podem regular o ciclo celular, a proliferação celular e a apoptose (FOSTER e XU, 2003).

A desregulação destes processos celulares tem sido descrita durante o desenvolvimento de vários tipos de tumores humanos, tais como câncer de mama, estômago e câncer colorretal (FOSTER e XU, 2003), e os dados descritos na literatura têm indicado alterações na expressão de fosfolipases-D em vários tipos de câncer, relacionados com o aumento da proliferação celular, transformação, sobrevivência, e invasão tumoral (FOSTER e XU, 2003). A atividade das fosfolipases-D são reguladas em resposta ao tratamento com diferentes fatores de crescimento, tais como o fator de crescimento derivado de plaquetas (PDGF) (PLEVIN *et al*., 1991), fator de crescimento epidérmico (EGF) (SONG *et al*., 1994), de crescimento de fibroblastos (FGF) (SA e DAS, 1999), fator-1 de crescimento tipo insulina (ILGF - 1) (BANNO *et al*., 2003) , e hormônio do crescimento (ZHU *et al*., 2002). Fibroblastos em cultura expostos a fosfolipase-D exógena (de *Streptomyces chromofuscus*) mostraram aumento da produção de ácido lisofosfatídico (LPA), gerado a partir de lisofosfatidilcolina na monocamada externa da membrana plasmática. Esta produção de LPA resultou na ativação de receptores de LPA ligados a proteína G e a subsequente ativação

da Ras, Rho e cascatas de sinalização intracelular dependentes de cálcio (van DIJK *et al*.,1998). Um aumento da atividade de fosfolipases-D tem sido descritas em diferentes células transformadas por oncogenes, tais como *v-Src, v-Ras, v-Fps e v-Raf* (FOSTER e XU, 2003).

Além das fosfolipases-D endógenas, a existência de várias fosfolipases-D exógenas produzidos por diferentes organismos vivos tem sido relatadas (RAGHU *et al*., 2009, LUCAS *et al*., 2010, MURPH *et al*., 2011). Entre os membros da família das fosfolipases-D exógenas, a fosfolipase-D de aranha marrom representa um bom exemplo de molécula biologicamente ativa, a participação destas moléculas e suas catálises foram observadas em vários aspectos fisiopatológicos do loxoscelismo, como dermonecrose, desrregulação de resposta inflamatória, nefrotoxicidade, agregação plaquetária e hemólise (CHAIM *et al*., 2006, da SILVEIRA *et al*., 2006, 2007a, APPEL *et al*., 2008, KUSMA *et al*., 2008, CHAVES-MOREIRA *et al*., 2011, CHAIM *et al*., 2011b).

No veneno de aranhas marrons existe uma mistura complexa de toxinas que exibem um amplo espectro de atividades biológicas, farmacológicas e bioquímicas, o que tornam estas moléculas importantes para a utilização biotecnológica como ferramentas bioativas em diversas metodologias. Recentemente, baseados na construção de uma biblioteca de cDNA e no estudo do perfil do transcriptoma da glândula de veneno da aranha-marrom *L. intermedia*, Gremski e colaboradores (2010) descreveram a diversidade das moléculas expressas neste veneno.

A análise do transcriptoma do mRNA da glândula de veneno de *L. intermedia* demonstraram que os mRNAs que codificam para fosfolipases-D representam 20,2 % do total do total dos transcritos que codificam estas toxinas neste órgão (GREMSKI *et al*., 2010). Utilizando técnicas de biologia molecular, tais como clonagem, expressão heteróloga, alinhamento de aminoácidos e análises filogenéticas, foram descritas as funções de seis isoformas de fosfolipases-D do veneno de *L. intermedia,* que foram denominadas LiRecDT1 (número de acesso ao GenBank DQ218155), LiRecDT2 (número de acesso ao GenBank DQ266399) , LiRecDT3 (número de acesso ao GenBank DQ267927),

LiRecDT4 (número de acesso ao GenBank DQ431848), LiRecDT5 (número de acesso ao GenBank DQ431849), e LiRecDT6 (número de acesso ao GenBank EF474482) (CHAIM *et al*., 2006; da SILVEIRA *et al*., 2006, 2007a, APPEL *et al*., 2008). Recentemente, foi identificada uma nova isoforma funcional de fosfolipase-D referida como LiRecDT7 (VUITIKA *et al*., 2013).

Com base nas atividades bioquímicas das fosfolipases-D dos venenos loxoscélicos, essas enzimas podem ser usadas em estudos sobre os constituintes da membrana celular, especialmente sobre esfingofosfolípidos, lisofosfolípidos, ácido lisofosfatídico e ceramida-1-fosfato e como um modelo para elucidar receptores de produtos lipídicos, vias de sinalização e atividades biológicas. A investigação nesta área pode levar à descoberta de reagentes que são úteis no diagnóstico e em novos medicamentos (SENFF-RIBEIRO *et al*., 2008; CHAIM *et al*., 2011a; MURPH *et al*., 2011).

A idéia de que isoformas exógenas de fosfolipases-D do veneno de aranhas marrons poderiam ser reagentes úteis em estudos de biologia celular surge a partir dos efeitos clínicos desencadeados após as picadas. As picadas podem provocar uma resposta inflamatória profunda e desregulada relacionada com loxoscelismo dermonecrótico e gangrenoso (histologicamente caracterizada como necrose de coagulação asséptica). O veneno também provoca agregação plaquetária, causando trombocitopenia, hemólise e é nefrotóxico (LUCIANO *et al*., 2004, da SILVA et al., 2004; SWANSON e VETTER, 2006). Todos esses eventos podem ser reproduzidos usando isoformas de fosfolipases-D recombinantes purificadas em condições de laboratório, fortalecendo a idéia de que as moléculas de fosfolipases-D do veneno desempenham um papel essencial nestas atividades e podem modular funções celulares (CHAIM *et al*., 2006; da SILVEIRA *et al*., 2006, 2007a; APPEL *et al*., 2008, KUSMA *et al*., 2008, CHAVES-MOREIRA *et al*., 2009, 2011, CHAIM *et al*., 2011b).

Neste trabalho, o estudo do veneno bruto de *Loxosceles intermedia* por meio de eletroforese bidimensional usando vários valores de pI (3,0-10,0) em primeira dimensão, SDS-PAGE na segunda dimensão e imunodetecção de fosfolipases-D no veneno com um anti-soro policlonal criado contra uma forma de

fosfolipase-D recombinante do veneno de aranha marrom (LiRecDT1), que reagiu de forma cruzada com anticorpos, mostrou que o veneno contém uma mistura heterogenea destas enzimas (pelo menos 25 spots) que variam em tamanho (30-35 kDa), apresentando níveis de pI que variam entre o ácido e o básico. Estes resultados estão de acordo com os dados da literatura que descrevem o veneno bruto como uma rica mistura de proteínas de baixa massa molecular (20-40 kDa) (VEIGA *et al*., 2000a). Estes resultados também corroboram com outros resultados encontrados na literatura que mostram que no veneno da aranha marrom existem vários membros da família de fosfolipases-D. Por exemplo, onze isoformas de fosfolipases-D têm sido observadas no veneno de *L. laeta* (MACHADO *et al*., 2005). Finalmente, os resultados obtidos com este trabalho fortaleceram as observações de Gremski *et al*., (2010) que baseados na análise do transcriptoma mostraram que o mRNA para fosfolipase-D é responsável por cerca 20,2% dos transcritos que codificam estas toxinas na glândula de veneno de *L. intermedia* e pelos relatos das clonagens de sete isoformas de fosfolipases-D da glândula de veneno, como mostrado acima.

Os resultados deste trabalho indicaram um alto perfil de expressão de proteínas que estão imunologicamente correlacionadas com isoformas fosfolipases-D e sugerem fortemente que esta classe de toxinas é de grande importância biológica no veneno e provavelmente desempenham um papel tanto quanto moléculas de defesa contra predadores ou moléculas letais durante a captura de alimentos pelas aranhas. Além disso, dados recentes têm indicado que as fosolipases-D do veneno de aranha marrom podem atuar como moléculas inseticidas (ZOBEL-THROPP *et al*., 2012).

Usando a microscopia confocal de imunofluorescência com anticorpos contra LiRecDT1 (CHAIM *et al*., 2006, da SILVEIRA *et al*., 2006), foi possível detectar a ligação desta fosfolipase-D exógena na superfície das células B16-F10. Além disso, a interação da fosfolipase-D com a membrana de células B16-F10 foi reforçada pela ligação com uma fosfolipase-D recombinante de fusão (LiRecDT1-GFP) (CHAVES-MOREIRA *et al*., 2009), mostrado por meio de microscopia de fluorescência e ensaios de competição.

Os resultados obtidos demonstraram a existência de sítios de ligação para a fosfolipase-D do veneno da aranha marrom na membrana das células B16-F10 e é possível sugerir que esta molécula exerça sua atividade enzimática sobre os constituintes da membrana dessas células. Corroborando esses dados, foi recentemente relatado que uma fosfolipase-D recombinante de *L. laeta* foi capaz de induzir mudanças nas estruturas laterais e morfologia das membranas alvo usando grandes e gigantes vesículas unilamelares (STOCK *et al*., 2012). Além disso, tem sido mostrado que células endoteliais, células epiteliais tubulares e eritrócitos são alvos para a ligação da fosfolipase-D recombinante de aranha marrom (KUSMA *et al*., 2008, CHAIM *et al*., 2011b, CHAVES-MOREIRA *et al*., 2011).

Para demonstrar que a catálise pela fosfolipase-D recombinante de aranha marrom e a degradação de fosfolípidos da membrana desempenham um importante papel na indução de alterações metabólicas das células B16-F10, foi avaliada a capacidade desta enzima em degradar esfingomielina e lisofosfatidilcolina sintéticas, que são importantes constituintes das monocamadas externas da membrana das células. Os resultados obtidos mostraram a preferência de LiRecDT1 por esfingomielina. Em um experimento de cinética de tempo, a esfingomielina foi hidrolisada mais rapidamente e mais eficientemente, mas os resultados mostraram que a fosfolipase-D recombinante de aranha marrom também tem atividade lisofosfolipase.

Também foram realizados ensaios da atividade enzimática de LiRecDT1 sobre os fosfolipídios da membrana de células B16-F10 em de extratos de “ghosts” e “ghosts” destas células. Os resultados observados por espectrofluorimetria, obtidos pela verificação da geração de colina demonstraram que LiRecDT1 tem acessibilidade e atividade tanto em “ghosts” quanto em extratos de “ghosts”, portanto, esta enzima tem atividade catalítica sobre os fosfolipídios da membrana destas células. Os resultados também indicaram que a atividade de LiRecDT1 sobre esfingomielina gera colina e consequentemente ceramida1-fosfato e que a atividade sobre lisofosfatidilcolina produz colina e ácido lisofosfatídico. Ceramida1-fosfato, ceramida, esfingosina, esfingosina 1-fosfato, ácido lisofosfatídico e ácido fosfatídicos são potentes moléculas bioativas

relacionadas com funções biológicas, incluindo sobrevivência, proliferação migração e diferenciação celular (OHANIAN e OHANIAN 2001; CHALFANT e SPIEGEL, 2005).

Estas observações indicam que a fosfolipase-D recombinante LiRecDT1 pode interagir com componentes da membrana das células B16-F10, tendo atividade hidrolítica aos fosfolípidos, e podem metabolizar diretamente fosfolípidos estruturalmente organizados nas membranas celulares ou obtidos a partir das membranas citoplasmáticas como os extraídos das membranas das células B16-F10 para gerar moléculas bioativas.

Apesar de se ligarem e atuarem no metabolismo dos fosfolípidos de membrana, até mesmo na maior concentração da fosfolipase-D testada e maior tempo de exposição (300µg por 72 horas), uma concentração suficiente para matar ratos e coelhos e ainda causar problemas graves no ser humano (da SILVA *et al*., 2004, KUSMA *et al*., 2008), as células B16-F10 não exibiram nenhuma alteração da viabilidade (pelo método de Azul de Tripan). Além disso, as células não sofreram qualquer tipo de alteração morfológica, como vacuolização citoplasmática, arredondamento, desadesão do substrato, agregação ou rompimento (observado por microscópio invertido). Estes resultados sugerem a ausência de efeitos deletérios de fosfolipase-D sobre as células B16-F10 como quebra de integridade da membrana.

Além disso, experimentos realizados na presença de Fluo-4 (um fluoróforo celular permeante sensível ao cálcio), indicaram um aumento da fluorescência após o tratamento das células B16-F10 com a toxina loxoscélica recombinante LiRecDT1 (detectado em dois ensaios experimentais individuais: espectrofluorimétrico e ensaio de microscopia de fluorescência), demonstrando que a atividade de LiRecDT1 sobre o metabolismo dos fosfolipídios de membranana das células B16-F10 podem estimular um influxo de cálcio para o citoplasma das células. Ensaios comparando o influxo de cálcio entre as células das linhagens B16-F10 e B16-F1 com o objetivo de relacionar o influxo de cálcio com o potencial metastático das duas diferentes linhagens, visto que já é conhecido que células B16-F10 têm maior índice metastático do que células B16-F1. Nas duas linhagens celulares ensaios com a toxina mutada

LiRecDT1 H12A foram realizadas com objetivo de verificar se o evento do influxo de cálcio era dependente da atividade catalítica da enzima, uma vez que na toxina mutada LiRecDT1 H12A com uma importante mutação no sítio catalítico tem a atividade enzimática diminuída (CHAVES-MOREIRA *et al*., 2011). Os resultados monstraram um discreto aumento na entrada de cálcio nas duas linhagens analisadas e de maneira pouco significativa quando comparadas aos controles. Tal fato, mostra que o influxo de cálcio desencadeado por esta toxina é dependente da atividade catalítica da enzima. A ocorrência de influxo de cálcio nas células está de acordo com dados da literatura, que relatam a ocorrência do influxo de cálcio no citoplasma de fibroblastos tratados com uma fosfolipase-D exógena (obtida de *Streptomyces chromofuscus*) (VAN DIJK *et al*., 1998). Além disso, a idéia de que ocorre um influxo de cálcio em células tratadas com LiRecDT1 é apoiada por resultados que apontam que o influxo de cálcio após o tratamento com fosfolipase-D de aranhas marrons induz hemólise, que relata que ácido lisofosfatídico (um produto gerado após o tratamento das células B16-F10 com LiRecDT1), induz entrada de cálcio em eritrócitos humanos (YANG *et al*., 2000, CHAVES-MOREIRA *et al*., 2011).

Finalmente, o influxo de íons cálcio para o interior de células após o tratamento com a fosfolipase-D recombinante de aranha marrom não foi consequência de furos na membrana da célula. Como mencionado acima a viabilidade das células não foi alterada, mesmo após a exposição a uma alta concentração de LiRecDT1 (como demonstrado pelo ensaio com azul de tripan não houve perda da integridade da membrana). O aumento na concentração de cálcio citossólico pode mediar um número de eventos celulares tais como desenvolvimento embrionário, contração e agregação celular, secreção, modulação do metabolismo de energia, entre muitos outros (CLAPHAM, 2007; BOOTMAN, 2012), desta forma especula-se que a fosfolipase-D do veneno de aranha marrom representa uma nova ferramenta para se estudar eventos intracelulares desencadedos pela modulação de cálcio dentro das células e abre a possibilidade para aplicações bioquímicas e biológicas para esta molécula.

Finalmente a atividade da fosfolipase-D recombinante da aranha marrom sobre as células B16-F10 foi confirmada pela sua capacidade em estimular a proliferação de uma maneira dependente da concentração e do tempo. Além disso, aumentos nas taxas de proliferação das células B16-F10 foram maiores quando as células foram incubadas na presença de esfingomielina exógena (que conforme foi relatado anteriormente é um bom substrato para a fosfolipase-D recombinante). Uma explicação hipotética para este evento é que a esfingomielina exógena aumenta a concentração e a acessibilidade aos substratos da enzima gerando mediadores lipídicos bioativos (como ceramida-1-fosfato ou lipídios interconvertíveis, tais como ceramida, esfingosina e esfingosina-1-fosfato) em comparação com substratos lipídicos organizados nas bicamadas lipídicas das membranas celulares.

Os resultados descritos indicam que uma fosfolipase-D recombinante de aranha marrom (LiRecDT1) ligou-se a superfície das células de melanoma B16-F10 porém não causou alterações na viabilidade e morfologia. A toxina loxoscélica recombinante LiRecDT1 atuou sobre o metabolismo de lipídios, gerando moléculas lipídicas metabolicamente ativas, desencadeando a mobilização de cálcio para o citoplasma das células e o aumento de sua proliferação, especialmente na presença de esfingomielina exógena. Os resultados indicam que a fosfolipase-D do veneno de aranha marrom, conhecida como “toxina dermonecrótica” porque está diretamente envolvida no loxoscelismo necrótico e gangrenoso, gera lipídios biativos tais como ácido lisofosfatídico e/ou ceramida-1-fosfato, que pode modular o metabolismo de fosfolipídios da membrana, regular a proliferação de células tumorais e modular o influxo de cálcio citossólico, abrindo a possibilidade para o uso desta enzima como uma nova bioferramenta nos estudos sobre fosfolipídios e metabolismo de cálcio.

7. CONCLUSÕES

Os resultados obtidos neste trabalho sobre a ação da fosfolipase-D recombinante do veneno de aranha marrom LiRecDT1 em células tumorais das linhagens murinas B16-F10 e B16-F1 permitem as seguintes conclusões:

I. Por meio de eletroforese bidimensional do veneno de *L. intermedia* seguido por imunoblotting com anticorpos que reconhecem LiRecDT1 foi possível detectar que no veneno das aranhas deste gênero existem várias isoformas de fosfolipases-D, o que evidencia a importância deste tipo de molécula para a biologia das aranhas;

II. Ensaios de imunofluorescência indireta com anticorpos contra LiRecDT1 ou fluorescência direta com a toxina de fusão LiRecDT1-GFP mostraram a existência de sítios de ligação para a fosfolipase-D do veneno da aranha marrom sobre a membrana das células B16-F10;

III. A fosfolipase-D recombinante de aranha marrom LiRecDT1 foi capaz de hidrolisar tanto fosfolipídios sintéticos como esfingomielina, lisofosfatidilcolina e fosfatidilcolina quanto fosfolipídios estruturalmente organizados nas membranas das células B16-F10 para gerar moléculas bioativas;

IV. Apesar de LiRecDT1 se ligar na membrana e atuar no metabolismo dos fosfolípidos, as células B16-F10 não exibiram nenhuma alteração da viabilidade (avaliada pelo método de Azul de Tripan) ou sofreram qualquer tipo de alteração morfológica, como vacuolização citoplasmática, arredondamento, desadesão do substrato, agregação ou rompimento (observado através do microscópio invertido) sugerindo a ausência de efeitos deletérios de fosfolipase-D sobre as células B16-F10;

V. A atividade catalítica de LiRecDT1 sobre as células das linhagens B16-F10 e B16-F1 foi capaz de estimular um influxo de cálcio para o interior das células, observado pela fluorescência do fluoróforo permeante sensível ao cálcio Fluo4 AM e por estudos comparativos entre LiRecDT1 e a toxina mutada LiRecDT1 H12A, com uma importante mutação no sítio catalítico que reduz drasticamente a atividade enzimática.

VI. A atividade da fosfolipase-D recombinante da aranha marrom sobre as células B16-F10 foi confirmada pela sua capacidade em estimular a proliferação de uma maneira dependente da concentração e do tempo e pela incubação das células na presença de esfingomielina exógena (substrato para a fosfolipase-D recombinante).

VII. Finalmente, a fosfolipase-D recombinante de aranha marrom (LiRecDT1) ligou- se à superfície das células de melanoma B16-F10 porém não causou alterações na viabilidade e morfologia, também atuou sobre o metabolismo de lipídios, gerando lipídios metabolicamente ativos, desencadeando a mobilização de cálcio para o citoplasma das células e aumento de sua proliferação, especialmente na presença de esfingomielina exógena. Por todas essas propriedades se abre a possibilidade do uso desta enzima como uma nova bioferramenta nos estudos sobre fosfolipídios e metabolismo de cálcio.

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8.ANEXOS

PARECER TÉCNICO Nº 542/2006

Processo nº: 01200.000022/1997-37
Requerente: Setor de Ciências Biológicas – UFPR.
CNPJ: 000.348.003/0055-03
Endereço: Centro Politécnico da UFPR- Setor de Ciências Biológicas - 2º Piso, Sala 295 - Caixa Postal 19031 Paraná/PR.
Assunto: Solicitação de Extensão do CQB 009/97
Extrato Prévio: 350/2006 Publicado no D.O.U. de 21 de fevereiro de 2006
Reunião: 90ª Reunião Ordinária da CTNBio, ocorrida em 19 de abril de 2006
Decisão: DEFERIDO

A CTNBio, após apreciação do processo de pedido de Parecer Técnico referente à Solicitação de Extensão do CQB (Certificado de Qualidade em Biossegurança) 009/97, conclui pelo DEFERIMENTO, nos termos deste Parecer Técnico.
No âmbito das competências dispostas na Lei 11.105/05 e seu decreto 5.591/05, a Comissão concluiu que o presente pedido atende às normas da CTNBio e à legislação pertinente que visam garantir a biossegurança do meio ambiente, agricultura, saúde humana e animal.

PARECER TÉCNICO

1) Fundamentação técnica

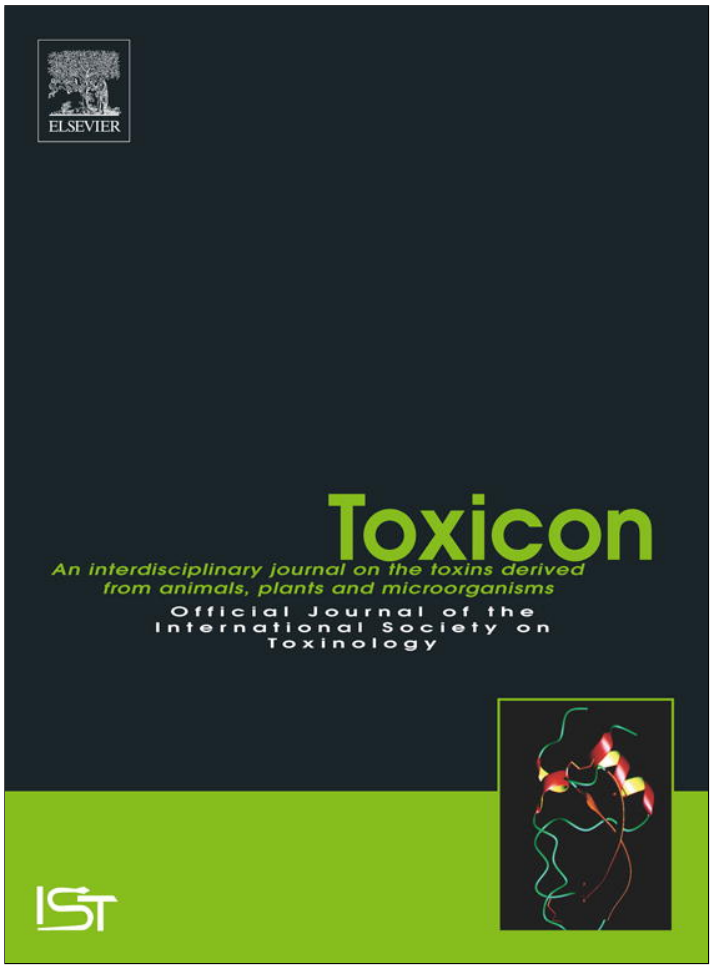
Solicita à CTNBio Parecer Técnico para a extensão de seu CQB do Setor de Ciências Biológicas (UFPR) para a sala de número 198B e sala Preparativa, ambas do departamento de Biologia Celular do mesmo setor. Foram apresentados no processo os currículos dos pesquisadpres, a planta baixa do laboratório, o projeto a ser desenvolvido nele assim como as medidas de biossegurança.

2) Medidas de biossegurança descritas no processo.

Os microrganismos a serem manipulados no projeto (bactérias e leveduras) são da classe de segurança I mas, como os genes a serem expressos (toxinas de animais peçonhentos), apresentam riscos à saúde humana o laboratório que solicita a extensão de CQB tem que ser do tipo NB-2. Embora no processo não haja menção específica sobre a classe laboratorial na qual se encaixa este laboratório, todos os equipamentos apresentados são perfeitamente compatíveis com a classe NB-2, portanto, adequados para as manipulações propostas no projeto. Também foram apresentados as medidas adequadas para o transporte dos microrganismos da sala de manipulação (198B) para a sala de esterilização (sala Preparativa). Além disso, foi informado que as instalações acima mencionadas têm acesso a serviço médico emergencial no próprio setor de Ciências Biológicas da UFPR que contém um centro de atendimento médico para alunos, funcionários e professores além do Hospital Universitário.

Atendidas as recomendações e as medidas de biossegurança, recomenda-se, contudo, que sejam devidamente observadas as práticas laboratoriais compatíveis com a classe laboratorial NB-2.

Dr. Walter Colli
Presidente da CTNBio



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Modulation of membrane phospholipids, the cytosolic calcium influx and cell proliferation following treatment of B16-F10 cells with recombinant phospholipase-D from *Loxosceles intermedia* (brown spider) venom

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ABSTRACT

The mechanism through which brown spiders (*Loxosceles* genus) cause dermonecrosis, dysregulated inflammatory responses, hemolysis and platelet aggregation, which are effects reported following spider bites, is currently attributed to the presence of phospholipase-D in the venom. In the present investigation, through two-dimensional immunoblotting, we observed immunological cross-reactivity for at least 25 spots in crude *Loxosceles intermedia* venom, indicating high expression levels for different isoforms of phospholipase-D. Using a recombinant phospholipase-D from the venom gland of *L. intermedia* (LiRecDT1) in phospholipid-degrading kinetic experiments, we determined that this phospholipase-D mainly hydrolyzes synthetic sphingomyelin in a time-dependent manner, generating ceramide 1-phosphate plus choline, as well as *lyso*phosphatidylcholine, generating *lyso*phosphatidic acid plus choline, but exhibits little activity against phosphatidylcholine. Through immunofluorescence assays with antibodies against LiRecDT1 and using a recombinant GFP-LiRecDT1 fusion protein, we observed direct binding of LiRecDT1 to the membrane of B16-F10 cells. We determined that LiRecDT1 hydrolyzes phospholipids in detergent extracts and from ghosts of B16-F10 cells, generating choline, indicating that the enzyme can access and modulate and has activity against membrane phospholipids. Additionally, using Fluo-4, a calcium-sensitive fluorophore, it was shown that treatment of cells with phospholipase-D induced an increase in the calcium concentration in the cytoplasm, but without altering viability or causing damage to cells. Finally, based on the known endogenous activity of phospholipase-D as an inducer of cell proliferation and the fact that LiRecDT1 binds to the cell surface, hydrolyzing phospholipids to generate bioactive lipids, we employed LiRecDT1 as an exogenous source of phospholipase-D in B16-F10 cells. Treatment of the cells was effective in increasing their proliferation in a time- and concentration-dependent manner, especially in the presence of synthetic sphingomyelin in the medium. The results described herein indicate the ability of brown spider phospholipase-D to induce the

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generation of bioactive phospholipids, calcium influx into the cytoplasm and cell proliferation, suggesting that this molecule can be used as a bioactive tool for experimental protocols in cell biology.

1. Introduction

Biological membranes are thin structures that are basically composed of lipids and proteins and are essential to the functions of cells. From studies in the literature, it is known that beyond simply enclosing and defining the boundary of cells, as in the case of the plasma membrane, or maintaining differences between the cytosol and within organelles, biological membranes are also involved in a number of other functions. These functions include acting as a barrier to polar molecules, providing sites for the attachment of distinct proteins, containing transmembrane proteins that are responsible for the transport of ions and other water-soluble molecules inside/outside of cells, presenting sites for receptors for extracellular/intracellular signals and binding enzymes involved in cell communication, metabolism or the transduction of signals. Additionally, constituents of biological membranes act as substrates that are subjected to biochemical modifications that are important for cell survival or death (Mukherjee and Maxfield, 2004; van Meer, 2005; Engelman, 2005; Alberts et al., 2008; Lodish et al., 2012).

An exciting biological function of membranes is the participation of phospholipids in cell signaling, such as through the phosphorylation of inositol phospholipids in the cytosolic monolayer in plasma membranes, which plays a role in intracellular signaling by activating the recruitment of cytosolic proteins. An additional process associated with these membranes is the activation of phospholipase-C isoforms in the cytosolic monolayer of plasma membranes by extracellular signals, generating fragments of phospholipids, such as diacylglycerol and inositol 1,4,5-triphosphate, which mediate intracellular signaling through the activation of protein kinase-C and the release of calcium from the smooth endoplasmic reticulum, respectively (Nishizuka, 1992; Mukherjee and Maxfield, 2004; van Meer, 2005; Engelman, 2005; Alberts et al., 2008; Lodish et al., 2012). Nevertheless, lipid signaling is not restricted to the constituents of the cytosolic monolayer of plasma membranes. Following cleavage, phospholipids in the outer monolayer are involved in the generation of diacylglycerol, sphingolipids, fatty acids, and molecules derived from such lipids, which act as important mediators of biological activities (Futerman, 2007; Alberts et al., 2008; Lodish et al., 2012). Additionally, membrane phospholipid asymmetry, together with the translocation of phosphatidylserine to the extracellular monolayer of the plasma membrane, acts as a cell surface signal for apoptotic cells to be phagocytosed (Verhoven et al., 1995; Alberts et al., 2008; Lodish et al., 2012). In several types of lipid-dependent cell signaling, phospholipids must be cleaved through the action of different classes of phospholipases, which cleave ester bonds (e.g., isoforms of phospholipase-A_1, phospholipase-A_2 and phospholipase-B) or phosphoester bonds (e.g., isoforms of phospholipase-C and phospholipase-D), generating modified phospholipid acyl or phospholipid head groups that are directly or indirectly modified and act as extracellular or intracellular mediators (Alberts et al., 2008; Nelson and Cox, 2009; Lodish et al., 2012; Aloulou et al., 2012).

Among the different classes of phospholipases, the phospholipase-D class has been receiving special attention in the literature based on the biological activities of these molecules. These enzymes exhibit a broad distribution in nature and have been described in different organisms, such as viruses, bacteria, plants, yeasts, invertebrates and mammals (Jenkins and Frohman, 2005; Raghu et al., 2009). Phospholipase-D catalyzes the hydrolysis of glycerophospholipids or sphingophospholipids, generating phosphatidic acid, lysophosphatidic acid, ceramide 1-phosphate plus choline or other hydrophilic molecules, such as serine, inositol, and ethanolamine. The phosphatidic acid originating in the cellular environment is metabolically converted into diacylglycerol and/or lysophosphatidic acid, while ceramide 1-phosphate is converted in sphingosine 1-phosphate. Both of these molecules can act as second messengers within cells, contributing to the effects of phospholipase-D (Anliker and Chun, 2004; Chalfant and Spiegel, 2005). Several signaling cascades have been described involving these lipid-derived metabolites and their specific membrane receptors. These bioactive lipids are known to activate different signaling pathways in different cells and stimulate various physiological and pathophysiological changes, such as inflammatory responses, platelet aggregation, increased vascular permeability, and cell proliferation and death, among other alterations (Anliker and Chun, 2004). In mammals, phospholipase-D regulates the organization of the actin cytoskeleton, G-protein-linked receptors, and the trafficking of vesicles via endocytosis (McDermott et al., 2004).

Among the living organisms that produce phospholipase-D, *Loxosceles* spiders (brown spiders) are remarkable in producing a mixture of isoforms of these molecules in their venom (da Silva et al., 2004; Kalapothakis et al., 2007). Among the different toxins found in brown spider venom, isoforms of phospholipase-D (referred to as dermonecrotic toxins because of the involvement of these molecules as a hallmark of dermonecrosis) are the most widely biologically and biochemically studied toxins. When purified under laboratory conditions, these molecules can reproduce the major biological effects triggered by crude venom, such as dermonecrosis, red blood lysis, dysregulated inflammatory responses, platelet aggregation, increased vessel permeability and acute renal failure (Chaim et al., 2006; da Silveira et al., 2006, 2007; Kusma et al., 2008; Chaves-Moreira et al., 2009, 2011; Chaim et al., 2011). Previous studies have characterized the dermonecrotic toxin found

in brown spider venoms as a sphingomyelinase D molecule based on its ability to hydrolyze the phospholipid sphingomyelin into choline and ceramide 1-phosphate (Kurpiewski et al., 1981). However, based on the hydrolysis of different purified phospholipids mediated by brown spider venom toxins, the term sphingomyelinase D has been replaced with phospholipase-D as a more accurate and broader denomination because these toxins hydrolyze not only sphingophospholipids but also lysoglycerophospholipids, generating ceramide 1-phosphate or lysophosphatidic acid (LPA) (Lee and Lynch, 2005; Chaim et al., 2011; Chaves-Moreira et al., 2011). It has been postulated that by hydrolyzing phospholipids that generate ceramide 1-phosphate or lysophosphatidic acid, dermonecrotic toxins activate signaling pathways in different cells causing pathophysiological changes, such as inflammatory responses, red blood cell hemolysis, acute renal disease, platelet aggregation, and increased blood vessel permeability (da Silveira et al., 2007; Kusma et al., 2008; Chaves-Moreira et al., 2009, 2011; Chaim et al., 2011).

The idea that there is a family of phospholipase-D proteins in the venoms of *Loxosceles* species was further supported by the cloning and expression of phospholipase-D toxins from a variety of *Loxosceles* spiders. Kalapothakis et al. (2002) performed studies with a recombinant phospholipase-D from *Loxosceles intermedia*. Ramos-Cerrillo et al. (2004) cloned, expressed and analyzed recombinant phospholipase-D proteins from *Loxosceles reclusa* and *Loxosceles boneti* venoms. Binford et al. (2005) reported three cDNA sequences of phospholipase-D in *Loxosceles arizonica*. Chaim et al. (2006), da Silveira et al. (2006, 2007), and Appel et al. (2008) used a cDNA library obtained from the venom gland of *L. intermedia* to clone and express these toxins and observed differential functionality for six related toxins classified as phospholipase-D proteins. Catalán et al. (2011) reported the cloning and heterologous expression of two venom phospholipase-D proteins from *Loxosceles laeta*. Finally, Zobel-Thropp et al. (2012) reported the cloning and heterologous expression of a phospholipase-D from *L. arizonica*. Additionally, proteomic analysis of *Loxosceles* species venoms demonstrated the existence of eleven isoforms of related phospholipase-D proteins in *Loxosceles gaucho* venom (Machado et al., 2005) and at least seven related phospholipase-D proteins in *L. intermedia* (dos Santos et al., 2009). The toxins characterized as phospholipase-D proteins have been grouped into a family (Kalapothakis et al., 2007). The noxious effects induced by crude *Loxosceles* venom may be a result of synergism among these toxins, strengthening the biological importance of these molecules in the biological cycle of *Loxosceles* spiders (Kalapothakis et al., 2007).

Here, using a recombinant isoform of *L. intermedia* phospholipase-D (LiRecDT1) and related biotools, such as polyclonal antibodies against LiRecDT1 and GFP-LiRecDT1 (Chaim et al., 2006; Kusma et al., 2008; Chaves-Moreira et al., 2011), we achieved immune detection of several expressed phospholipase-D isoforms in crude venom and found that they exhibit modulatory activities, such as affecting membrane binding, phospholipid hydrolysis, calcium influx and proliferative activity, in the mouse melanoma cell line B16-F10. The results open the possibility of using this toxin as an exogenous biotool to modulate cellular processes and in studies addressing calcium and phospholipid metabolism.

2. Materials and methods

2.1. Reagents

Polyclonal antibodies against recombinant phospholipase-D (LiRecDT1) were produced in rabbits following the procedure in Chaim et al. (2006). Adult rabbits weighing approximately 3 kg from the Central Animal House of the Federal University of Paraná were used. All experimental protocols involving animals were performed according to the Principles of Laboratory Animal Care (NIH Publication n° 85-23, revised 1985), Brazilian Federal Laws, and ethical committee agreement number 566 of the Federal University of Paraná. Crude *L. intermedia* venom was extracted from wild-caught spiders following Feitosa et al. (1998), in accordance with the Brazilian Federal System for Authorization and Information on Biodiversity (SISBIO-ICMBIO, N° 29801-1). DAPI, AlexaFluor-conjugated anti-rabbit IgG, Fluo-4 AM and the CyQUANT Cell proliferation assay kit were purchased from Molecular Probes (Eugene, Oregon, USA).

2.2. Recombinant protein cloning and expression

A venom gland cDNA library was constructed previously (Chaim et al., 2006; Gremski et al., 2010). The GenBank designation for the deposited data on the cloned *L. intermedia* LiRecDT1 cDNA sequence is DQ218155.1. The cDNA sequence corresponding to the mature phospholipase-D LiRecDT1 protein was amplified via PCR. The forward primer for amplification was 30Rec sense (5′-CTCGAG GCAGGTAATCGTCGGCCTATA-3′), which was designed to contain an Xho I restriction site (underlined) plus the sequence related to the first seven amino acids of the mature protein. The reverse primer was 30Rec antisense (5′-CGGGATCC**TTA**TTTCTTGAATGTCACCCA-3′), which contains a BamH I restriction site (underlined) and a stop codon (bold). The obtained PCR product was cloned into the pGEM-T vector (Promega, Madison). The pGEM-T vector containing the cDNA encoding the mature protein was then digested with the Xho I and BamH I restriction enzymes. The excised insert was gel purified using the QIAquick Gel 74 Extraction Kit (Qiagen, Valencia) and subcloned into a pET-14b vector (Novagen, Madison) digested with the same enzymes. The recombinant protein GFP-LiRecDT1 was obtained by subcloning the previously constructed LiRecDT1 sequence and the enhanced green fluorescence protein (EGFP) sequence into pET-14b using a Blunt-Cut-Cut strategy at the *Nde* I site of pET-14b and two *Bam*H I sites (between LiRecDT1, EGFP and the vector) (Chaves-Moreira et al., 2009). All recombinant constructs were expressed as fusion proteins with a 6x His-Tag at the N terminus and a 13 amino acid linker (including a thrombin site) between the 6x His-Tag and mature protein (N-terminal amino acid sequence before the mature protein: MGSSHHHHHHSSGLVPRGSHMLE). pET-14b/*L. intermedia* cDNA constructs were transformed into

One Shot *E. coli* BL21(DE3)pLysS competent cells (Invitrogen, Carlsbad) and plated on LB agar plates containing 100 mg/mL ampicillin and 34 mg/mL chloramphenicol. A single colony was inoculated into 50 mL of LB broth (100 mg/mL ampicillin and 34 mg/mL chloramphenicol) and grown overnight at 37 °C. A 10 mL aliquot of this overnight culture was grown in 1 L of LB broth/ampicillin/chloramphenicol at 37 °C until an OD of 0.5 at 550 nm was reached. IPTG (isopropyl b-D-thiogalactoside) was added to a final concentration of 0.05 mM, and the culture was induced by incubation for an additional 3.5 h at 30 °C (with vigorous shaking). Cells were harvested via centrifugation (4000 g, 7 min), and the pellet was frozen at −20 °C overnight.

2.3. Protein purification

Cell suspensions were thawed and then disrupted via 6 cycles of 10 s of sonication at low intensity. The lysed materials were centrifuged (20,000 × *g*, 20 min), and the supernatants were incubated with 1 mL of Ni^{2+}-NTA agarose beads for 1 h at 4 °C (with gentle agitation). The suspensions were loaded into a column, and the packed gel was thoroughly washed with the appropriate buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 20 mM imidazole) until the OD at 280 nm reached 0.01. The recombinant protein was eluted with 10 mL of elution buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 250 mM imidazole), and 1 mL fractions were collected and analyzed via 12.5% SDS-PAGE under reducing conditions. The fractions were pooled and dialyzed against phosphate-buffered saline (PBS).

2.4. Gel electrophoresis and western blotting

Protein concentrations were determined using the Coomassie Blue method. Five replicates were performed. Protein analysis was conducted using an IEF system (Ettan IPGphor 3, GE Healthcare) for the first dimension and 12.5% SDS-PAGE under reducing conditions for the second dimension. After electrophoresis, samples of *L. intermedia* crude venom (100 μg) were transferred to nitrocellulose membranes, which were dyed with Ponceau-S and examined via western blotting using hyperimmune antisera against LiRecDT1 (Phospholipase-D) diluted 1:1000.

2.5. Sphingomyelinase activity assay

Phospholipase activity was measured using the Amplex Red Assay Kit (Molecular Probes). In this assay, phospholipase-D activity is monitored using 10-acetyl-3,7-dihydroxyphenoxazine (the Amplex Red reagent), a sensitive fluorogenic probe for H_2O_2 (Giganti et al., 2008). First, recombinant phospholipase-D hydrolyzes sphingomyelin to yield ceramide 1-phosphate and choline. Choline is then oxidized by choline oxidase to betaine and H_2O_2. Finally, in the presence of horseradish peroxidase, H_2O_2 reacts with the Amplex reagent in a 1:1 stoichiometry to generate the highly fluorescence product resorufin. In our experiments, recombinant phospholipase-D (10 μg, in three trials) was added to the Amplex Red reagent mixture. The reaction tubes were incubated at 37 °C for 5 min to 24 h, and fluorescence was measured in a Tecan Infinite® M200 spectrofluorometer (Tecan, Männedorf, Switzerland) with excitation at 540 nm and emission detection at 570 nm. The same method was used to test the ability to hydrolyze other phospholipids, such as Egg SM (Sphingomyelin Egg, Chicken), 16:0 *Lyso* PC (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine) and 16:0–18:0 PC (1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine), with the exception that the sphingomyelin in the kit was exchanged with other phospholipids, and choline generation was measured. All phospholipids were acquired from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA).

2.6. Cell culture

B16-F10 cells were purchased from the American Type Culture Collection (Rockville, MD). The cells were grown in RPMI medium containing 40 μg/mL gentamicin sulfate supplemented with 10% fetal calf serum (FCS). The cultures were maintained at 37 °C in a humidified atmosphere under 5% CO_2. Release of the cells was achieved via treatment with a 10 mM solution of ethylenediaminetetraacetic acid (EDTA) in cation-free/PBS for 10 min. After cell counting, the cells were resuspended in medium supplemented with FCS and allowed to adhere and grow for 24 h. The cells were then evaluated in the presence or absence of the recombinant LiRecDT1 phospholipase under the different concentrations indicated. During the experiments, the plates were photographed at 24, 48 and 72 h using an inverted microscope (Leica-DMIL, Wetzlar, Germany), and changes in cell morphology were evaluated. Additionally, cytotoxicity assays were carried out in 24-well plates (TPP, Trasadingen, Switzerland). Cells (4×10^4 cells/well) were plated and allowed to adhere and grow for 24 h prior to incubation with recombinant toxins at concentrations of 100, 200, and 300 μg/mL for 24, 48 and 72 h in hexaplicate. After toxin incubation, cell viability was estimated according to the Trypan blue exclusion method (Merck, Darmstadt, Germany) as described by Freshney (2000). The same experimental conditions were applied to a control group, except that the medium contained PBS in place of the toxins. The cell viability of the control group (in the absence of toxins) was set as 100%.

2.7. Detection of choline release from B16-F10 cell ghosts and a B16-F10 cell ghost extract

B16-F10 cell membrane ghosts were obtained from approximately 5×10^8 cells. B16-F10 cells were lysed and washed in Hypo-osmotic Buffer (NaH_2PO_4 5 mM, PMSF 2 mM and pH 8.0). The lysed cells were collected via centrifugation (12,000 × *g*, 10 min, 4 °C); this procedure was repeated four times. The B16-F10 cells ghosts were resuspended in 1 mL of cold extraction buffer (Tris–HCl 50 mM, NaCl 150 mM, Triton X-100 0.5%). After gentle homogenization for 10 min at 4 °C, the suspension was centrifuged at 20,000 × *g* for 20 min at 4 °C, and the supernatants were collected for subsequent use. The ghosts and extracts (50 μg of protein) were utilized as substrates for LiRecDT1 (10 μg) in a total final volume of 250 μL for 5, 15 or 30 min or 1, 3, 6, 12 or 24 h, at 37 °C, followed by gentle mixing using a rotational shaker in a BOD incubator.

The contents of the treated tubes were then added to a 250 μL reaction mixture adapted from the Amplex Red Sphingomyelinase Assay Kit (Molecular Probes) containing choline oxidase (4 U), alkaline phosphatase (80 U), horseradish peroxidase (20 U), and the Amplex Red reagent (100 μM), excluding the sphingomyelin substrate. After incubation in a water bath for 30 min at 37 °C, fluorescence was measured in a Tecan Infinite® M200 spectrofluorometer (Tecan) with excitation at 540 nm and emission detection at 570 nm.

2.8. Immunofluorescence and GFP fluorescence binding

B16-F10 cells (0.5×10^3) were incubated with LiRecDT1 for 5 h (10 μg/mL), and 100 μL of the cell suspension was applied to coverslips for adhesion. Unbound cells were removed by washing 10 times with PBS, and adherent cells were fixed with 4% paraformaldehyde in PBS for 30 min at 4 °C. The cells were then incubated with 0.1 M glycine for 3 min and washed with PBS; then, prior to incubation with antibodies, the non-specific binding sites were blocked by incubating the coverslips in blocking buffer (PBS containing 1 mg/mL BSA, 0.1 M glycine) for 20 min. The samples were stained to detect phospholipase-D via indirect immunofluorescence using antibodies incubations at a 1:1000 dilution in PBS (anti-LiRecDT1) for 2 h. After washing with PBS, the slides were incubated for 1 h with the secondary antibody Alexa-Fluor-594 conjugated anti-rabbit IgG (1:500) in PBS. For the antigen competition assays, the immunofluorescence protocol was the same as described above, except that hyperimmune IgG against recombinant LiRecDT1 phospholipase-D was previously incubated with 100 μg/mL of LiRecDT1 diluted in PBS for 1 h at 37 °C. Then, the mixture was incubated with treated B16-F10 cells as described above. To analyze nuclear fluorescence, cells were incubated with DAPI (4′-6-Diamidino-2-phenylindole) (300 nM diluted in PBS) for 5 min. Coverslips were mounted on glass slides using Fluoromount-G (Southern Biotechnology, Birmingham, AL). LiRecDT1-GFP binding was evaluated as described above, except that B16-F10 cells (0.5×10^3 cells) were incubated with 10 μg/mL of the recombinant fluorescent toxin (5 h, 37 °C). Non-specific binding of GFP alone to the cells was evaluated as a negative control. For binding competition assays, the fluorescence protocol was the same as described above, except that B16-F10 cells were previously incubated with an excess of LiRecDT1 (100 μg/mL) for 1 h at 37 °C and then with 10 μg/mL LiRecDT1-GFP. The samples were observed using a Zeiss Axio Observer.Z1 inverted microscope (Carl Zeiss, Germany). Single images were obtained using a 63× oil lens for differential interface contrast (DIC) microscopy and a monochromatic camera (AxioCam HRm, Carl Zeiss) to examine fluorescence intensity. Finally, AxioVision LE software was used for image processing and morphometric measurements in the Zeiss image format (ZVI).

2.9. Measurement of Ca^{2+} influx into B16-F10 cells containing fluo-4

B16-F10 cells (1×10^8 cells/mL) were prepared in Ringer's Solution (122.5 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 10 mM HEPES, 11 mM glucose, 1 mM NaH_2PO_4, pH 7.4) containing 5 mM $CaCl_2$ and treated according to Kaestner et al. (2006) and Haase et al. (2009). B16-F10 cells were loaded with Fluo-4 AM (10 μM) in buffer with Pluronic F-127 (0.01%) for 30 min at 37 °C. This indicator exhibits high-affinity binding to Ca^{2+} (K_d = 345 nM) and shows a large increase in fluorescence intensity in response to Ca^{2+} binding (>100 fold). Subsequently, the cells were washed twice with Ringer's Solution and equilibrated for de-esterification for 30 min at room temperature. Then, the cells were incubated with 25 μg/mL recombinant phospholipase-D (LiRecDT1) for 5, 15, 30, 45, 60 or 90 min. Cells incubated under the same laboratory conditions but in the absence of phospholipase-D for 90 min were used as a control. Following this reaction, the cells were transferred to Black 96-well plates at a density of 1×10^6 cells/well in a total volume of 200 μL, and the resulting fluorescence was recorded on a Tecan Infinite M200 spectrofluorometer (Tecan) using an excitation wavelength of 485 nm and measuring emission at 535 nm. Additionally, Fluo-4 dye-loaded B16-F10 cells were allowed to settle onto coverslips, and images of calcium-dependent fluorescence were obtained using an Axio Observer.Z1 inverted microscope Zeiss (Carl Zeiss, Germany). Fluo-4 AM was excited at 488 nm, with emission detected using an LP 505 nm filter (green channel). Single images were obtained using a 63× oil lens for differential interface contrast (DIC) microscopy and a monochromatic camera (AxioCam HRm, Zeiss, Carl Zeiss, Germany) to measure the fluorescence intensity. Finally, AxioVision LE software was used for image processing and to perform morphometric measurements in the Zeiss image format (ZVI).

2.10. Proliferation assays

B16-F10 cells were initially cultivated as described above, in RPMI medium containing 40 μg/mL gentamicin sulfate supplemented with 10% fetal calf serum (FCS). The cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Prior to conducting the proliferation assays, B16-F10 cells (5×10^3 cells/well) were plated in 96-well plates (TPP) and allowed to adhere and grow for 24 h under the same conditions as described above. Subsequently, the culture medium was removed and replaced with RPMI without serum to synchronize the cell cycle for an additional 24 h. Cells were then incubated with LiRecDT1 at concentrations of 10 and 25 μg/mL for 48 h in pentaplicate. The same experimental conditions were used in the control group, except that the medium contained an adequate amount of vehicle (PBS) rather than LiRecDT1. Additionally, an evaluation of the proliferation of B16-F10 cells following LiRecDT1 exposure was performed, but by using a concentration of 10 μg/mL, with a time of exposure of 24, 48 or 72 h after the addition of phospholipase-D. Finally, proliferation assays were conducted with cells in the presence of synthetic sphingomyelin (5 and 10 mM) and LiRecDT1 at a concentration of 10 μg/mL for 48 h. After phospholipase-D incubation, measurement of cell proliferation was performed via the CyQUANT cell proliferation assay (Molecular Probes), as described by the manufacturer. This method is based on the use of a green

fluorescent dye that exhibits fluorescence when bound to cellular nucleic acids. The resulting fluorescence was recorded on a Tecan Infinite M200 spectrofluorometer (Tecan) using an excitation wavelength of 480 nm and measuring emission at 520 nm.

2.11. Statistical analysis

Statistics were performed using analysis of variance (ANOVA) and a post-hoc Tukey's test for comparisons of means with the GraphPad InStat program, version 5.00 for Windows 7 and Vista. Statistical significance was set at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. Native dermonecrotic toxin (phospholipase-D) profile of crude L. intermedia venom

Brown spiders (*Loxosceles* genus) are responsible for necrotic or gangrenous arachnidism. Their venoms are remarkable due to their inflammatory and dermonecrotic activities, as previously reported, and data in the literature have indicated phospholipase-D toxins as being responsible for these deleterious effects (da Silva et al., 2004, Kalapothakis et al., 2007). Fig. 1 shows the phospholipase-D profile of *L. intermedia* crude venom processed through two-dimensional electrophoresis, followed by immunoblotting using a polyclonal antibody raised against the recombinant toxin LiRecDT1 (Chaim et al., 2006). The results indicated the existence of an intra-species family of antigenically and structurally related toxins (as indicated by the visualization of at least 25 spots), strengthening the hypothesized biological importance of this family of toxins in the biology of this spider and supporting transcriptome data showing that phospholipase-D mRNAs contribute approximately 20% of the total toxin-encoded transcripts in *L. intermedia* venom (Gremski et al., 2010).

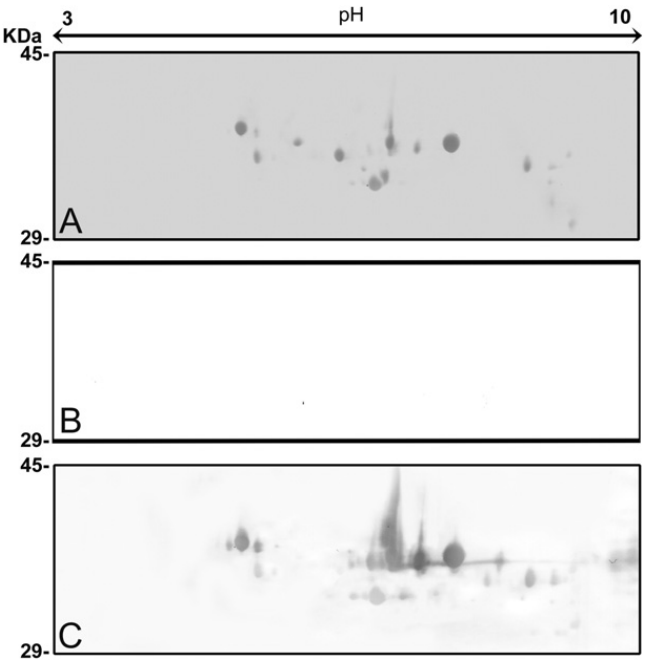


Fig. 1. Two-dimensional electrophoresis profile of native phospholipase-D proteins from *L. intermedia* crude venom. Crude venom from *L. intermedia* was separated via two-dimensional electrophoresis using isoelectric focusing (IEF) electrophoresis (pH 3–10) and 12.5% SDS-PAGE under reducing conditions. Proteins were immunoblotted with pre-immune serum (B), or polyclonal serum raised against LiRecDT1 (C). Panel (A) shows crude venom dyed with Ponceau-S. The positions of the molecular masses of standard proteins are depicted on the left of the figure, and the pH gradient is indicated at the top. The result indicated that at least 25 spots reacted with the serum, strengthening the idea of an intraspecies family of related phospholipase-D proteins and suggesting the importance of this molecule for the biology of brown spiders.

3.2. The recombinant brown spider venom phospholipase-D LiRecDT1 exhibits both sphingomyelinase-D and a lysophospholipase-D activities

To validate the model of recombinant brown spider venom phospholipase-D as a tool for studying the biological modulation of cells by generating bioactive lipids, we present the results of a kinetic digestion experiment performed against different synthetic phospholipids, including sphingomyelin (SM), which is a sphingophospholipid, and lysophosphatidylcholine (LPC) and phosphatidylcholine (PC), which are glycerophospholipids. The phospholipids (5 mM) were treated with LiRecDT1 (10 μg) under the same experimental conditions (examining the kinetics from 5 min to 24 h), and choline generation was then evaluated using a fluorimetric method. As shown in Fig. 2, SM was preferentially hydrolyzed compared to LPC, which was also hydrolyzed but to a lower degree, while PC was only residually hydrolyzed; this degradation occurred in a time-dependent manner. Under the applied conditions, recombinant brown spider phospholipase-D preferentially hydrolyzes SM and LPC and can be considered both a sphingomyelinase-D and a lysophospholipase-D. Following the LiRecDT1 treatments, the results indicated the generation of at least two bioactive

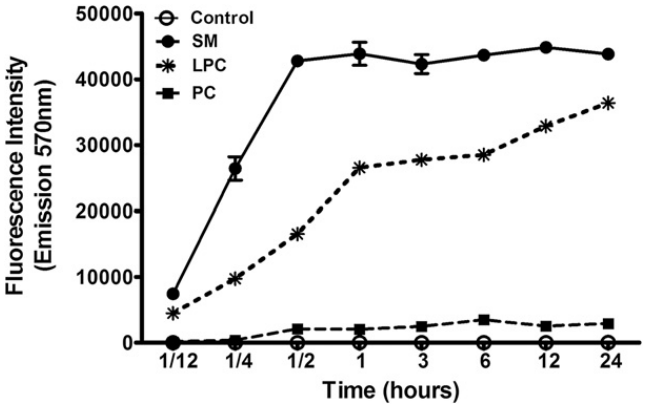


Fig. 2. The recombinant phospholipase-D LiRecDT1 hydrolyzes synthetic phospholipids. The ability of recombinant phospholipase-D to hydrolyze synthetic phospholipids such as sphingomyelin (SM), lysophosphatidylcholine (LPC), and phosphatidylcholine (PC) was tested verifying the production of choline after phospholipase-D in a kinetic protocol. As a negative control, sphingomyelin was incubated in the absence of phospholipase-D. The results are presented as the average of three experiments ± SEM. According to the time of exposure, recombinant phospholipase-D presents a preference for SM as a substrate and also exhibited hydrolytic activity against LPC, but only residual activity against PC under the evaluated conditions.

lipids: ceramide 1-phosphate from SM and lysophosphatidic acid from LPC. Although, SM is hydrolyzed at first 30 min with a higher intensity when compared to LPC.

3.3. Evidence that recombinant phospholipase-D binds to the B16-F10 cell membrane

Additionally, we demonstrated that there are attachment sites for recombinant brown spider phospholipase-D on the B16-F10 cell membrane. B16-F10 cells were used as a melanoma model because melanoma cells produce and secrete autotaxin-like phospholipase-D molecules, which have been found to be involved in the stimulation of tumor cell growth and several other metabolic changes (Umezu-Goto et al., 2002; Okudaira et al., 2010). We investigated B16-F10 cells treated with LiRecDT1 based on an immunofluorescence reaction using an antibody that reacts with brown spider phospholipase-D (Chaim et al., 2006; da Silveira et al., 2006). As shown in Fig. 3A, the antibody reaction produced a positive signal at the B16-F10 cell surface. To confirm antibody specificity, we employed the same immunofluorescence approach with the following modifications: incubating the antibody with an excess of LiRecDT1 (100 μg/mL) in solution and then exposing B16-F10/LiRecDT1-treated cells to this mixture (antigen competition assay). The results supported the direct binding of LiRecDT1 to the B16-F10 cell surface. Moreover, B16-F10 cells were incubated with the recombinant fusion toxin

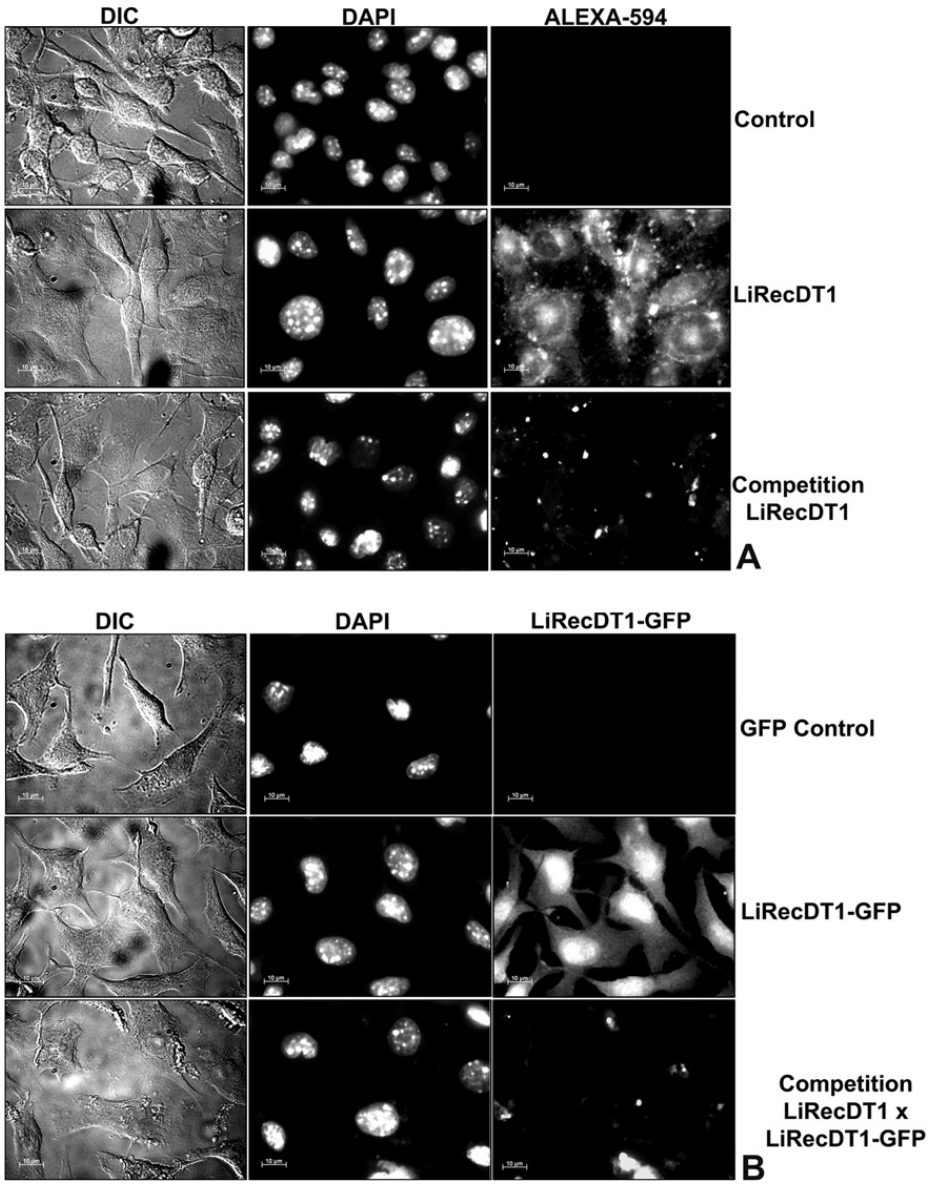


Fig. 3. Recombinant phospholipase-D binds to the B16-F10 cell membrane. (A) B16-F10 cells were immunostained with antibodies against recombinant phospholipase-D or to detect antibody specificity in a competition assay using antibodies against LiRecDT1 that had previously been incubated with an excess of LiRecDT1 in solution. As a negative control, cells were processed identically in the absence of toxin treatment. The cells were observed under differential interference contrast (DIC) microscopy or analyzed based on nuclear staining using DAPI (DAPI) or through immunofluorescence. Scale bars are shown at the left of the figure. (B) Alternatively, B16-F10 cells were treated with the recombinant fusion toxin GFP-LiRecDT1 or with a competition solution for examining the competition between LiRecDT1-GFP and an excess of LiRecDT1; as a negative control, the cells were exposed to recombinant GFP alone. The cells were observed under DIC microscopy or analyzed based on nuclear staining using DAPI or fluorescence. Scale bars are shown at the left of the figure at the bottom. The results supported the presence of "planted phospholipase-D" and its binding to the B16-F10 cell surface.

GFP-LiRecDT1 (Chaves-Moreira et al., 2009) using GFP alone as a negative control. The cells were evaluated via fluorescence microscopy. As depicted in Fig. 3B, the recombinant phospholipase-D fusion protein bound to B16-F10 cells, whereas the signal for GFP alone was negative. These findings were strengthened by the results of binding competition assays, as described in the Materials and Methods. Our results support the direct binding of brown spider phospholipase-D to the membrane of B16-F10 cells and suggest a possible enzyme catalytic domain-dependent manner of modulating biological activities via generating bioactive lipids from membrane phospholipids in these cells.

3.4. The recombinant brown spider venom phospholipase-D LiRecDT1 hydrolyzes plasma membrane phospholipids of B16-F10 cells

Exogenous recombinant brown spider phospholipase-D binds to the surface of B16-F10 cells and hydrolyzes synthetic phospholipids such as sphingomyelin and lysophosphatidylcholine that are normally constituents of cell membranes. To ascertain whether this recombinant phospholipase-D is able to alter the levels of phospholipids that are present and organized as a lipid bilayer in the cytoplasmic membrane of cells, likely containing different hydrophobic tails among their fatty acids compared to synthetic molecules, ghosts of B16-F10 cells or detergent extracts of ghosts (Fig. 4) (washed ghosts of cells were used to avoid cytoplasmic phospholipids being used as substrates for recombinant phospholipase-D) were treated with LiRecDT1, and the generation of choline was examined in a fluorimetric assay. As depicted in the figures, choline production was detected following LiRecDT1 treatment both in the presence of ghosts and detergent extracts of ghosts, supporting the accessibility and activity of recombinant brown spider phospholipase-D with respect to plasma membrane phospholipids of B16-F10 cells.

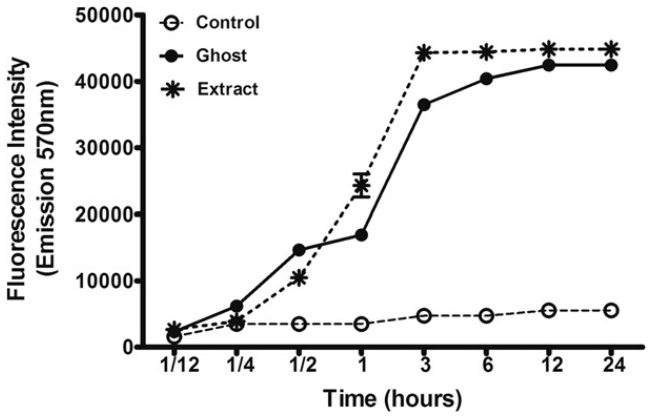


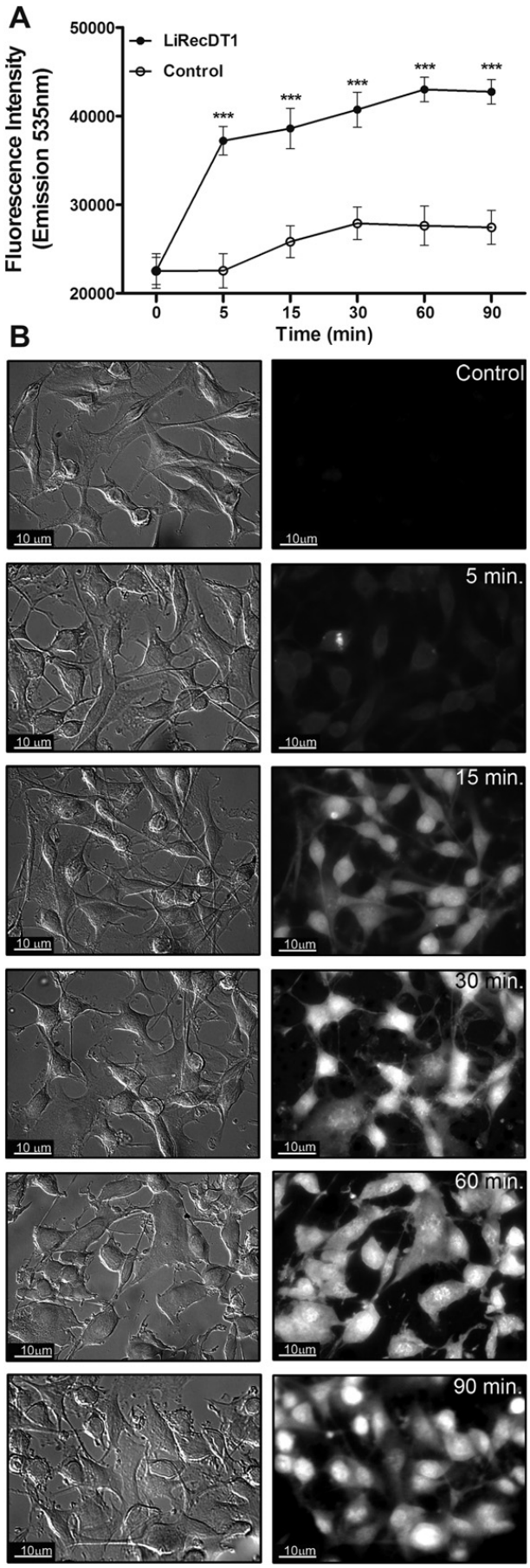
Fig. 4. Recombinant phospholipase-D hydrolyzes phospholipids in the plasma membrane of B16-F10 cells. Additionally, ghosts of B16-F10 cells (Ghost) or detergent extracts of ghosts of B16-F10 cells (Extract) were treated with recombinant phospholipase-D. As a negative control, extracts of B16-F10 cells incubated without the toxin. The generation of choline was examined in a spectrofluorimetric assay. The data are presented as the average of three experiments ± SEM. LiRecDT1 hydrolyzed phospholipids in both ghosts and detergent extracts from ghosts of B16-F10 cells compared to the negative control, which did not generate choline. The results indicate the accessibility and activity of recombinant phospholipase-D with respect to B16-F10 membrane phospholipids.

3.5. Recombinant phospholipase-D causes a cytoplasmic influx of calcium in B16-F10 cells but does not trigger cytotoxicity

Because lysophosphatidic acid, which is a lipid-derived product generated following exogenous autotaxin activity in various cell types, can mobilize calcium in several cell types (Stunff et al., 2004; Itagaki et al., 2005), we studied the involvement of recombinant brown spider phospholipase-D activity on calcium mobilization in B16-F10 cells. We examined the calcium influx into B16-F10 cells following recombinant phospholipase-D treatment in the presence of Fluo-4, a cell-permeant, calcium-sensitive fluorophore, via spectrofluorimetry. As shown in Fig. 5A, phospholipase-D treatment caused increases in fluorescence and in the calcium influx in B16-F10 cells in a time-dependent manner. Additionally, Fluo-4-loaded B16-F10 cells were treated with recombinant phospholipase-D (LiRecDT1) in different time intervals and observed using an inverted microscope for differential interface contrast (DIC) microscopy and to observe the fluorescence intensity (see details in the Materials and Methods). There was increased fluorescence and Calcium uptake observed according to the time following phospholipase-D treatment (Fig. 5B), strengthening the idea that exposure to exogenous recombinant brown spider phospholipase-D induced an acute ionic response associated with Calcium influx into the B16-F10 cells. To avoid the possibility that the Calcium influx into B16-F10 cells was a consequence of the deleterious effect of toxins on the plasma membrane of cells, thereby causing a change in membrane integrity and an artificial Calcium influx, the viability of cells was assayed through the Trypan blue exclusion method, and the morphology of the cells was evaluated using inverted microscopy. As indicated in Fig. 6A and B, even when recombinant phospholipase-D was applied at concentrations 10× higher than those that induced a Calcium influx, phospholipase-D treatment did not alter the viability or morphology of the cells or their adhesion to the culture substrate, indicating that under these conditions, recombinant phospholipase-D did not alter the integrity of the membrane or damage the health of the cells.

3.6. Recombinant brown spider phospholipase-D stimulates B16-F10 cell proliferation

Finally, we studied the impact of recombinant brown spider phospholipase-D on the proliferation of B16-F10 cells because it has been demonstrated that exogenous autotaxin is a powerful inducer of cell proliferation. To this end, B16-F10 cells (5×10^3 cells/well) were treated with recombinant brown spider phospholipase-D (10 and 25 μg/mL for 48 h), and their cell proliferation was evaluated using the CyQUANT method and spectrofluorimetry. As shown in Fig. 7A, exogenous treatment of B16-F10 cells with the recombinant phospholipase-D led to an increase in cell growth in a concentration-dependent manner. Additionally, cells (5×10^3 cells/well) were treated with



recombinant phospholipase-D (10 μg/mL) for 24, 48 or 72 h, and their proliferation was examined under conditions identical to those described above. It was observed that exogenous treatment with recombinant brown spider phospholipase-D induced proliferation in a time-dependent manner (Fig. 7B), strengthening the idea that the lipid-modulating and other activities of this molecule in cells stimulate increases in proliferation. The putative lipid substrates that are targeted following brown spider phospholipase-D exposure include sphingomyelin, which produces ceramide 1-phosphate following phospholipase-D treatment, and other interconvertible bioactive molecules, such as ceramide and sphingosine 1-phosphate (both of which are bioactive lipids involved in increasing cell proliferation) (Chalfant and Spiegel, 2005). Therefore, we repeated the proliferation assays (5×10^3 cells/well), but using exogenous sphingomyelin (5 and 10 mM) in the culture medium together with the recombinant phospholipase-D LiRecDT1 at a concentration of 10 μg/mL for 48 h. As depicted in Fig. 7C, cells incubated with exogenous sphingomyelin showed a higher proliferation index, indicating that brown spider phospholipase-D can act as an exogenous factor that stimulates proliferation.

4. Discussion

Phospholipase-D proteins have been described as important regulators of several critical physiological processes (Exton, 2002). These enzymes catalyze the hydrolysis of various phospholipids, generating bioactive molecules that play a role in distinct events in intracellular signaling cascades. Phospholipase-D proteins have also been shown to regulate the cell cycle, cell proliferation and apoptosis (Foster and Xu, 2003).

Dysregulation of these cellular processes has been reported during the development of several types of human tumors, such as breast, stomach, and colorectal cancers (Foster and Xu, 2003), and data reported in the literature have indicated changes in the expression of phospholipase-D proteins in several types of cancers related to increases in cell proliferation, transformation, survival, and tumor invasiveness (Foster and Xu, 2003). The activity of phospholipase-D proteins are up regulated as response to treatment with different growth factors, such as platelet-derived growth factor (PDGF) (Plevin et al., 1991), epidermal growth factor (EGF) (Song et al., 1994), fibroblast

Fig. 5. Treatment of B16-F10 cells with recombinant phospholipase-D causes an influx of calcium. (A) B16-F10 cells were incubated with LiRecDT1 in the presence of Fluo-4 in a buffer containing calcium and the fluorescence of Fluo-4 was measured after various periods. As a negative control, B16-F10 cells were also incubated without phospholipase-D treatment. The presented values are the average of five experiments ± SEM. Significance is defined as $^{***}p < 0.001$. There was an increase in the uptake of calcium following LiRecDT1 exposure. (B) Additionally, B16-F10 cells were exposed to recombinant phospholipase-D (LiRecDT1) for different times and then observed via transmission microscopy together with DIC and fluorescence imaging of Fluor-4-loaded cells. The control was B16-F10 cells cultured without phospholipase-D, but still loaded with Fluor-4. Phospholipase-D treatment induced an increase of calcium inside the cells in a time-dependent manner. Scale bars are shown at the left and bottom of the figures.

growth factor (FGF) (Sa and Das, 1999), insulin-like growth factor-1 (ILGF-1) (Banno et al., 2003), and growth hormone (Zhu et al., 2002). Fibroblasts in culture exposed to exogenous phospholipase-D (from *Streptomyces chromofuscus*) showed increased production of lysophosphatidic acid (LPA) generated from lysophosphatidylcholine in the external monolayer of the plasma membrane. This LPA production resulted in the activation of the G-protein-linked LPA receptor and subsequent activation of the *Ras*, *Rho* and Calcium-dependent intracellular signaling cascades (van Dijk et al., 1998). An increase of phospholipase-D activity has been described in different cells transformed by oncogenes, such as *v-Src*, *v-Ras*, *v-Fps e v-Raf* (Foster and Xu, 2003).

In addition to endogenous phospholipase-D proteins, the existence of several exogenous phospholipase-D proteins produced by distinct living organisms has been reported (Raghu et al., 2009; Lucas et al., 2010; Murph et al., 2011). Among the members of the exogenous phospholipase-D family, brown spider phospholipase-D represents a prominent example of a biologically active molecule, and the participation of these molecules and their catalysis have been observed associated with several pathophysiological aspects of loxoscelism, such as dermonecrosis, dysregulated inflammatory responses, nephrotoxicity, platelet aggregation and hemolysis (Chaim et al., 2006; da Silveira et al., 2006, 2007; Appel et al., 2008; Kusma et al., 2008; Chaves-Moreira et al., 2011; Chaim et al., 2011).

Brown spider venom contains a complex mixture of toxins that exhibit a broad spectrum of biological, pharmacological and biochemical activities, supporting the putative biotechnological use of these molecules as bioactive tools for multipurpose methodologies. Recently, based on constructing a cDNA library and studying the transcriptome profile of the venom gland of the brown spider *L. intermedia*, we described the diversity of molecules expressed by this venom (Gremski et al., 2010). Transcriptome analysis of venom gland mRNA from *L. intermedia* demonstrated that phospholipase-D mRNAs represent 20.2% of the total toxin-encoding transcripts in this organ (Gremski et al., 2010). Using molecular biology techniques, such as cloning, heterologous expression, amino acid alignment and phylogenetic analysis, we were able to describe the functions of six isoforms of phospholipase-D in the *L. intermedia* venom, which were designated LiRecDT1 (GenBank accession number DQ218155), LiRecDT2 (GenBank accession number DQ266399), LiRecDT3 (GenBank accession number DQ267927), LiRecDT4 (GenBank accession number DQ431848), LiRecDT5 (GenBank accession number DQ431849) and LiRecDT6 (GenBank accession number EF474482) (Chaim et al., 2006; da Silveira et al., 2006, 2007; Appel et al., 2008). Recently, we identified a novel functional isoform of phospholipase-D referred to as LiRecDT7 (L. Vuitika personal communication, 2012).

The idea that exogenous brown spider venom phospholipase-D isoforms could be useful reagents for cell biology studies and can interact with exposed cells arises from the clinical effects triggered following spider bites accidents. Bites evoke a deep and dysregulated inflammatory response related to gangrenous and dermonecrotic loxoscelism (histopathologically characterized as an aseptic coagulative necrosis). The venom also triggers platelet aggregation, causing thrombocytopenia, induces hemolysis and is nephrotoxic (Luciano et al., 2004; da Silva et al., 2004; Swanson and Vetter, 2006). All of these events can be reproduced using purified recombinant brown spider phospholipase-D isoforms under laboratory conditions, strengthen the idea that phospholipase-D molecules in the venom play an essential role in such as activities and could modulate cellular functions (Chaim et al., 2006; da Silveira et al., 2006, 2007; Appel et al., 2008; Kusma et al., 2008; Senff-Ribeiro et al., 2008; Chaves-Moreira et al., 2009, 2011; Chaim et al., 2011).

Herein, studying crude *L. intermedia* venom through a two-dimensional electrophoresis approach using a wide range of pI values (3.0–10.0) in the first dimension, SDS-PAGE for the second dimension, and immunodetection of venom phospholipase-D with a polyclonal antiserum raised against a recombinant form of brown spider venom phospholipase-D (LiRecDT1), we showed that the venom contains a heterogeneous mixture of proteins (at least 25 spots) ranging in size from 30 kDa to 35 kDa and presenting pI levels ranging from acidic to basic that cross-reacted with antibodies. This result is in agreement with data reported in the literature, which have described crude venom as a mixture of proteins enriched in the low molecular mass range (20–40 kDa) (Veiga et al., 2000). Our findings also corroborate results in the literature indicating that brown spider venom contains several members of the phospholipase-D family. For instance, eleven intraspecies isoforms of phospholipase-D have been observed in *L. laeta* venom (Machado et al., 2005). Finally, our results strengthened the observations of Gremski et al. (2010), who showed that phospholipase-D mRNA accounts for approximately 20.2% of the toxin-encoding transcripts in the *L. intermedia* venom gland based on transcriptome analysis, and the reported cloning of seven phospholipase-D isoforms from the *L. intermedia* venom gland, as noted above. The results indicating a high expression profile of proteins that are immunologically correlated with phospholipase-D isoforms strongly suggest that this class of toxins is of great biological importance in the venom, as they most likely play a role either as defense molecules against predators or lethal molecules during food capture by spiders. Additionally, recent data have indicated that brown spider venom phospholipase-D proteins might act as insecticidal molecules (Zobel-Thropp et al., 2012).

Using confocal immunofluorescence microscopy with antibodies against LiRecDT1 (Chaim et al., 2006; da Silveira et al., 2006), we were able to detect the binding of this exogenous phospholipase-D on to B16-F10 cell surface. Additionally, the interaction of phospholipase-D with the B16-F10 cell membrane was supported by the binding of a recombinant fusion phospholipase-D (GFP-LiRecDT1) (Chaves-Moreira et al., 2009), as shown via fluorescence microscopy and competition assays. Our results demonstrated the existence of sites of attachment for brown spider phospholipase-D on the B16-F10 cell membrane and suggested that this molecule could exert its enzymatic activity on membrane constituents in these cells, which is the first

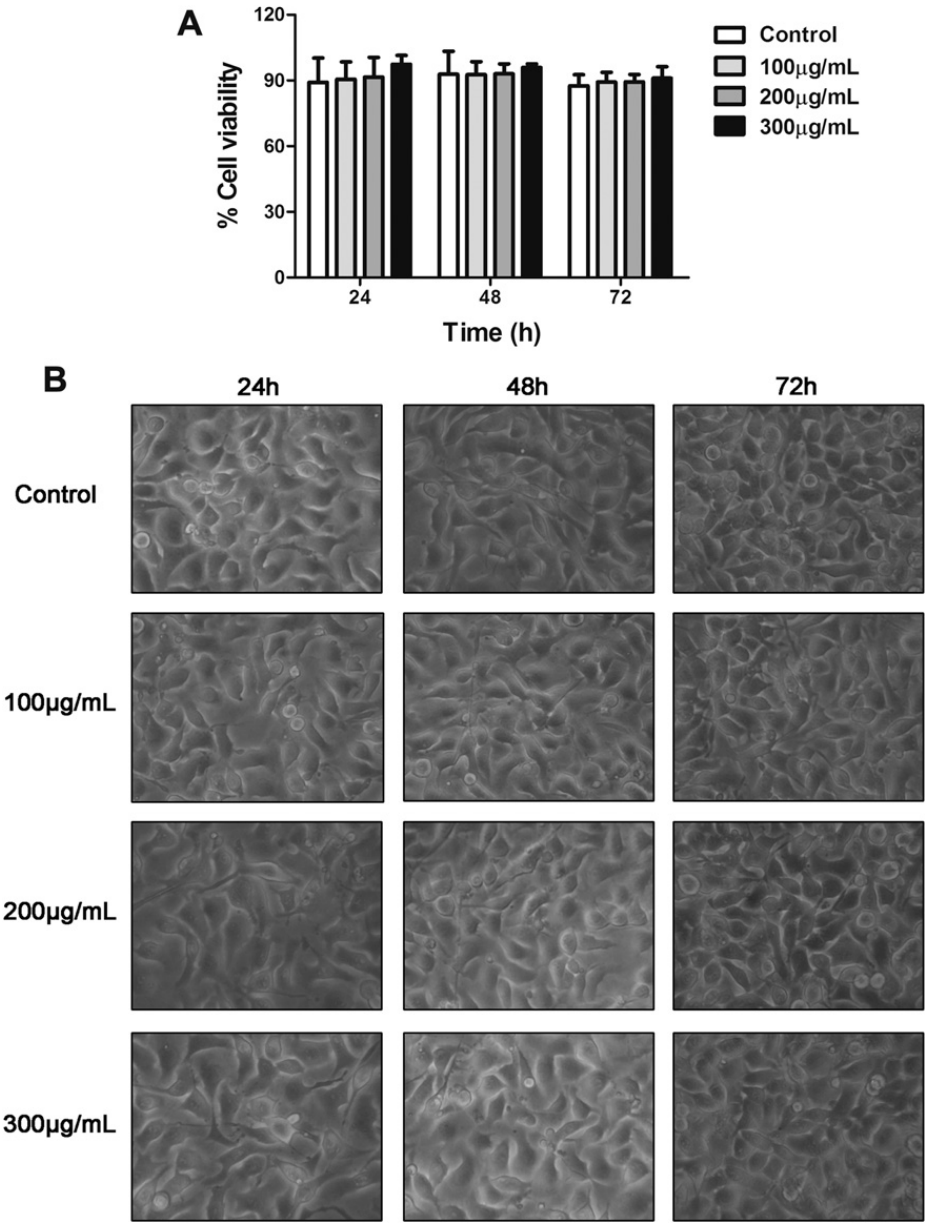


Fig. 6. Cytotoxicity assays. Effect of LiRecDT1 on the viability and morphology of B16-F10 cells. (A) The cytotoxic effect of LiRecDT1 on B16-F10 cells was analyzed via a Trypan blue dye exclusion assay. The cytotoxic effects of LiRecDT1 were determined after 24, 48 and 72 h under the indicated concentrations of purified phospholipase-D. The experiments were performed in hexaplicate, and the presented values given the mean ± SEM. Even at the highest tested concentration used, phospholipase-D exposure did not cause cytotoxicity under the applied conditions. (B) Morphological analysis of B16-F10 cells exposed to LiRecDT1 showed no signals of cytotoxicity such as vacuolated cytoplasm, changes in cell adhesion and spreading under culture conditions or altered morphology.

condition for being classified as an exogenous cellular modulator. Furthermore, our results supported the direct binding of phospholipase-D to the membrane of B16-F10 cells and suggest that the effects on plasma membrane constituents may occur in a manner that is dependent upon the enzyme catalytic domain. Corroborating these data, it has recently been reported that a recombinant phospholipase-D from *L. laeta* was able to induce changes in lateral structures and morphology of target membranes using large and giant unilamellar vesicles (Stock et al., 2012). Additionally, it has been shown that endothelial cells, tubular epithelial cells and erythrocytes are targets for the binding of recombinant brown spider phospholipase-D (Kusma et al., 2008; Chaim et al., 2011; Chaves-Moreira et al., 2011).

To demonstrate that phospholipase-D catalysis and the degradation of membrane phospholipids play a role in inducing metabolic changes in cells, we showed that recombinant phospholipase-D (LiRecDT1) was able to hydrolyze synthetic sphingomyelin and *lyso*phosphatidylcholine, which are important membrane constituents of the outer monolayers of cells. The results showed a preference of LiRecDT1 for sphingomyelin and to *lyso*phosphatidylcholine as substrates compared to phosphatidylcholine. Sphingomyelin was hydrolyzed more rapidly and efficiently in a time kinetics experiment, but the data supported the idea that brown spider phospholipase-D proteins have both sphingomyelinase-D and lysophospholipase-D activities. We also observed that detergent extracts of ghosts of B16-F10 cells and B16-F10 ghosts treated with LiRecDT1 both

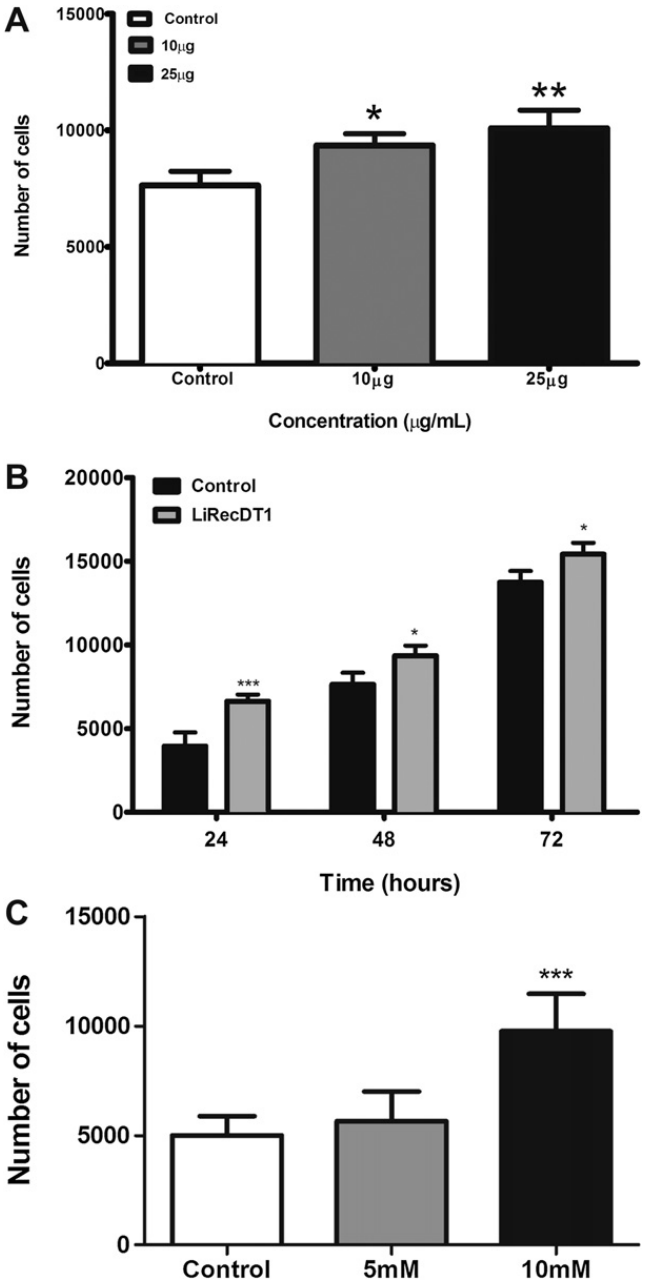


Fig. 7. Recombinant brown spider phospholipase-D stimulates B16-F10 cell proliferation. (A) The B16-F10 cells were plated onto 96-well plates at a dilution of 5×10^3 cells/well and incubated with recombinant phospholipase-D at concentrations of 10 and 25 μg/mL for 48 h in pentaplicate. The control group contained an appropriate amount of vehicle (PBS) rather than LiRecDT1. Measurements of cell proliferation were performed using the CyQUANT cell proliferation assay. (B) B16-F10 cells were treated with 10 μg/mL of LiRecDT1 for 24, 48 and 72 h after phospholipase-D exposure. (C) B16-F10 cells were plated in medium containing synthetic sphingomyelin (5 and 10 mM) with recombinant phospholipase-D (10 μg/mL, for 48 h). The resulting fluorescence was recorded on a spectrofluorometer. The *Y*-axis indicates the number of cells, and the *X*-axis provides the concentration of phospholipase-D (A), the different lengths of the experiments (B) and the concentration of synthetic sphingomyelin (C). The experiments were performed in pentaplicates, and the presented values are the mean ± SEM. Significance is defined as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

generated choline production, as detected in a fluorimetric assay. Therefore, LiRecDT1 stimulates the hydrolysis of important synthetic phospholipid constituents of cell membranes and shows accessibility and activity related to both the membrane detergent extract and ghost phospholipids from B16-F10 cells (demonstrated by choline generation). The results also indicated that the enzymatic activity of LiRecDT1 on sphingomyelin generated choline and, consequently, ceramide 1-phosphate and that the activity on *lyso*phosphatidylcholine produced choline plus *lyso*-phosphatidic acid. These observations indicated that the recombinant phospholipase-D LiRecDT1 can interact with B16-F10 membrane constituents, exhibits hydrolytic activity toward phospholipids, and can directly metabolize phospholipids that are structurally organized on cell membranes or are extracted from B16-F10 cytoplasmic membranes to generate bioactive molecules.

In spite of binding to and causing metabolism of membrane phospholipids, even under the highest purified tested phospholipase-D concentration and longest exposure time (300 μg for 72 h; a concentration sufficient to kill mice and rabbits and even cause serious problems in humans; da Silva et al., 2004; Kusma et al., 2008), the B16-F10 cells exhibited no change in viability (using Trypan blue assay). Additionally, they did not suffer any type of morphological modification, such as cytoplasmic vacuolation, rounding up of cells and detaching from the substrate, cell aggregation, or cell lysis (observed through inverted microscope). These findings suggested an absence of deleterious effects of phospholipase-D on these cells as well as a lack of cellular damage, such as a breakdown of membrane integrity, under the assayed experimental conditions.

Additionally, experiments using Fluo-4, which is a cell-permeant, Calcium-sensitive fluorophore, indicated an increase in fluorescence after LiRecDT1 treatment (detected in two individual experimental assays: a spectrofluorimetric assay and fluorescence microscopy), demonstrating that the activity of LiRecDT1 on membrane phospholipid metabolism in B16-F10 cells could stimulate a calcium influx into the cytoplasm of the cells. This finding is in agreement with data in the literature indicating that treatment of fibroblasts with another exogenous phospholipase-D (obtained from *S. chromofuscus*) resulted in a cytoplasmic calcium influx (van Dijk et al., 1998). Moreover, the occurrence of an influx of Calcium ions inside cells following phospholipase-D treatment is supported by results showing that Calcium is required for brown spider phospholipase-D-induced hemolysis and by those of Yang et al. (2000), who reported that *lyso*phosphatidic acid (a product generated following LiRecDT1 treatment of B16-F10 cells) induces calcium entry in human erythrocytes. Finally, the influx of ions Calcium inside cells following recombinant brown spider phospholipase-D treatment was not a consequence of leakage at the cell membrane because, as noted above, the viability of cells was unchanged, even following exposure to a high concentration of purified LiRecDT1 (as demonstrated by a Trypan blue assay detecting the breakdown of membrane integrity). Because increases in the cytosolic Calcium concentration can mediate a number of cellular events, such as playing roles in embryonic development, cell contraction, cell aggregation, secretion, and the modulation of energy metabolism, among many others (Clapham, 2007; Bootman, 2012), we speculate

that brown spider venom phospholipase-D represents a novel extracellular tool for studying intracellular events triggered via the modulation of Calcium inside cells and opens the possibility of biochemical or cell biology applications for this molecule. Moreover, the generation time of the final product (ceramide 1-phosphate), is compatible with the cellular early response (before 30 min) observed for Calcium influx.

Finally, the activity of recombinant brown spider phospholipase-D on B16-F10 cells was further confirmed by its ability to stimulate cell proliferation in a concentration and time-dependent manner. Additionally, the increases in the cell proliferation rate in B16-F10 cells following LiRecDT1 exposure were higher when the cells were incubated in the presence of exogenous sphingomyelin (which was, as reported, a good substrate for recombinant phospholipase-D). A putative explanation for this event is that exogenous sphingomyelin increases the concentration and accessibility of enzyme substrates, generating bioactive lipid mediators following treatment with recombinant brown spider proteins (such as ceramide 1-phosphate or interconvertible lipids such as ceramide, sphingosine and sphingosine 1-phosphate), compared to offering lipid substrates organized in the lipid bilayer of cell membranes.

The results described herein indicated that B16-F10 melanoma cells subjected to exogenous treatment with a recombinant phospholipase-D from brown spider venom (LiRecDT1) bound phospholipase-D at the cell surface, did not suffer changes in viability, experienced metabolism of their phospholipids by the enzyme, generated metabolically bioactive lipids, triggered Calcium mobilization inside the cytosol, and had their proliferation stimulated, especially in the presence of exogenous sphingomyelin. The data indicated that venom phospholipase-D, which is also referred to as “dermonecrotic toxin” because it is directly involved on gangrenous and necrotic loxoscelism due to generating bioactive lipids such as lysophosphatidic acid and/or ceramide 1-phosphate, can also modulate membrane phospholipid metabolism, regulate tumor cell proliferation, and modulate the cytosolic Calcium influx, opening the possibility of using this enzyme as a novel biotool in studies addressing phospholipid and calcium metabolism.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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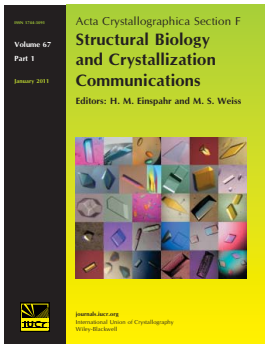

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Crystallization and preliminary X-ray diffraction analysis of a class II phospholipase D from *Loxosceles intermedia* venom

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Phospholipases D are the major dermonecrotic component of *Loxosceles* venom and catalyze the hydrolysis of phospholipids, resulting in the formation of lipid mediators such as ceramide-1-phosphate and lysophosphatidic acid which can induce pathological and biological responses. Phospholipases D can be classified into two classes depending on their catalytic efficiency and the presence of an additional disulfide bridge. In this work, both wild-type and H12A-mutant forms of the class II phospholipase D from *L. intermedia* venom were crystallized. Wild-type and H12A-mutant crystals were grown under very similar conditions using PEG 200 as a precipitant and belonged to space group $P12_11$, with unit-cell parameters $a = 50.1$, $b = 49.5$, $c = 56.5$ Å, $\beta = 105.9^\circ$. Wild-type and H12A-mutant crystals diffracted to maximum resolutions of 1.95 and 1.60 Å, respectively.

1. Introduction

Envenomation by members of the genus *Loxosceles* (brown spiders), considered to be the most dangerous form of arachnidism, is a serious public health problem in both North and South America (Santi Ferrara *et al.*, 2009). *Loxosceles* venom can cause local dermonecrosis with gravitational spreading and systemic manifestations such as thrombocytopaenia, haemolysis and acute renal failure that can lead to death (Futrell, 1992; da Silva *et al.*, 2004).

Several toxic proteins present in *Loxosceles* spp. venoms have been identified and biochemically characterized (da Silva *et al.*, 2004; Gremski *et al.*, 2010). Members of the phospholipase D family are abundant in the venoms of several *Loxosceles* spp. and contribute significantly to the typical response after envenomation (Kalapothakis *et al.*, 2007; Senff-Ribeiro *et al.*, 2008).

Phospholipases D (30–35 kDa), also referred to as dermonecrotic toxins, catalyze the hydrolysis of sphingomyelin and (lyso) glycerophospholipids, resulting in the formation of bioactive mediators such as ceramide-1-phosphate and lysophosphatidic acid which play a role in several pathological and biological responses (Van Meeteren *et al.*, 2004; Moolenaar *et al.*, 2004; Lee & Lynch, 2005). As proposed by Murakami *et al.* (2006), spider-venom phospholipases D can be classified into two classes. Members of class I possess a single disulfide bridge and contain an extended hydrophobic loop, whereas class II proteins contain an additional intra-chain disulfide bridge and display decreased catalytic activity towards phospholipids. To date, only the phospholipase D from *L. laeta* venom, a member of class I, has been structurally characterized (Murakami *et al.*, 2005), despite the clinical importance of phospholipases D in loxoscelism. Based on its crystal structure, an acid–base catalytic mechanism was proposed in which His12 and His47 play key roles and are supported by a network of hydrogen bonds between Asp34, Asp52, Trp230, Asp233 and Asn252 (Murakami *et al.*, 2005).

The recombinant dermonecrotic toxin (LiRecDT1) obtained from a cDNA library of the *L. intermedia* venom gland is able to directly induce renal injuries in mice and the haemolysis of human erythrocytes *in vitro*, suggesting that this protein is directly involved in the

the nephrotoxicity and haematological disturbances evoked during envenomation by *Loxosceles* spiders (Chaim *et al.*, 2006; Chaves-Moreira *et al.*, 2009). Mutation of the catalytic residue His12 to Ala abolishes both the nephrotoxic effect of LiRecDT1 in mice and the haemolysis of human erythrocytes (Kusma *et al.*, 2008; Chaves-Moreira *et al.*, 2009).

The present report describes the crystallization and preliminary crystallographic analysis of recombinant wild-type (LiRecDT1) and mutant (LiRecDT1 H12A) dermonecrotic toxin from *L. intermedia* venom, which belongs to the class II phospholipases D. The structural characterization of LiRecDT1 will be essential to shed light on the structural determinants of the functional differentiation between members of the class I and class II phospholipases D.

2. Materials and methods

2.1. Expression and purification

DNA corresponding to the wild-type (LiRecDT1) and mutated (LiRecDT1 H12A) forms of the mature phospholipase D was cloned into pET-14b vector (Novagen, Madison, USA) as described by Chaim *et al.* (2006) and Kusma *et al.* (2008). Both recombinant constructs were expressed as fusion proteins with a 6×His tag at the N-terminus and a 13-amino-acid linker including a thrombin site between the 6×His tag and the mature protein. pET-14b/*L. intermedia* cDNA constructs were transformed into One Shot *Escherichia coli* BL21 (DE3) pLysS competent cells (Invitrogen) and plated on LB agar plates containing 100 mg ml^{-1} ampicillin and 34 mg ml^{-1} chloramphenicol. A single colony was inoculated into 50 ml LB broth (plus antibiotics) and allowed to grow overnight at 310 K. A 10 ml portion of this overnight culture was grown in 1 l LB broth/ampicillin/chloramphenicol at 310 K until the OD at 550 nm reached 0.5. IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to a final concentration of 0.05 m*M* and the culture was induced by incubation for an additional 3.5 h at 303 K. Cells were harvested by centrifugation (400*g*, 7 min) and the pellet was frozen at 253 K overnight.

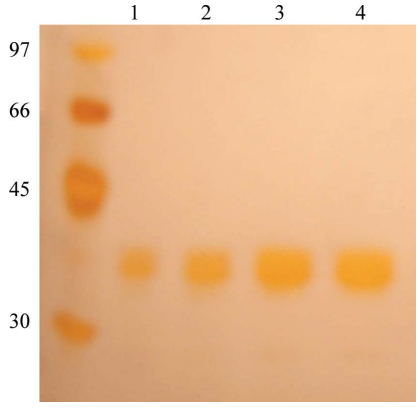

Figure 1
Silver-stained SDS–PAGE (12%) of purified samples of wild-type and H12A-mutant dermonecrotic toxin from *L. intermedia*. Lane 1, molecular-weight markers (kDa); lanes 2 and 4, purified LiRecDT1 (18 and 34 µg, respectively); lanes 3 and 5, purified LiRecDT1 H12A (18 and 34 µg, respectively).

The cell suspensions were thawed and were additionally disrupted by six 10 s cycles of sonication at low intensity. The lysed materials were centrifuged (20 000*g*, 20 min) and the supernatants were incubated with 1 ml Ni^{2+}–NTA agarose beads for 1 h at 277 K (with gentle agitation). The suspensions were loaded onto a column and the packed gel was exhaustively washed with 50 m*M* sodium phosphate pH 8.0, 500 m*M* NaCl, 20 m*M* imidazole until the OD at 280 nm reached 0.01. The recombinant proteins were eluted with 10 ml elution buffer (50 m*M* sodium phosphate pH 8.0, 500 m*M* NaCl, 250 m*M* imidazole) and 1 ml fractions were collected and analyzed by 12.5% SDS–PAGE under reducing conditions (Fig. 1). The fractions were pooled and dialyzed against phosphate-buffered saline (PBS). Site-directed mutagenesis did not alter the correct folding of the brown spider phospholipase D as assessed by circular-dichroism and fluorescence experiments (results not shown).

2.2. Crystallization

The wild-type and mutant proteins were initially crystallized by vapour diffusion in sitting drops using a Cartesian HoneyBee 963 system (Genomic Solutions) at 291 K. For initial screening, 1 µl protein solution at a concentration of 17 mg ml^{-1} for the wild type and of 9 mg ml^{-1} for the H12A mutant was mixed with 1 µl screening solution and equilibrated over a reservoir containing 100 µl of the latter solution. Small crystals of wild-type LiRecDT1 were observed in the condition 0.1 *M* Tris–HCl pH 8, 35%(*v*/*v*) PEG 200, which was refined by varying the PEG 200 concentration *versus* the pH using the hanging-drop method. The best wild-type LiRecDT1 crystals were observed in drops consisting of 2 µl protein solution (17 mg ml^{-1}) and 2 µl reservoir solution equilibrated over 1 ml reservoir solution [0.1 *M* Tris–HCl pH 7.5, 40%(*v*/*v*) PEG 200] (Fig. 2*a*). Crystals of the H12A mutant were grown in a very similar condition consisting of 0.1 *M* Tris–HCl pH 7.5 and 35%(*v*/*v*) PEG 200 (Fig. 2*b*).

2.3. X-ray diffraction analysis

LiRecDT1 and LiRecDT1 H12A crystals were directly flash-cooled in a 100 K nitrogen-gas stream. X-ray diffraction data were collected on the W01B-MX2 beamline at the Brazilian Synchrotron Light Laboratory (Campinas, Brazil). The LiRecDT1 crystal was exposed for 60 s per 2° rotation in φ with the crystal-to-detector distance set to 100 mm. The LiRecDT1 H12A crystal was exposed for 20 s per 1° rotation in φ with the crystal-to-detector set to 69 mm. A total of 180 and 300 images were collected from the LiRecDT1 and the LiRecDT1 H12A crystals, respectively. The data were indexed,

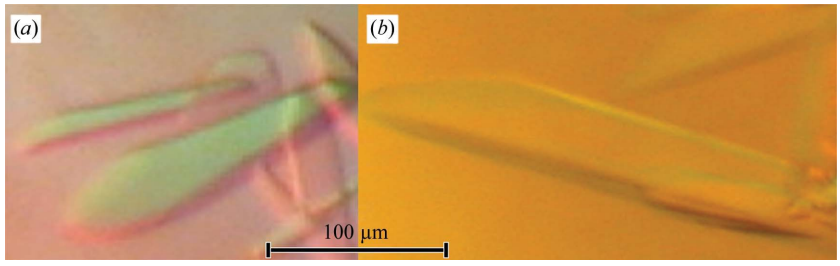

Figure 2
Microphotograph of crystals of (*a*) wild-type and (*b*) mutant dermonecrotic toxin from *L. intermedia*.

Table 1
Data-collection statistics.

Values in parentheses are for the last resolution shell.

	LiRecDT1	LiRecDT1 H12A
Data collection		
Temperature (K)	100	100
Radiation source	Brazilian Synchrotron Light Laboratory	Brazilian Synchrotron Light Laboratory
Beamline	W01B-MX2	W01B-MX2
Wavelength (Å)	1.458	1.458
Detector	MAR Mosaic 225 mm	MAR Mosaic 225 mm
Space group	$P12_11$	$P12_11$
Unit-cell parameters (Å, °)	a = 50.08, b = 49.43, c = 56.59, β = 105.88	a = 49.58, b = 49.46, c = 56.40, β = 105.56
Resolution range (Å)	30.0–2.0 (2.07–2.00)	30.0–1.60 (1.66–1.60)
R_{merge} (%)†	12.1 (49.4)	7.3 (37.0)
$\langle I/\sigma(I)\rangle$	9.3 (2.4)	19.5 (2.9)
Data completeness (%)	99.5 (98.1)	98.9 (92.4)
No. of unique reflections	18148 (1765)	34632 (3241)
Multiplicity	3.1 (2.7)	5.1 (3.3)
Data analysis		
V_M (Å^3 Da^{-1})	2.25	2.22
Solvent content (%)	45.25	44.63
Molecules per asymmetric unit	1	1

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations I of reflection hkl.

integrated and scaled using the *DENZO* and *SCALEPACK* programs from the *HKL*-2000 package (Otwinowski & Minor, 1997). Data-processing statistics are summarized in Table 1.

3. Results and discussion

LiRecDT1 and LiRecDT1 H12A crystals diffracted to resolutions of 1.95 and 1.60 Å, respectively. Although the mutant protein crystal diffracted better than the native protein crystal, the H12A mutation did not alter the crystal packing or the unit-cell symmetry and parameters. The LiRecDT1 and LiRecDT1 H12A data sets were indexed in the monoclinic crystal system. The presence of systematic absences indicated that the crystals belonged to space group $P12_11$. Both crystals possessed highly similar unit-cell parameters (Table 1). The Matthews coefficient calculated for the LiRecDT1 crystal was 2.25 Å^3 Da^{-1}, corresponding to a solvent content of 45% (Matthews, 1968). Considering the molecular weight of 30 000 Da, one molecule is present in the asymmetric unit of both crystals. Data-processing statistics for both data sets are presented in Table 1.

The atomic coordinates of the phospholipase D from *L. laeta* venom (PDB code 1xx1; Murakami *et al.*, 2005), which displays a sequence identity of 58% with LiRecDT1, were used to generate a search model and molecular-replacement calculations were carried out using the program *MOLREP* in the resolution range 15.0–3.0 Å (Vagin & Teplyakov, 2010). A solution was obtained for one molecule in the asymmetric unit in space group $P12_11$. Analysis of the packing contacts and steric clashes clearly showed that this was the correct solution. *REFMAC*5 (Murshudov *et al.*, 1997) was used for rigid-body refinement of this solution in the resolution range 30.0–1.95 Å (excluding 5% of reflections for R_{free} calculations), resulting in a correlation coefficient of 49.2, a score of 0.675 (the score of the next highest peak was 0.338) and an R factor of 45.6% (R_{free} = 49.1%). Structure refinement and analysis are currently in progress. Determination of the LiRecDT1 H12A crystal structure will be performed using the final model of wild-type LiRecDT1.

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Structure of a novel class II phospholipase D: Catalytic cleft is modified by a disulphide bridge

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ABSTRACT

Phospholipases D (PLDs) are principally responsible for the local and systemic effects of *Loxosceles* envenomation including dermonecrosis and hemolysis. Despite their clinical relevance in loxoscelism, to date, only the SMase I from *Loxosceles laeta*, a class I member, has been structurally characterized. The crystal structure of a class II member from *Loxosceles intermedia* venom has been determined at 1.7 Å resolution. Structural comparison to the class I member showed that the presence of an additional disulphide bridge which links the catalytic loop to the flexible loop significantly changes the volume and shape of the catalytic cleft. An examination of the crystal structures of PLD homologues in the presence of low molecular weight compounds at their active sites suggests the existence of a ligand-dependent rotamer conformation of the highly conserved residue Trp230 (equivalent to Trp192 in the glycerophosphodiester phosphodiesterase from *Thermus thermophofilus*, PDB code: 1VD6) indicating its role in substrate binding in both enzymes. Sequence and structural analyses suggest that the reduced sphingomyelinase activity observed in some class IIb PLDs is probably due to point mutations which lead to a different substrate preference.

1. Introduction

Envenomation by brown spiders (*Loxosceles* spp.) leads to a strong local dermonecrotic effect (cutaneous loxoscelism) and systemic manifestations, whose symptoms include hematuria, hemoglobinuria, jaundice and fever [1–3]. Although, the systemic loxoscelism occurs in a minority of cases, it can be fatal, especially in children [1–3].

Phospholipases D (PLD) are considered the main components responsible for the local and systemic effects of *Loxosceles* venom including dermonecrosis, renal toxicity and hemolysis [4–9]. *Loxosceles* PLDs (30–35 kDa), also referred to as dermonecrotic toxins, were primarily designated as sphingomyelinases D (SMases D) due to their ability to convert sphingomyelin to choline and ceramide 1-phosphate (*N*-acylsphingosine1-phosphate) [4]. As some *Loxosceles* PLDs have broad substrate specificity, being able to hydrolyze not only sphingophospholipids but also lysoglycerophospholipids, they are now classified as phospholipases D [10–12].

PLDs can be grouped into two classes based on sequence, structural and biochemical data [13]. The class I, represented by PLD I from *Loxosceles laeta*, is characterized by the presence of a single disulphide bridge (Cys51–Cys57) and an extended hydrophobic loop (variable loop). Class II comprises PLDs that contain an additional intra-chain disulphide bridge linking the flexible loop and the catalytic loop. Depending on their ability to hydrolyze sphingomyelin, they are further subdivided into classes IIa (more active) and IIb (less active or inactive), respectively [13].

Despite the clinical importance of these enzymes in loxoscelism, to date, only the PLD I from *L. laeta*, a class I member, had been structurally characterized [14]. Based on its crystal structure, an acid–base catalytic mechanism was proposed, where His12 and His47 play key roles and are supported by a network of hydrogen bonds between Asp34, Asp52, Trp230, Asp233, and Asn252 [14]. However, the vast majority of *Loxosceles* PLDs belongs to the class II, and none of their three-dimensional structures have been determined. A representative member of class II PLD is the dermonecrotic toxin isoform 1 from *Loxosceles intermedia* [11]. The recombinant protein

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(LiRecDT1) is able to hydrolyze sphingomyelin and presents dermonecrotic and hemolytic activities, characteristics of the whole venom [10,11,15,16]. Moreover, LiRecDT1 is able to directly induce renal injuries in mice and its nephrotoxic effects are dependent on its catalytic activity [11]. It has also been demonstrated that LiRecDT1 released choline from endothelial and kidney cell membrane extracts and bound to various lipids, such as sphingomyelin, lysophosphatidylcholine and cholesterol [12].

This work presents the first crystal structure of a *Loxosceles* venom class II PLD, the dermonecrotic toxin isoform 1 from *L. intermedia*. A comparison of the three-dimensional structures of class I and II PLDs, indicates the effect of an additional disulfide bridge which links the surface loops on the volume and shape of the catalytic cleft. Sequence and structural analyses suggest that the reduced or absent sphingomyelinase activity of some class IIb PLDs [15,17,18] is probably due to mutations of amino acid residues affecting substrate affinity or protein stability, since all catalytically-relevant residues are fully conserved. Moreover, the mutations observed at the catalytic pocket of these proteins are the same encountered in the active PLD 2 from *L. laeta* [19].

2. Materials and methods

2.1. Expression and purification

Mature dermonecrotic toxin isoform 1 from *L. intermedia* (LiRecDT1) was cloned into a pET-14b vector (Novagen, Madison, USA) and expressed in *Escherichia coli* BL21(DE3)pLysS cells (Invitrogen) as described previously [20]. Expression was induced by the addition of 0.05 mM IPTG (isopropyl β-D-thiogalactoside) during 3.5 h at 303 K after the cell culture had reached an OD_{550} of 0.5.

Cell suspension was disrupted by six 10 s-cycles of sonication. Lysed material was centrifuged (20,000*g*, 20 min) and the supernatant was incubated with 1 ml Ni^{2+}-NTA agarose beads for 1 h at 277 K. The suspensions were loaded onto a column and the packed gel was washed with 50 mM sodium phosphate pH 8.0, 500 mM NaCl, 20 mM imidazole. The recombinant protein was eluted with 10 ml of the above buffer which additionally contained 250 mM imidazole and 1 ml fractions were collected and analyzed by 12.5% SDS–PAGE. Fractions were pooled and dialyzed against phosphate buffer saline (PBS).

2.2. Crystallization

The LiRecDT1 protein was crystallized by vapor diffusion in sitting drops using a Cartesian HoneyBee 963 system (Genomic Solutions) at 291 K as described in [20]. Optimal crystals were observed in drops containing 2 μl of the protein solution (17 mg ml^{-1}) and 2 μl of the reservoir solution equilibrated over 1 ml of reservoir solution (0.1 M Tris–HCl pH 7.5, 40% (v/v) PEG200).

2.3. Data collection and processing

LiRecDT1 crystals were directly flash-cooled in a 100 K nitrogen-gas stream. X-ray diffraction data were collected on the W01B-MX2 beamline at the Brazilian Synchrotron Light Laboratory (Campinas, Brazil) as described in earlier [20]. The data were indexed, integrated and scaled using the DENZO and SCALEPACK programs from the HKL-2000 package [21]. Data collection and refinement statistics are summarized in Table 1.

2.4. Structure solution and refinement

The initial structural model of LiRecDT1 was determined by molecular-replacement using the program MOLREP [22] and the atomic coordinates of PLD 1 from *L. laeta* as the template (PDB code 1XX1; [14]). Model refinement was carried out alternating cycles of REFMAC5 [23] with visual inspection of the electron density maps and manual rebuilding with COOT [24]. A total of 278 residues were modeled, comprising LiRecDT1 residues from Gly2 to Lys284, numbered according to the class I phospholipase D structure (PDB code 1XX1; [14]). Water molecules were added using COOT [24] during the last refinement cycles. One magnesium ion (Mg^{2+}), two ethylene glycol (EDO), four diethylene glycol (PEG) and one triethylene glycol (PGE) were modeled based on the difference Fourier map of LiRecDT1. The final model consists of one monomer and was established after the convergence of R_{factor} and R_{free} values to 17.2% and 21.4%, respectively. Analyses of monomer–monomer interfaces using the PISA web server [25] did not indicate any biologically relevant quaternary structure in the crystal. Stereochemistry of the model was analyzed with Molprobity [26]. No outlier was observed in the Molprobity Ramachandran plot and more than 99% of the residues are in its favored region. Data collection and structure refinement statistics are summarized in Table 1. Figures were produced using the program Pymol [27].

Table 1
Data collection and refinement statistics.

Data collection[a]	
Temperature (K)	100
Radiation source	W01B-MX2 (LNLS, Brazil)
Wavelength (Å)	1.458
Detector	MarMosaic 225
Space group	$P12_11$
Unit-cell parameters (Å, °)	a = 49.81, b = 49.30 and c = 56.30; β = 105.83
Resolution range (Å)	24.65–1.72 (1.81–1.72)
R_{merge}[b] (%)	9.7 (46.8)
$\langle I/\sigma(I)\rangle$	8.1 (2.0)
Data completeness (%)	99.9 (99.9)
No. of measured reflections	102,021 (14,085)
No. of unique reflections	28,041 (4040)
Multiplicity	3.6 (3.5)
Structure refinement statistics	
R_{factor}	17.2
R_{free}	21.4
r.m.s.d. Bond distances (Å)	0.023
r.m.s.d. Bond angles (°)	1.930
Ramachandran outliers (%)	0
Ramachandran favored (%)	99.3
Average *B*-factors (Å^2)	17.74

[a] Values in parentheses are for the last resolution shell.
[b] $R_{merge} = \sum_{hkl}\sum_i|I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations I of reflection hkl.

2.5. Molecular modeling and quality analysis

The atomic coordinates of LiRecDT1 (PDB code: 3RLH) was used as the initial model to obtain three-dimensional models of LiRecDT3 from *L. intermedia* (ABB71184.1) and Lb3 from *L. boneti* (AAT66074.1), using the modeling option of the Swiss-Model server [28]. For energy minimization, the final models were submitted under explicit solvent molecular dynamics (MD) simulations using YASARA until the convergence of root mean square deviation (r.m.s.d.) (Fig. S1). The overall and local quality analysis of the final model was assessed by ProSA-web [29] and Molprobity [26]. More than 99% of the residues of both models are in the favored region of the Ramachandran plot. ProSA-web analyses indicated that the referred models present *Z*-scores within the range typically observed for native proteins of similar size.

3. Results and discussion

3.1. Overall structure

LiRecDT1 is a single domain protein which folds into a TIM $(\alpha/\beta)_8$-barrel with the insertion of additional β-strands and α-helices (Fig. 1). FastSCOP [30] analysis indicates that this class II PLD belongs to the phospholipase C (PLC) like phosphodiesterases superfamily, suggesting that PLDs share a common ancestor with PLC like domains encountered in mammalian phospholipase C isozyme D1 [30], bacterial phosphatidylinositol-specific phospholipase C [31] and glycerophosphodiester phosphodiesterases [32].

The catalytic (blue), variable (green), flexible (red) and other short loops surround the active site cleft (Fig. 1). The catalytic loop, which contains the catalytically important residue, His47, forms a hairpin due to the presence of a disulphide bridge (Cys51–Cys57). A network of hydrogen bonds ensures the correct relative orientation of the hairpin in relation to the core of the protein.

3.2. Structural comparison between class I and II phospholipases D

LiRecDT1 superimposes on the class I PLD from *L. laeta* (SMase I) (PDB code: 1XX1; [19]) with a r.m.s. deviation of 0.938 Å for 268 Cα atoms and a sequence identity of 61.6 %. The main conformational changes are observed at the flexible and variable loops (Fig. 1). LiRecDT1 possesses a disulfide bridge between Cys53 and Cys201, which causes a large displacement of the flexible loop towards the catalytic loop (Fig. 1). This disulfide bridge is well conserved in class II PLDs, but absent in class I PLD due to the mutation C201F (Fig. 2).

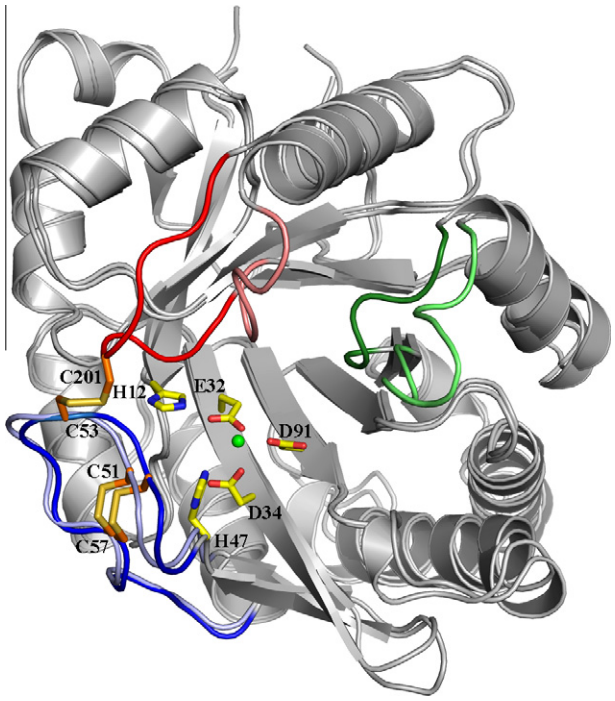

Fig. 1. Structural alignment between LiRecDT1 (class II) and PLD I from *L. laeta* (class I). The LiRecDT1 residues involved in metal–ion binding and catalysis are presented in atom colors (PDB code: 3RLH). The Mg^{2+} ion is shown as a green sphere. The catalytic, flexible, and variable loops are colored in blue, red, and green, respectively. Dark and light colors refer to LiRecDT1 and *L. laeta* PLD, respectively. The disulphide bridges are presented by orange sticks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Regarding the variable loop, a five-residue insertion (YLPSL), which protrudes from the core structure of class I PLD, is absent in the class II PLD (Figs. 1 and 2). Moreover, the three last residues that compose the variable loop are not conserved between class I and class II PLDs (Fig. 2). The mean *B*-factors for the main-chain atoms of the variable loops are 26.39 $Å^2$ and 15.86 $Å^2$ for class I (PDB code: 1XX1, chain A) and class II respectively, indicating that the class I variable loop is significantly more flexible than that of class II.

In the class II PLD, the flexible loop is displaced towards the catalytic loop and partially occludes an electronegative cleft that leads to the active site in the class I PLD (Fig. 3). This results in a volumetric reduction of the cavity that forms the catalytic site of the class II PLD. Based on SURFNET analysis [33], the volume and average depth of the cleft observed at the class I PLD catalytic face are 4339.4 $Å^3$ and 15.2 Å, respectively. These values are considerably larger than those estimated for the corresponding cavity at the class II PLD (volume 1468.1 $Å^3$, average depth 10.1 Å). Besides the lack of the disulfide bridge connecting the catalytic and flexible loops, the protrusion of variable loop and the substitution N137G also account for the enlargement of the cleft surrounding the catalytic site of the class I PLD (Fig. 3).

A large electronegative patch, which includes the variable loop, is observed surrounding the right side of the class II PLD catalytic pocket. However, in the class I PLD, this patch is interrupted by the longer variable loop that forms a protrusion with neutral potential (Fig. 3). Interestingly, an electronegative cleft, hidden by the neutral catalytic and flexible loops in the class II PLD, is exposed in the class I PLD, due to the absence of a disulfide bridge connecting these loops. Thus, in the class I PLD the impairment in the electronegative potential of a patch at the right side of the catalytic pocket seems to be compensated by the exposure of another electronegative patch on the left side.

Together, these differences observed in the topography and electrostatic potential distribution of class I and II PLDs catalytic faces suggest that these enzymes might have different ways to guide the substrate into the catalytic pocket.

3.3. Active site

The Mg^{2+} binding site and the two catalytic histidine residues (His12 and His47), which compose the active-site pocket are strictly conserved in both classes of spider venom PLDs (Fig. 2). Interestingly, at the active site of LiRecDT1, the difference Fourier map contained residual density. However, neither substrates nor products could account for this density. This residual density was modeled as two PEG molecules with partial occupancy.

In the LiRecDT1 structure, the Mg^{2+} ion (B factor of 14.26 $Å^2$ and a mean Mg–O bond distance of 2.1 Å) is hexacoordinated by the carboxyl oxygens of Glu32, Asp34, Asp91, one water molecule and two PEG4 oxygens. The same geometric coordination is observed for the Mg^{2+} ion in class I PLD structure bound to a sulfate ion [13]. As pointed out earlier, *Loxosceles* PLDs and glycerophosphodiester phosphodiesterases (GDPDs) share a similar divalent metal dependent catalytic mechanism and probably evolved from a common ancestor [14]. In spite of their different specificities, structural comparisons of the PLD and GDPD active sites suggest that they share not only residues involved in metal binding and catalysis but also in substrate binding such as Lys93 and Trp230 (Fig. 4A). Murakami et al. have suggested a role for Lys93 and Trp230 in the orientation of the substrate in both spider and bacterial PLDs [14]. Structural analysis indicates that the Lys residue might interact with the phosphate moiety of GDPDs substrates. Moreover, since the mutation K121A greatly reduces the enzymatic activity of the *Thermoanaerobacter tengcongensis* GDPD, suggesting that this Lys residue affects catalysis via electronic

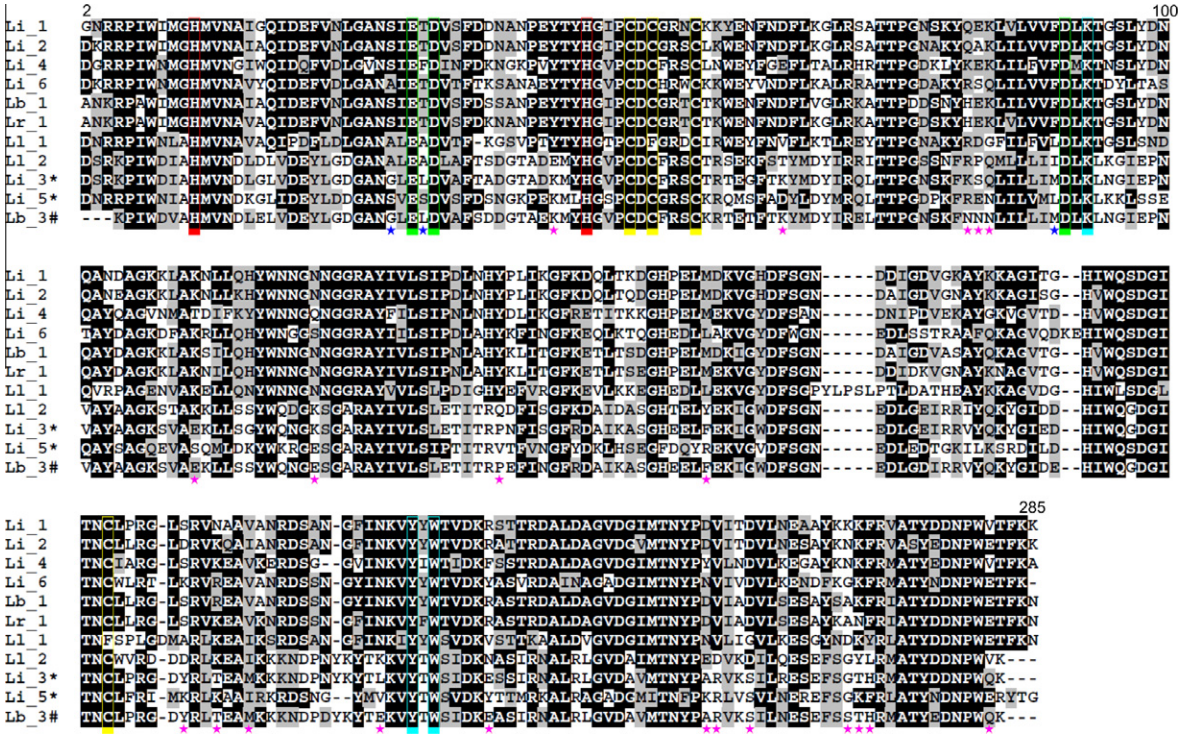

Fig. 2. Multiple sequence alignment of class I and II PLDs. Li_1 (LiRecDT1, PDB code: 3RLH), Li_2 (LiRecDT2, ABB69098.1), Li_3 (LiRecDT3, ABB71184.1), Li_4 (LiRecDT4, ABD91846.1), Li_5 (LiRecDT5, ABD91847.1) and Li_6 (LiRecDT6, ABO87656.1) are PLDs paralogs from *L. intermedia*. Ll_1 (AAM21154.1, PDB code: 1XX1) and Ll_2 (AAM21156.1) are isoforms from *L. laeta*, Lb_1 (AAT66073.1) and Lb_3 (AAT66074.1) are PLDs from *L. boneti* and Lr_1 (AAT66075.1) is a PLD from *L. reclusa*. The symbols ∗ and # indicate PLDs with reduced or abolished sphingomyelinase activity, respectively. Residues involved in metal–ion binding and catalysis are boxed in green and red, while cysteines are boxed in yellow. Residues possibly involved in substrate binding and orientation are boxed in cyan. The numbers represent the class I PLD sequence. Stars indicate amino acid substitutions occurred specifically in PLDs with reduced or abolished sphingomyelinase activity. Blue stars refer to buried residues near to the metal-binding site and pink stars refer to surface-exposed residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

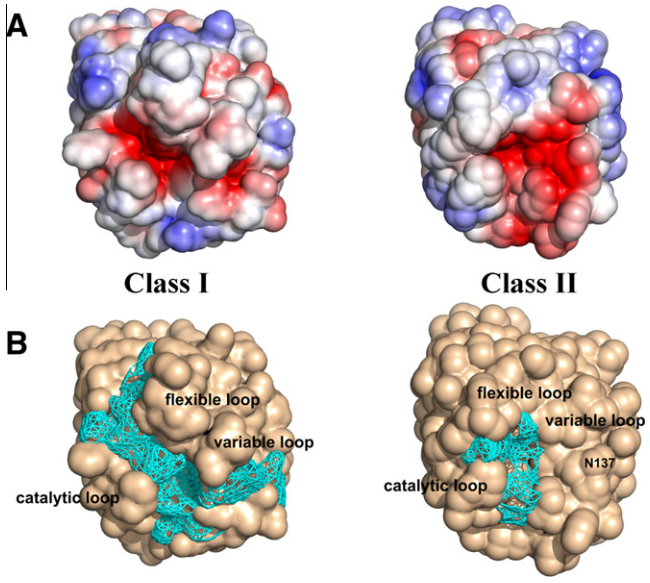

Fig. 3. Solvent accessible surface analyses of class I and II PLDs. (A) Electrostatic surface of class I (left) and II (right) PLDs are colored by charge, from red (−2 kV) to blue (+2 kV). Electrostatic potential was calculated using PBEQ solver [38]. (B) Representation of the largest cavities (*cyan*) in the respective class I and II PLD structures. Models are oriented according to Fig. 1. Class I PLD = PDB code: 1XX1, chain A. Class II PLD = PDB code: 3RLH. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

interaction with the phosphate moiety of the substrate [34]. A similar role might be attributed to the corresponding Lys residue in spider PLDs.

Trp230, strategically located at the bottom of the active site, adopts different rotamer conformations depending on the presence of ligands (Fig. 4B and C). The equivalent in the GDPD from *Thermus thermophofilus* is Trp192 (PDB code: 1VD6). In the presence of PEG (PDB code: 3RLH) or glycerol (PDB code: 1VD6) molecules, Trp230(192) forms one of the walls of the active site pocket, freeing space for ligand binding. The presence of a sulfate ion in the active site of class I PLD (PDB code: 1XX1) induces another conformation of Trp230 by the formation of a hydrogen bond between the NE1 nitrogen of Trp230 and the sulfate O1 atom. Contrastingly, in the ligand free form of class I PLD (PDB code: 2F9R), the Trp230 ring adopts a third conformation in which the torsion angle $\chi 2$ changes by $-90°$ in relation to the Trp230 conformation in presence of PEG or glycerol. These findings suggest that Trp230 motions might play a pivotal role in substrate binding in both *Loxosceles* venom PLDs and bacterial GDPDs.

3.4. Functional diversification in spider venom PLDs

The spider venom PLDs encompass a gene family with multiple orthologs and paralogs, which differ in catalytic efficiency, substrate specificity or intensity of biological effects [7,10,11,15–19,35,36]. A structure-based classification segregates these enzymes into two major groups as mentioned in the introduction. This classification is in agreement with recent phylogenetic analysis of a large set of Sicariid spider venom PLDs, which grouped members of different classes into distinct branches belonging to

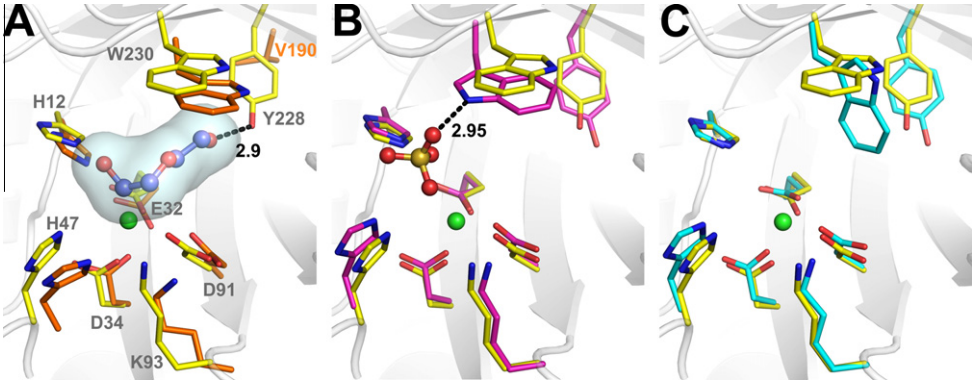

Fig. 4. The active site. Structural alignment among class I and II PLDs and a glycerophosphodiester phosphodiesterase (GDPD) from *Thermus thermophilus*. (A) Representation of class II PLD (carbon atoms in yellow) and GDPD (carbon atoms in orange C) superposed structures highlighting the PEG molecule (carbon atoms in violet) from the class II PLD structure. (B) Representation of class II PLD and class I PLD (carbon atoms in magenta) superposed structures showing the sulfate ion from the class I structure. (C) Representation of class II PLD and the sulfate-free class I (cyan C atoms) superposed structures. Nitrogen, oxygen, sulfur and magnesium atoms are colored blue, red, wheat and green, respectively. PDB codes: GDPD (1VD6:A), class I PLD (1XX1:D), sulfate free class I PLD (2F9R:B), class II PLD (3RLH). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

two major clades (α and β). Clade α includes class I and IIa PLDs whereas clade β includes class IIb PLDs [36].

In an attempt to understand the structural determinants for the functional divergence of β clade members with reduced sphingomyelinase activity [15], we analyzed mutations exclusively observed in these enzymes (Fig. 2). Three of these mutations (S30G, T33L, F90M), are observed at the protein hydrophobic core and prediction of protein stability by the PoPMuSiC program [37] suggests that the substitutions S30G and F90M may have a destabilizing effect. The other substitutions are sparsely distributed on the LiRecDT1 surface relatively far from the catalytic pocket. Considering the long aliphatic tail of sphingomyelin, changes in the charge distribution and surface shape of the catalytic face induced by some of these mutations may affect the protein–lipid interaction and, consequently, their substrate affinity.

This analysis of the active site pockets in the structures of the class I, the class II and the modeled structure of Lb3 did not provide a clear explanation for the reduced or absent sphingomyelinase activity of LiRecDT3 and Lb3. These proteins conserve the Mg^{2+} binding site residues along with Lys93, Tyr228, Trp230 and the two catalytic histidine residues (His12 and His47) (Fig. 2 and Fig. S2). Moreover, the substitutions observed at their catalytic site are the same encountered in the active Ll2 enzyme. This suggests that LiRecDT3, LiRecDT5 and Lb3 may not be devoid of catalytic activity but might possess affinity to other substrates. This hypothesis is supported by the fact that some spider venom PLDs demonstrates broad substrate selectivity [13–15].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.053.

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Review

Brown Spider (*Loxosceles* genus) Venom Toxins: Tools for Biological Purposes

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Abstract: Venomous animals use their venoms as tools for defense or predation. These venoms are complex mixtures, mainly enriched of proteic toxins or peptides with several, and different, biological activities. In general, spider venom is rich in biologically active molecules that are useful in experimental protocols for pharmacology, biochemistry, cell biology and immunology, as well as putative tools for biotechnology and industries. Spider venoms have recently garnered much attention from several research groups worldwide. Brown spider (*Loxosceles* genus) venom is enriched in low molecular mass proteins (5–40 kDa). Although their venom is produced in minute volumes (a few microliters), and contain only tens of micrograms of protein, the use of techniques based on molecular biology and proteomic analysis has afforded rational projects in the area and permitted the

discovery and identification of a great number of novel toxins. The brown spider phospholipase-D family is undoubtedly the most investigated and characterized, although other important toxins, such as low molecular mass insecticidal peptides, metalloproteases and hyaluronidases have also been identified and featured in literature. The molecular pathways of the action of these toxins have been reported and brought new insights in the field of biotechnology. Herein, we shall see how recent reports describing discoveries in the area of brown spider venom have expanded biotechnological uses of molecules identified in these venoms, with special emphasis on the construction of a cDNA library for venom glands, transcriptome analysis, proteomic projects, recombinant expression of different proteic toxins, and finally structural descriptions based on crystallography of toxins.

Keywords: *Loxosceles*; brown spider; venom; recombinant toxins; biotechnological applications

1. The Spiders of Genus *Loxosceles* and Loxoscelism

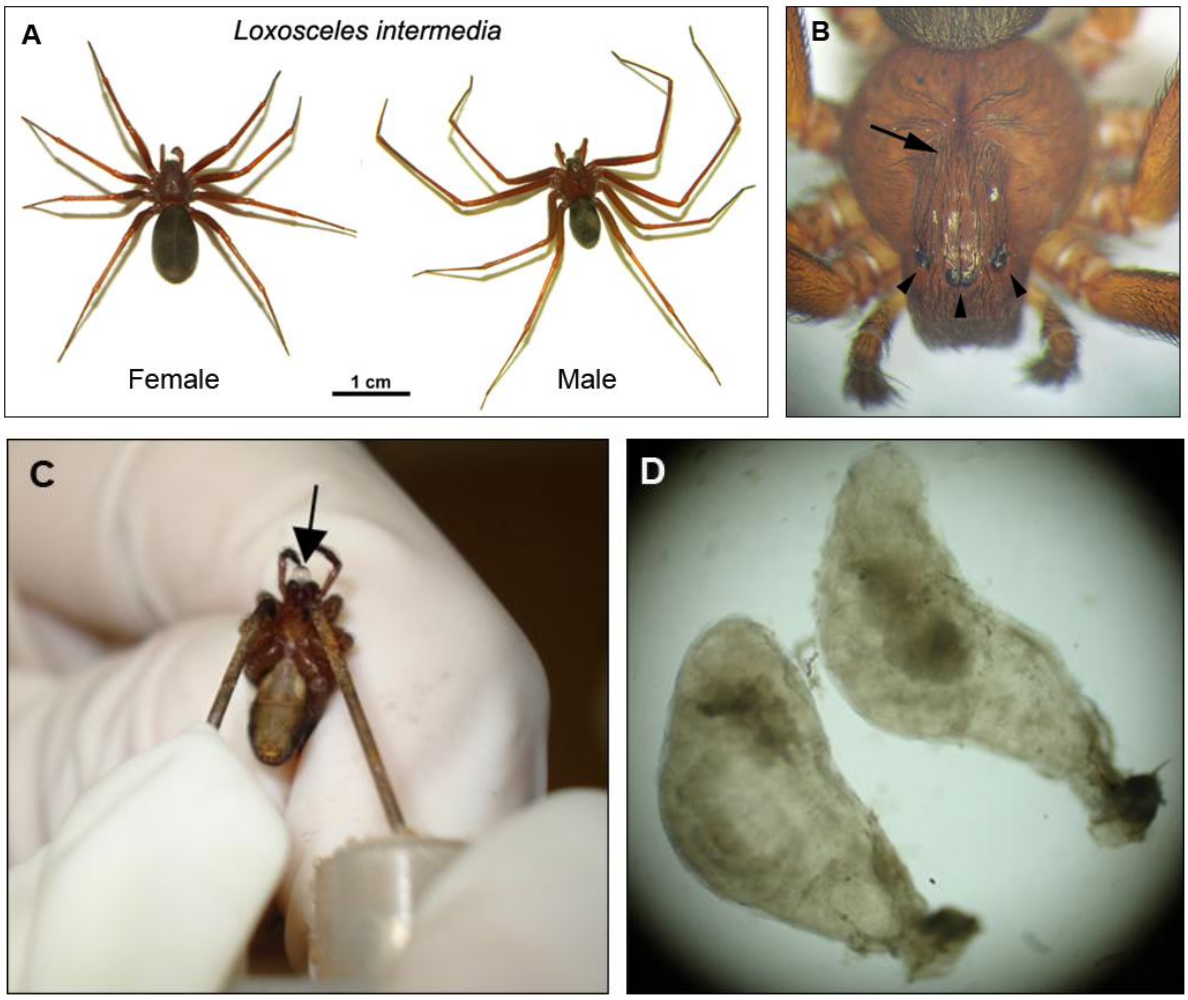
The spiders of the *Loxosceles* genus, commonly denoted as brown spiders, belong to the family *Sicariidae*, sub-order *Labidognatha*, order *Araneida*, class *Arachnida,* and phylo *Arthropoda* [1,2]. The Sicariidae family also comprises the spiders of *Sicarius* genus. Strong evidences show that the genera *Loxosceles* and *Sicarius* are old, having originated from a common sicariid ancestor and diversified on Western Gondwana, before the separation of the African and South American continents. Both sicariid genera are diverse in Africa and South/Central America. *Loxosceles* spiders are also distributed in North America and the West Indies, and have species described from Mediterranean Europe and China. Apparently African and South American *Sicarius* have a common ancestor and South African *Loxosceles* are derived from this group. New World *Loxosceles* also have a common ancestor and fossil data is consistent with the hypothesis of North America colonization by South American *Loxosceles via* a land bridge predating the modern Isthmus of Panama [3].

The color of spiders of this genus ranges from a fawn to dark brown (Figure 1A). *Loxosceles* spiders have a violin-shaped pattern on the dorsal surface of their cephalothorax, vary in length from 1 cm to 5 cm, including legs, and have six eyes arranged in non-touching pairs in a U-shaped pattern (Figure 1B). This positioning of eyes has been described as the best means of identifying these brown spiders [4–8]. The brown spiders are sedentary, non-aggressive, have nocturnal habits and prefer to inhabit dark areas. In human habitats, brown spiders are often found behind furniture, pictures and associated with clothes.

Accidents involving *Loxosceles* genus spiders occur mainly in the warmest months of the year, predominantly during spring and summer [4,6]. The condition caused by brown spiders, categorized as Loxoscelism, is associated with a series of clinical symptoms including cutaneous lesions, which spread gravitationally from the spider bite. The lesions are characterized by necrotizing wounds that are dark blue-violet in color and become indurated, leading to the formation of scar tissue. Surrounding the lesion, there is also erythema and edema. At the systemic level (less frequent than the appearance of skin lesions), patients may experience fever, weakness, vomiting, pruritic reactions, renal failure,

and hematologic disturbances that may include thrombocytopenia, disseminated intravascular coagulation and hemolytic anemia [5,6,8,9].

Figure 1. Brown spider aspects. (**A**) *Loxosceles intermedia* adult specimens—female and male. (**B**) Violin-shaped pattern (arrow) on the dorsal surface of cephalothorax from *Loxosceles intermedia* adult spider, and its six eyes arranged in pairs as a semi-circle (arrowheads). (**C**) Venom harvesting by electric shock applied to the cephalothorax. Arrow points for a drop of *Loxosceles intermedia* venom. Briefly, venom is extracted using an electric shock of 15 V applied to the cephalothorax of the spider and the venom from the tips of the fang is collected and diluted in phosphate buffered saline (PBS) or dried and stored at -80 º C until use. (**D**) Brown spider venom glands of *Loxosceles intermedia* observed by stereo dissecting microscope (40X). Venom can be harvested directly from venom glands: the removed glands are washed in PBS and the venom is obtained by gentle compression of the glands.



2. The *Loxosceles* Venoms

Over recent years, *Loxosceles* genus spider venoms have been studied by several scientific research groups worldwide, and many different toxins have been identified in the venoms. The corresponding biological and biochemical properties of these toxins have been reported, yielding insights into the pathophysiology of envenomation [4,5,7]. The venom of *Loxosceles* spiders is a complex mixture of protein and peptide toxins with a molecular mass profile ranging from 1 to 40 kDa [5]. To date, several molecules in the *Loxosceles* spider crude venoms have been described, including alkaline phosphatase [5,10], 5'-ribonucleotide phosphohydrolase [5], sulfated nucleosides [11], hyaluronidase [5,12–14], fosfolipases-D [5,15–17], metalloproteases, serine proteases [12,13,18–22] and insecticide toxins [23]. Table 1 contains a brief collection of main features from proteic toxins described in *Loxosceles* genus.

Low molecular weight components, such as neurotoxic and non-neurotoxic peptides, polyamines and other components are poorly studied in *Loxosceles* venom. Using NMR-spectroscopy, Schroeder and colleagues (2008) showed that sulfated guanosine derivatives comprise the major small-mollecule components of the brown recluse spider. They detected cross-peaks corresponding to 2,5-disulfated guanosine and 2-sulfated guanosine. It appears that sulfated nucleosides occur in several spider superfamilies, such as Agelenoidea and Amaurobioidea. The physiological properties of the sulfated nucleosides remain largely unexplored [11].

Serine proteases were already described in *Loxosceles* venom as high molecular weight enzymes (85–95 kDa) with gelatinolytic activity activated by trypsin [19]. Proteome and transcriptome analyses of *Loxosceles* venom also described this family of proteases [24,25]. Serine proteases generally are among the best characterized venom enzymes affecting the hemostatic system. However, the exact role of serine proteases in envenomation still remains to be clarified.

Recently, by using a cDNA library and transcriptome analysis, a novel expression profile has been elaborated for *Loxosceles intermedia* gland venom. This recently developed profile has allowed the identification of additional toxins as components of the venom, including insecticidal peptides similar to knottins (molecules that form an inhibitor cystin knot), astacin-like metalloproteases, venom allergen, a translationally controlled tumor protein family member (TCTP), serine protease inhibitors, and neurotoxins similar to Magi 3 [26,27]. Brown spider venoms display a broad diversity of toxin isoforms, including members of the phospholipase-D family and astacin-like toxins, even in the same sample [17,27–29]. Such features, which represent an adaptation to increase the survival of the spiders and the effectiveness of venoms, confer advantages to the spider predator. To confirm the existence of a new family of toxin isoforms, it is necessary to further characterize their biological properties. Recently, a spider toxin database called Arachnoserver which was manually curated [30], has cataloged 54 toxins from Sicariidae spiders family. It was elaborated, based on information gleaned through studies on complex venom mixtures, and has resulted in an exponential increase in the identification of peptide-toxins. King *et al.* [31] recommend a rational nomenclature for naming toxins from spiders and other venomous animals to avoid the continued use of *ad hoc* naming schemes that introduce confusion and make it difficult to compare toxins among species and establish evolutionary relationships.

Table 1. An overview of toxin families in *Loxosceles* genus.

Toxins	MW (kDa)	Characteristics and actions described	No. Seq *
Phospholipases-D (SicTox family members, such as LiRecDTs)	30–35	Several isoforms with variant features such as: - Dermonecrosis [12,13,16,32–38] - Lipids hydrolysis [33,39–42] - Hemolysis [38,43–45] - *In vitro* platelet aggregation [34,36,37] - Infiltration of inflammatory cells [35–37,42] - Edema [34,38] - Renal disturbances [35,46] - Lethality [34,38,46,47] - *In vitro* cytotoxicity [35,42,46] - Cytokine activation [41,48–50]	335
Insecticidal peptides	5–8	- LiTx family members [23,27] and Magi 3-related peptides [23,27,51] - LiTx: Lethal to *S. frugiperda* (flaccid paralysis) [23] - LiTx3: appears to act upon Na^+ channels [23]	8
Metalloproteases	28–35	- Astacin-like Metalloprotease (LALPs) [29,52] - Present in the venom of different species of *Loxosceles* genus [12,13,27,51,53] - Activity upon gelatin, fibronectin, fibrinogen and entactin [18,52–54]	4
Hyaluronidases	41–43	- Classified as endo-beta-N-acetyl-d-hexosaminidases hydrolases [14] - Activity upon hyaluronic acid and chondroitin sulphate [13,14] - Present in the venom of different species of *Loxosceles* genus[12–14,24,27,51,55]	-
Serine-proteases	85–95	- Gelatinolytic activity [19] - Activated *in vitro* by trypsin [19] - Present in the venom of *L. intermedia* and *L. laeta* [27,51]	-
Serine/Cysteine protease inhibitors	N.D.	- Belongs to Serpin superfamily [27] - Identified in *Loxosceles* spp. transcriptomes and proteome [24,27,51] - May be related to coagulation processes, fibrinolysis and inflammation [51]	-

Table 1. *Cont.*

TCTP (translationally controlled tumour protein)	~46	- Identified in *Loxosceles* spp. transcriptomes [27,51] - Putative functions: Histamine releasing factor in extracellular environment; several intracellular roles such as embryonic development, cell proliferation, stabilization of microtubules [56]	-
Lectin-like	N.D.	- Putative features: carbohydrate-binding molecules; involved in extracellular matrix organization, endocytosis, complement activation, *etc*. [51]	-
Alkaline-phosphatase	N.D.	- Degrades the synthetic substrate *p-nitrophenyl phosphate*[10]	-
ATPase	N.D	- ATP hydrolysis [10]	-

N.D.: not determined. *Number of sequences deposited in PUBMED protein database.

3. The Rational Use of Venom Toxins as Biotechnological Tools

The idea of using venom toxins as tools for biological purposes is currently gaining acceptance worldwide, as researchers incorporate the use of novel technologies to overcome old obstacles such as low venom volumes. Technological advancement has led to better techniques for protein purification; different models for synthesis of recombinant toxins; structural views of molecular domains, binding sites or catalytic sites of molecules of interest; design of synthetic inhibitors or agonists; and finally, cellular and animal models for testing the products obtained. The use of toxins directly as a source of materials to produce medicines or similar products has been receiving much attention from the pharmaceutical industry and experts in the field of applied research. Examples of toxin-derived biomedicines derived from venoms of different animals are abundant. Venoms from snakes, perhaps the best studied example of biotechnological applications among animal venoms, with biologically active toxins in the cardiovascular system, central nervous system, membrane lipids and proteins, hemostatic system, and muscular system, have led to the discovering of several products used in the treatment of various diseases. These drugs include Captopril (blood pressure), Integrilin (acute coronary syndrome), Aggrastat (myocardial infarct and ischemia), Ancrod (stroke), Defibrase (acute cerebral infarction and angina pectoris), Hemocoagulase (hemorrhage), and Exanta (anti-coagulant). Toxin-derived products from snake venoms have also been used for diagnosis. This group of compounds includes Protac (protein C activator, diagnosis of hemostatic disorders), Reptilase (diagnosis of blood coagulation disorder) and Ecarin (diagnostic of hemostatic disorder) (for review, see [57,58]).

Other toxin-derived medicines have been prepared from components of marine cone snail venoms, called conotoxins, which are potent ion channel modulators, and have facilitated the discovery of a novel analgesic agent named ziconide, used in the treatment of pain syndromes [59,60]. The honeybee venom toxin, called tertiapin (TPN), is an inhibitor of potassium channels, has generated TPNLQ, a variant and a potential novel model for the treatment of hypertension [61]. Exenatide (synthetic exendin-4) is a toxin-derived medicine from the venom of Gila monster lizard that stimulates the

production of insulin by pancreatic cells and has the potential to treat type 2 diabetes [62,63]. Scorpion venom toxins have been studied as well, and a large number of molecules with biological activities as pain-killers, agents that control the spread of cancer, and natural insecticides can be generated. Scorpion venom, such as kurtoxin and anuroctoxin, can target specific mammalian cell ion channels and their isolation has opened possibilities for drug design in the context of neurologic and autoimmune diseases [64,65]. Other scorpion venom toxins (beta-toxins) can selectively interact with insect voltage-gated sodium channels and can be used as toxin-based pesticides [66]. Sea anemone venom toxins have been reported as potential agents for the treatment of autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and type I diabetes [67]. These toxins, such as Shk, a 35-residue polypeptide toxin that is a potassium channel blocker, have proven to be very useful sources of pharmacological tools. Furthemore, the molecule's analogs have been evaluated with regard to the development of new biopharmaceuticals for autoimmune disorders [68,69].

With regard to spider venoms, researchers are involved in the study of insecticidal toxins, which can be used as tools in the elaboration of environmentally safe pesticides. Notably, the venom of the Australian funnel web spider has been analyzed, with emphasis on the toxin omega-atracotoxin (ALTX) HV1, a 37-residue peptide molecule. One model proposes the use of baculoviruses to express spider toxin to act as a pesticide [59,70]. Additionally, spider venom toxins can be used as models for the development of transgenic plants expressing insecticidal toxins. One example of this situation is the case of omega-ACTH-Hvt1 toxin from the venom of *Hadronyche versuta*, which protects the tobacco plant against insects. Another rational use of spider venom toxin as a model for design of therapeutic agents involves use of the toxin from *Phoneutria nigriventer* venom as a tool for the treatment of erectile dysfunction. The toxin Tx2–6 causes an improvement in the level of nitric oxide in penile tissue in rats [71,72]. Additionally, antibacterial peptides were identified in the venom of the *Cupiennius salei* spider. These peptides appear to act as channel-forming toxins within the bacteria wall. Analogous synthetic molecules would be expected to have great potential, especially in the age of multiple-antibiotic-resistant bacteria and related threats to human health [59,73].

The biotechnological uses of *Loxosceles* spider venoms have received increased attention over recent years. Notably, a spider toxin-derived product (ARACHnase) was proposed for the diagnosis of lupus anticoagulant. Also, antisera produced with *Loxosceles* venom has been used as bioproducts for serum therapy after spider accidents (for more information, see [74]). Recently, several recombinant toxins from *L. intermedia, L. laeta*, *L. boneti*, *L .gaucho,* and *L. reclusa* have been described. These include members of the phospholipase-D family [32–37,39,43], members of metalloprotease/astacin family [29,52], a member of translationally controlled tumor protein family (TCTP), a hyaluronidase, a serine protease inhibitor, a venom allergen, an insecticide toxin, member of neurotoxin/Magi 3 family , and an insecticidal toxin [75]. Recombinant molecules will not only expand our knowledge of spider biology and the pathophysiology of Loxoscelism, but as we shall discuss in the next chapters, they will also provide additional molecules for biotechnological purposes [74].

4. Phospholipase-D

Phospholipase-D is the most studied type of molecule present in the venom from *Loxosceles* species. In the general literature, these toxins are referred to as sphingomyelinase-D, due to their first

biochemical description as enzymes capable to hydrolyze sphingomyelin substrate. Based on the IUBMB recommendations, these molecules are biochemically classified as sphingomyelin phosphodiesterases D (E.C. 3.1.4.41) [5,6] Dermonecrotic toxin is a biological term widely applied by toxinologists to *Loxosceles* phospholipase-D, due to the hallmark of brown spider bites, which trigger dermonecrosis *in vivo.* Kalapothakis *et al.* [17] have organized dermonecrotic toxins of *L. intermedia* into a protein family, denoted LoxTox, by using cDNA coding sequences of several dermonecrotic/sphingomyelinase proteins from *Loxosceles intermedia*. The authors present at least six distinct groups (LoxTox 1 to 6) based on similarities among the molecules. At the present moment, Arachnoserver [30] includes 49 toxins from the *Loxosceles* genus with biological activity patterns characterized by dermonecrosis; these toxins were denoted as brown spider phospholipase-D proteins or partial sequences following the phylogenetic analyses of sicariid SMases by Bindford *et al.* [1].

The *Loxosceles* and *Sicarius* genera uniquely share the dermonecrotic venom toxin phospholipase D within the Haplogyne lineage. The most prospective evolutionary scenario for the origin of this enzyme is a single origin in the most recent ancestor of the Sicariidae family [76]. Phospholipases-D vary in molecular mass between species of North American *Loxosceles* (31–32 kDa), Old World species (32–33.5 kDa) and South American *Loxosceles* (32–35 kDa) [76]. Sphingomyelinase-D activity can be detected in all (36) *Loxosceles* and *Sicarius* species already tested. Binford and colleagues (2008) proposed to call this specific gene family *SicTox* towards a rational nomenclature. Based on Bayesian analyses they also resolved two clades of SMD genes, labeled α and β. Sequences in the α clade are exclusively from New World *Loxosceles* and *Loxosceles rufescens* and include published genes for which expression products have SMase D and dermonecrotic activity. The β clade includes paralogs from New World *Loxosceles* that have no, or reduced, SMase D and no dermonecrotic activity and also paralogs from *Sicarius*. In the context of structural position and proposed active sites [40], α and β clades differ only in conservation of key residues surrounding the apparent substrate binding pocket [3].

The pathological mechanisms of brown spider phospholipase-D have been continuously investigated, Van Meeteran [48] and Lee and Lynch [41] observed that recombinant *Loxosceles* SMaseD isoforms are able to hydrolyze lysophospholipids, generating bioactive lipid mediators such as lysophosphatidic acid (LPA). These researches extended the boundary of knowledge, which had depended upon sphingomyelin as a well-known substrate molecule. Furthermore, Lee and Lynch [41] also postulate that the term **phospholipase-D** (**PLD**) would more effectively represent the broad range of hydrolysable phospholipids than previously supposed to be applied for dermonecrotic toxins from *Loxosceles* genus [48]. Nomenclature of these toxins should be updated to account for the recent accumulation of knowledge regarding the biological and biochemical properties of these compounds.

The great interest of toxinologists in PLD proteins, to the neglect of other toxins present in the venom (most of them also enzymes or bioactive peptides), is due to the ability of these proteins to reproduce many effects of necrotic arachnidism or Loxoscelism. The PLDs from the *Loxosceles* genus are described as being responsible for several biological properties ascribed to whole venom, including the following: dermonecrosis, massive inflammatory response with neutrophil infiltration and complement activation, platelet aggregation, immunogenicity, edema and increased blood vessel wall permeability, hemolysis, renal failure, toxicity for several cultured cell types, and animal lethality [4,38,74,77].

Clinical investigations by Futrell [5] indicated that a dermonecrotic factor was responsible for histopathological observations resembling those of the cutaneous Arthus reaction, as observed in victims of accidents with brown spiders. Futrell [5] also reported the native toxin from *L. reclusa* (32 kDa) was an enzyme that hydrolyzes sphingomyelin and releases choline and N-acylsphingosine phosphate (or ceramide 1-phosphate). Various isoforms of phospholipase D were already reported for different species. Using SDS-PAGE analysis and chromatography methods, a range of molecular mass between 30–35 kDa was determined for PLD toxins that have hemolytic, necrotic and platelet aggregation activity, from *L. reclusa*, *L. rufescens*, *L. gaucho*, *L. laeta* and *L. intermedia* venoms [5,15,16,44,47,78,79]. Advances in proteomic studies have facilitated the description of many more PLD-related proteins in whole venom. Luciano *et al.* [80] performed two-dimensional electrophoresis and observed enriched levels of a 30-kDa molecule as well as cationic properties in *L. intermedia* whole venom, indicating the presence of several PLD-related protein spots. Furthermore, proteomic analysis of *L. gaucho* whole venom led to the identification of at least eleven PLD proteins (30–32 kDa 'loxnecrogin' isoforms) by Edman chemical sequencing and capillary liquid chromatography-mass spectrometry [25]. In summary, PLDs are dermonecrotic toxins that comprise a family of toxins with different related isoforms that have biological, amino acid and immunological similarities and which are found in diverse *Loxosceles* species [4,27,38,74]. This variation in phospholipase-D molecules may be due to post-translational modification and the expression of paralogous genes, since recent data demonstrate that gene duplications are frequent and that PLD genes lie in a region with high recombination within the genome [3].

Nowadays, heterologous systems based on cDNA sequences encoding mRNA transcripts from the brown spiders are a very useful tool for the production of recombinant PLD proteins (mainly in prokaryotic models). Using extracts of the venom gland, which is the tissue that is specialized for the production and secretion of venom toxins, molecular biology techniques were optimized to obtain several sequences as template for the identification, characterization and recombinant expression of PLD proteins [74].

At present, a new generation of molecules developed through cloning techniques still remains under investigation by researchers aiming to determine molecular and cell mechanisms of PLDs by biological approaches. *L. intermedia* LiD1 recombinant protein (31.4 kDa) is a sphingomyelinase D family molecule without dermonecrotic activity but with antigenic activity [32]. *L. laeta* recombinant protein (33 kDa) is a sphingomyelinase isoform able to degrade sphingomyelin [43]. *L. laeta* recombinant phospholipase-D generates lysophosphatidic acid and induces lysis of red blood cells [41]. Keratinocyte apoptosis was induced by recombinant PLD (SMaseD P2) from *L. intermedia* [81]. Global gene expression changes in fibroblast cells induced by PLD recombinant protein from *L. recluse* (SMD) are related to components of inflammatory response, such as human cytokines, genes involved in the glycosphingolipid metabolism pathway, and proteins known to impact transcriptional regulation [49]. Six isoforms of phospholipase-D were cloned from a cDNA library of *L. intermedia* gland venom and then expressed; they were shown to have similar toxic effects to those of native venom toxins [34–38]. *L. intermedia* recombinant protein (LiRecDT1, 34 kDa) displays dermonecrotic activity and was able to directly induce nephrotoxicity in mice and cultured tubular epithelial cells [42,46]. It could also induce non-complement-dependent hemolysis *in vitro* and inflammatory response using endothelial cell membrane as target [42,45]. Nephrotoxicity and hemolysis are both toxic effects

that depend directly on catalytic enzyme activity. In the same way, LiRecDT2 (ABB69098), LiRecDT3 (ABB71184), LiRecDT4 (ABD91846), LiRecDT5 (ABD91847), and LiRecDT6 (ABO87656) were identified, cloned and characterized as PLD proteins with high similarity to each other based on sequence alignment; this similarity is due primarily to conserved amino acids at the catalytic site [34–37]. The results of this alignment corroborated with the crystal structure analysis of a dermonecrotic toxin [40] from *L. laeta*, which suggested there were conserved residues at the proposed catalytic site for SMase D. The recent transcriptome analysis of *L. intermedia* venom gland identified at least two clusters (annotated as PLD-related ESTs) as new possibilities for a novel PLD isoform in *L. intermedia* venom, adding a new group to the LoxTox family classification [17,27].

The knowledge of structural, biochemical and biological properties of PLD toxins could be employed in design studies for the development of new drugs, biopharmaceuticals, diagnostic tests and other biotechnological and industrial applications. Immunoassays using brown spider PLDs as probes have been tested [50,82] because differential diagnosis of brown spider bites can often lead to misdiagnosis [83,84]. Moreover, therapeutic serum development and vaccination have been studied to ascertain the benefits of antivenom [85,86]. Synthetic peptides designed based on PLDs toxins with specific biological/protective effects have also been utilized [87,88]. Additionally, brown spider PLDs could be employed in the development of a vaccine derived from the phospholipase-D-mutated toxin from *L. intermedia* (substitution of the Histidine12 for Alanine in the catalytic site—LiRecDT1H12A) for the immunization of people living in regions that are endemic for accidents involving *Loxosceles* spiders. This method may be useful because enzyme activity of LiRecDT1H12A is dramatically decreased and has neither hemolytic activity nor nephrotoxicity [45,46]. Another possible application for PLD is as reagent of immunodiagnostic assays for identification and quantification of phospholipase-D in the sera of patients bitten by *Loxosceles* spider because diagnosis of Loxoscelism is very controversial and is commonly based on clinical signs and symptoms [89]. Brown spider venom may be detected in hair, wound aspirates, and skin biopsy for at least seven days after inoculation [90].

PLD enzyme activity triggers the degradation of the cell membrane phospholipids, loss of membrane asymmetry, phosphatidylserine exposure and membrane reorganization [91–93]. Sphingomyelin degradation changes membrane properties, such as lipid raft organization and membrane fluidity, triggering intracellular pathways [94,95]. Phospholipid metabolites induce the release of prostaglandins, activate the complement cascade, stimulate platelet aggregation, and enhance neutrophil chemotaxis and inflammation. Brown spider PLD toxins could be used in lipid protocols for cell membrane studies related to biological effects of lipid metabolites, with emphasis on sphingolipid-derived bioactive molecules and their signaling pathways. The activity and expression of some phospholipases are increased in several human cancers, suggesting that these enzymes may have central roles in tumor development and progression [96,97]. This involvement raises the possibility of considering phospholipid metabolism as a potential target for the development of new antitumoral agents by using brown spider PLDs as a novel model for tumor cell studies.

Further studies improving the understanding of PLD catalysis are relevant not only for comprehension of phospholipases mechanisms in basic sciences, but also for related pharmaceutical and biotechnological applications [98]. The catalytic activity of brown spider PLD plays a role in the pathological activity of this toxin and therefore cannot be dismissed as a rational target for new

strategies to treat Loxoscelism. Degradation of the phospholipid head-groups by brown spider PLDs changes membrane surface potential and affects the functional properties of some cation channels. Brown spider PLDs can offer an effective pharmacological way to activate voltage-gated channels that could be useful for "channelopathy" studies [99]. Certainly, elucidation of the roles of PLDs in a variety of molecular and cell biology mechanisms might be the greatest value of brown spider PLDs as a biotechnological product, which depends on their continuous characterization with regard to the details of pathogenesis and biochemistry.

5. Hyaluronidase

Hyaluronidases are enzymes that mainly degrade hialuronic acid (HA), and which may have activity upon chondroitin, chondroitin sulfate (CS) and, to a limited extent, dermatan sulfate (DS) [14,100,101]. The hyaluronidases are a group of enzymes that are distributed widely throughout the animal kingdom. They were discovered through the observation that extracts of some tissues contained a "spreading factor", which facilitated the diffusion of dyes and subcutaneous antiviral vaccines [102]. These enzymes are present in the venoms of multiple organisms, such as lizards, scorpions, spiders, bees, wasps, snakes and stingrays [103–105].

Hyaluronidases in venoms have been described as "spreading factors" due to their ability to degrade extracellular matrix components and to increase the diffusion of other toxins in tissues adjacent to the inoculation site [103]. Data from crystallography and X-ray diffraction suggested the evolutionary conservation of many poison hyaluronidases in a comparative study of several animal venoms [106,107]. Tan and Ponnudurai [108] reported that all venoms exhibit a wide range of hyaluronidase and protease activities. With regard to spider venoms, Kaiser [109] was the first to report hyaluronidase activity, from Brazilian *Lycosa raptoral* spiders, now known as *Phoneutria nigriventer* [110]. Shortly after that report, hyaluronidase activity was detected in the venom of European window spider *L. tredecimguttatus* and of the tarantula *D. hentzi* venom. This enzyme was isolated from the funnel web *A. robustus* and the tarantula *E. californicum venom* [111]. Spider venom hyaluronidases have been described more recently in *Lycosa godeffroy*, *Lympona cylindrata/murina* [110] and *Cupiennius salei*[112]. The *Hipassa* genus showed similar hyaluronidase activity to that of *H. agelenoides, H. lycosina* and *H. partita* species [110,113]. Moreover, venom obtained from *Vitalius dubius*, a spider found in southeastern Brazil, showed high levels of hyaluronidase activity [114]. With regard to necrotizing Australian spiders, hyaluronidase activity was demonstrated in *Badumna insignis*, *Loxosceles rufescens*, and *Lampona cylindrata* [12].

In 1973, Wright *et al.* were the first to describe hyaluronidase activity in venom of the genus *Loxosceles* [55]. This work was performed with *L. recluse* venom, and the purified enzymes, which were estimated to have molecular weights of 33 and 63 kDa by SDS-PAGE [115], exhibited activity against HA and CS types A, B, and C. The authors also showed that rabbit anti-venom inhibited the spreading effect exhibited by whole venom *in vivo* and completely inhibited hyaluronidase activity *in vitro* [55]. Young and Pincus [12], analyzing *L. recluse* venom, described hyaluronidase activity for a protein determined to be 32.5 kDa by HA-substrate SDS-PAGE [12,115]. Barbaro *et al.* [13] studied venoms from five *Loxosceles* species of medical importance in the Americas (*L. deserta, L. gaucho, L intermedia, L. laeta and L. recluse*).

Hyaluronidase activity was detected in all species of *Loxosceles* spider venom tested by HA zymogram. All venom samples contained an enzyme with molecular weight of approximately 44 kDa, which was able to digest HA and which may contribute to the characteristic gravitational spread of the dermonecrotic lesion in patients suffering from the effects of these venoms [13,115]. da Silveira *et al.* [14] reported that zymography showed *L. intermedia* venom included hyaluronidase molecules of 41 and 43 kDa molecular weight. The activity of these enzymes is pH-dependent, with optimal activity between 6 and 8, and was able to degrade HA in rabbit skin. Pedrosa *et al.* [51] studying *L. laeta* transcriptome found transcripts with similarity to *Bos Taurus* 'hyaluronidase' (gb|AAP55713.1): 4 clones and 1 cluster (LLAE0048C), representing 0.13% of the total sequence. In addition, hyaluronidase represents only 0.1% of all total toxin-encoding transcripts in the venom gland of *L. intermedia* [27]. This result may explain the difficulty associated with purification this enzyme from *Loxosceles* venoms. To obtain the recombinant hyaluronidase from *L. intermedia* venom, through the use of appropriate molecular biology techniques, an isoform was cloned and showed to have a theoretical molecular mass of about 46.1 kDa [75].

Hyaluronidase-mediated degradation of HA increases membrane permeability, reduces viscosity and renders tissues highly permeable to injected fluids. This degradation process is involved in bacterial pathogenesis, the spread of toxins and venoms, fertilization, and cancer progression [102]. Therefore, brown spider hyaluronidase could be used therapeutically in many fields, including orthopedics, surgery, ophthalmology, internal medicine, oncology, dermatology and gynecology [74]. There are several studies showing that hyaluronidases can be used to promote resorption of excess fluids, to increase the effectiveness of local anesthesia and to diminish tissue destruction by subcutaneous and intramuscular injection of fluids [100,102]. For example, hyaluronidase has been used to reduce the extent of tissue damage following extravasation of parental nutrition solution, electrolyte infusions, antibiotics, aminophyline, mannitol and chemotherapeutic agents, including Vinca alkaloids [116].

Additionally, recombinant human hyaluronidase (rHuPH20) has been used in chronic pain management, to improve systemic absorption and bioavailability of drugs [117–120]. In the context of cancer therapy, testicular hyaluronidase (HAase) has been added to drug regimens to improve drug penetration. In limited clinical studies, HAase has been used to enhance the efficacy of vinblastin in the treatment of malignant melanoma and Kaposi's sarcoma, among other cancers [121]. Furthermore, when the level of HA decreases under conditions in which hyaluronidase activity increases, the moisture and tension of the skin are reduced, and histamine is released from mast cells [122]. Therefore, the identification and characterization of hyaluronidase inhibitors could be relevant to the development of contraceptives, as well as anti-tumor, anti-microbial, and anti-venom, anti-wrinkle, and anti-aging agents, and allergy and inflammation suppressors [14,122–124]. Therefore, *Loxosceles* recombinant hyaluronidases are associated with numerous potential applications [27,74,125,126].

6. Translationally Controlled Tumor Protein (TCTP)

Loxosceles intermedia TCTP protein was identified during an *L. intermedia* venom gland transcriptome study [27], although another spider TCTP had already been described from the venom gland of *Loxosceles laeta* by transcriptome analysis [51]. Proteins of the TCTP superfamily were first

identified in the late eighties by research groups studying translationally regulated genes. These proteins were named *translationally controlled tumor proteins* when the discovery of human cDNA was published [127]. This name was based on the protein's tumoral origin, a human mammary carcinoma, and on the observation that TCTP is regulated at the translational level. The translationally controlled tumor protein (TCTP), which was initially named P21, Q23 and P23 by three different groups and is also called HRF (histamine-releasing factor), represents a large family of proteins that are highly conserved and ubiquitous in eukaryotes [56,128].

Sequence alignment studies of TCTP sequences revealed that nearly 50% of all amino acid residues are preserved. Among species from the same genus, TCTPs are completely conserved [56]. When the TCTP sequence found in the *L. intermedia* venom gland transcriptome was compared with the one described in the venom gland of *L. laeta*, 97% similarity was observed. *L. intermedia* TCTP also presented important similarities with the other arthropod TCTPs, such as *Ixodes scapularis* and *Amblyomma americanum* from mites [27]. The scientific community's understanding of TCTP's biological function is growing. The compound possesses a wide range of functions, and different biochemical roles are currently being established [56,129].

Although TCTP participates in various biological functions, the primary physiological roles of this protein are still unknown [130]. TCTP is widely expressed in many tissues and cell types, and its protein levels are highly regulated in response to a wide range of extracellular signals and cellular conditions [56]. Interactions between TCTP and other cellular proteins have already been reported for tubulin [131], actin-F [132], the mammalian Plk [133], translation elongation factors eEF1A and eEF1Bbeta [134], Mcl-1 [135,136], TSAP6 [137], Na,K-ATPase [138], Bcl-XL [139] and Chrf [140]. Studies have already shown that TCTP is essential for embryonic development and cell proliferation in mice and *Drosophila* [141,142]. Moreover, the protein has calcium-binding activity and is capable of stabilizing microtubules, a property that may be related to a possible role of TCTP in cell cycle control, as it was also shown that TCTP interacts with a checkpoint protein (Chrf) [56,140].

Loxosceles intermedia transcriptome analysis highlighted TCTP transcript as a toxin-coding messenger due to TCTP extracellular activities already described above [27]. TCTP was described as a protein that triggers histamine release in basophil leukocytes and was therefore called 'histamine release factor' (HRF) [128]. Then, other studies reported that TCTP presents more general 'cytokine-like' activity, as it also induces the production of interleukins from basophils and eosinophils [143]. TCTP itself is induced by certain cytokines and acts as a growth factor for B-cells [144]. Studies demonstrate that TCTP triggers histamine release in basophile leukocytes by mechanisms that may be dependent on or independent of the presence of IgE. It is believed that a specific TCTP receptor may participate in the process, leading to mast cell activation [56]. Although TCTP protein was found in biological fluid of asthmatic or parasitized patients and in saliva from ticks, TCTP mRNAs do not code for a signal sequence and no precursor protein has been described [56,145]. TCTP secretion from cells proceeds via an endoplasmic reticulum/Golgi-independent or non-classical pathway, probably mediated by secreted vesicles called exosomes, which have been suggested as possible pathways for non-classical secretion [137,145]. In the case of the *Loxosceles* venom gland, TCTP is secreted via holocrine secretion [27]. TCTPs have been described in gland secretions of many arthropods, such as ixodid ticks and in the venom gland of the wolf spider [146–148].

L. intermedia TCTP is very similar to *Dermacentor variabilis* TCTP, which is expressed in diverse tissues from the tick, including its salivary gland. When this TCTP was cloned and expressed as a recombinant protein, it was able to release histamine from a basophilic cell line [27,146]. Based on these data, it is possible to suggest that *L. intermedia* TCTP may act as a histamine release factor. The presence of a component in *L. intermedia* venom related to the histaminergic activity of venom supports with this hypothesis [149]. Recently, some authors have called attention to the role of histamine and its receptors in the development of edema, involving increased vascular permeability and vasodilatation [150], which occurs in Loxoscelism. Histamine had been described as the principal pharmacological component in the venom of the wolf spider (*Lycosa godeffroyi*) [148,151]. Proteins of the TCTP family were described to be expressed in human parasites suggesting that could be related to the survival mechanisms of parasites in the host and to the onset of pathological processes [152–154]. The antimalarial drug artemisin [155], probably acts on *Plasmodium* TCTP, confirming its important function in the development of pathology [153,154].

Recently, an increasing number of researchers have focused their attention on the cellular and extracellular activities of TCTP, as it has been implicated in the promotion of cell growth and tumorigenesis as well as in protection against apoptosis and other consequences of cell stress [56,156–158]. TCTP protein levels are upregulated in cancer cells and in human tumors [159–161]. Downregulation of TCTP has been implicated in biological models of tumor reversion [159,162], and the protein is the target of various anticancer drugs [159,163]. TCTP has been proposed as a potential cancer biomarker [160,164,165] and therapeutic target [166].

TCTP has enormous biotechnological potential; this toxin presents a wide range of putative applications: from a biological tool at research laboratories to clinical oncology, as a biomarker and/or a model for drug design to cancer treatment. Drugs that cause inhibition of TCTP activity resulted in tumor growth inhibition both *in vitro* and *in vivo* [159]. TCTP and its biological tools (e.g., antibodies against TCTP) can also be used in experimental oncology to study tumor cell behavior and metabolism, as well as in the screening of anticancer drugs. Still in the field of cell proliferation, TCTP and its related biological tools could also be used to study cell cycle regulation and the microtubule cytoskeleton, as well as its role in cell physiology and organelle transport.

Calcium metabolism and signaling are other issues that could be explored using TCTP and its derived biological tools. Antiapoptotic activities were also described for TCTP: this protein potentiates MCL1 and BCL-X_L inhibits BAX [158]. These effects highlight TCTP as a candidate for apoptosis studies, as an apoptotic drug and as a model for anti-apoptotic reagents. Another possible application of this toxin could be its employment in allergic screening tests, due to TCTP's histaminergic activity. Inhibitors of TCTP are putative anti-histaminic drugs and other TCTP-derived biological tools could be useful at research laboratories that study histamine release, mast cell metabolism and activation, immediate hypersensitivity reactions and the allergy process in general. Protocols that involve proliferation of B cells represent other potential applications for TCTP. TCTP secretion to the extracellular milieu is mediated by a non-classical pathway involving exosomes [137]; therefore, it is a good reagent with which to study this type of cellular secretion. TCTP has a surprising number of different functions as described here, but how these different functions might be interrelated remains to be determined [167]. Therefore the putative applications suggested herein are just the first insights into the potential uses and applications of TCTP in the field of biotechnology.

7. Astacin-Like Metalloproteases

Metalloproteases in *Loxosceles* venom were first characterized in *L. intermedia* venom. Feitosa *et al.* [18] described two metalloproteases, Loxolisin A (20–28 kDa, with fibronectinolytic and fibrinogenolytic activity) and Loxolisin B (32–35 kDa, with gelatinolytic activity). Zanetti *et al.* [168] purified a 30 kDa molecule with fibrinogenolytic activity from *L. intermedia* crude venom. Furthermore, da Silveira *et al.* [53] showed that venom gland extracts from brown spiders possess proteolytic activity, and this activity could be inhibited by bivalent chelators. This study proved that metalloproteases are components of *L. intermedia* and *L. laeta* venoms, and eliminated the possibility that electrostimulated venom could have been contaminated with digestive hydrolytic enzymes during extraction [53].

Metalloproteases were also identified as components of different *Loxosceles* species venoms, such as *L. rufescens, L. gaucho, L. laeta, L. deserta* and *L. reclusa* [12,13,51,168]. Recently, a recombinant metalloprotease from the *L. intermedia* venom gland, named LALP (*Loxosceles* astacin-like metalloprotease), was characterized as an astacin-like enzyme. This functional characterization supported previous data describing metalloproteases in *Loxosceles* venom [52]. The identification of LALP in *L. intermedia* venom was the first report in the literature of the presence of an astacin family member as an animal venom constituent. Trevisan-Silva *et al.* [29] described two new astacin-like toxin isoforms from *L. intermedia* venom (LALP2 and LALP3) and found that metalloproteases in *L. laeta* and *L. gaucho* venoms are also members of the astacin family. This study described the presence of a gene family of astacin-like toxins in three *Loxosceles* species suggesting that these molecules will be found in all South America *Loxosceles* species [29]. Astacin-like proteases are the second most commonly expressed class of toxins in the *L. intermedia* venom gland, comprising 9% of all transcripts [27].

The astacin family enzymes are zinc-dependent metalloproteases, which are considered as part of the metzincin superfamily [54,169]. Members from the astacin family are ubiquitous, existing more than 200 described astacins, which are found in some bacteria species and in all animal kingdoms [169–173]. Astacins are characterized by the zinc-binding motif (**H**EXX**H**XX**G**XX**H**EXXRXDR), which contains three histidine residues that are responsible for the complexation of zinc. Below the active site, all astacins have a methionine residue within a typical Met-turn (SX**M**X**Y),** with a tyrosine residue that might be involved in substrate fixation [54,169,174–176]. This protease family was named after the identification of astacin from freshwater crayfish, *Astacus astacus*. Astacin is the prototypical digestive collagenolytic enzyme of the astacin family [177,178]. Astacin family members are reported to have a wide range of functions, playing roles in digestion, in peptide and matrix molecules processing, in the activation of growth factors and in the degradation of distinct proteins [169,174,175].

We have little information about the biochemical and biological function of *Loxosceles* venom astacins because astacin members have distinct functions and the study of astacins from *Loxosceles* venoms is just beginning. Previous studies of *Loxosceles* metalloproteases have shown that they degrade some matrix proteins (fibronectin, fibrinogen, gelatin and entactin), but the mechanism involved in the noxious effect of the venom is until unclear [18,20,21,52]. It has been suggested that astacin toxins could be involved in gravitational spreading of dermonecrosis, in hemorrhagic

disturbances observed in accidents, imperfect platelet adhesion and increased vascular permeability, which can occur near bite sites after brown spider accidents [13,29,52]. Also, astacin proteases could act as a spreading factor for other venom toxins and could serve as important agents, in the processing of other venom toxins, by cleaving inactive proteins and generating active peptides that may be involved in Loxoscelism effects [29,52].

Astacin-like proteases are biologically active enzymes that have potential applications in pharmaceutical studies and could be used as tools for research protocols [74]. The enzymatic activities of astacins upon different proteins highlight these molecules as useful tools in studies involving protein degradation, especially the degradation of extracellular matrix (ECM) components. Considering the physiological and pathological events related with ECM degradation, astacins can be used in protocols for medical and pharmaceutical research, such as ECM assembly and remodeling (including collagen processing and the healing process). Drug administration (as a co-adjuvant), cell membrane metabolism, embryogenesis, cellular differentiation (including stem cells), tumorigenesis and metastasis, enzymatic activation (latency and activation of zymogens), cell signaling based on proteolysis, inflammatory response and vascular permeability are other potential applications for these molecules.

Astacins from *L. intermedia* could also be used as starting materials to design new drugs/molecules, as agonists and/or inhibitors. One possible therapeutic use of astacins from *L. intermedia* is the context of vascular diseases (acute myocardial infarction, acute ischemic stroke, thrombosed aortic aneurysms, pulmonary embolism, etc.) and as thrombolytic agents. At present, intravenously administered tissue plasminogen activator (IV-TPA) remains the only FDA-approved therapeutic agent for the treatment of ischemic stroke within three hours of symptom onset. Although intra-arterial delivery of the thrombolytic agent seems effective, various logistic constraints limit its routine use and, as yet, no lytic agent has received full regulatory approval for intra-arterial therapy [179]. Moreover, astacin inhibitors may be therapeutically useful in atherosclerosis prevention. Meprins, which are members of the astacin family, hydrolyze and inactivate several endogenous vasoactive peptides, some of which could alter various functions of cells in the arterial wall. Recent studies have shown that a meprin inhibitor suppresses the formation of atherosclerotic plaques [180]. The recombinant astacins could also be used as reagents for laboratorial tests to diagnose Loxoscelism, as well as anti-loxosceles serum production, in the treatment of envenomation.

8. Insecticidal Peptides

Spider venoms are functionally related to defense against predators and primarily used to paralyze and capture natural prey, especially insects [89,181–183]. To execute these functions, spiders developed an arsenal of insecticidal molecules in their venoms, resulting in a combinatorial peptide library of insecticidal peptides that has been improved over the course of evolution [184]. Such peptides consist of single-chain, low molecular weight molecules of 3–10 kDa, with a high number of cysteine residues that form intramolecular disulfide bridges [185,186]. Over the last decade, these peptides have been investigated extensively through identification, purification, characterization and cloning studies [23].

The insecticidal peptides act in the nervous system of prey or predator, causing paralysis or even death, by interacting with specific neuronal ion channels of the excitable membranes [183]. These peptides can be classified depending on their mode of action, such as effects on sodium (Na^+), calcium (Ca^{2+}), potassium (K^+) and chloride (Cl^-) ion channels [111,187]. Many of these peptides present a structural motif designated as an inhibitory cystine knot (ICK), and therefore these molecules are named *knottins*. The ICK motif is composed of a triple-stranded, anti-parallel β-sheet, stabilized by a cystine knot containing three disulphide bridges [188,189], which confer rigidity to the molecules in addition to a stabilization of their secondary structures and relative resistance to denaturation [190].

Although there are a great number of insecticidal peptides characterized in several spider species, little is known about insecticidal molecules in *Loxosceles* spiders. By studying *L. intermedia* venom, de Castro *et al.* [23] first described and characterized three isoforms of insecticidal peptides named LiTx1, LiTx2 and LiTx3 which contain ICK motif and act on specific ion channels. The chromatographic fraction containing these peptides showed potent insecticidal activity against the agricultural pests *Spodoptera* species. LiTx1 (7.4 kDa) presents some sites to possible post-translational modifications, such as N-myristoylation, protein kinase C phosphorylation, amidation and casein kinase II phosphorylation. With regard to its specificity, the study was not able to determine whether LiTx1 interacts with Na^+ or Ca^{2+} channels. LiTx2 (7.9 kDa) and may present N-myristoylation, protein kinase C phosphorylation and amidation sites. Its specificity to ion channels was not determined. LiTx3 peptide (5.6 kDa) has also sites for N-myristoylation and protein kinase C phosphorylation. Based on bioinformatic analyses, de Castro, *et al.* hypothesized that LiTx3 may interact with Na^+ channels. In 2006, a new isoform, LiTx4, was identified (GenBank n ºDQ388598.1).

Transcriptome analysis of the *L. intermedia* venomous gland revealed ESTs with similarity to LiTx peptides described by de Castro *et al.* [23]. LiTx3 was the most abundant sequence in the *L. intermedia* transcriptome, comprising 32% of toxin-encoding messengers. LiTx2 had a representativeness of 11% in relation to the toxin-encoding transcripts. [27]. The transcriptome analysis of *L. intermedia* venomous gland additionally revealed the presence of another class of ion channel-binding peptides. These peptides present similarity to neurotoxin Magi 3, a peptide isolated by Corzo *et al.* [26] from the venom of the *Macrothele gigas* spider. Magi 3 peptide is able to paralyze insects, although the authors did not confirm whether Magi 3 is specific for insect sodium channels or also acts on calcium channels [191].

The specificity of insecticidal peptides for ion channels provides an important tool to understand their dynamic activity. Ion channels are transmembrane proteins involved in the control of ion fluxes across the membrane, regulating membrane potential and ion balance. Their activity is also related to the coordination of diverse cellular functions such as excitation-contraction coupling, hormone and neurotransmitter secretion and gene expression. Thus, the comprehension of the interaction between peptide-ionic channels allows a more refined investigation of the physiological role of ion channels, as well as the determination of possible therapeutic applications [192].

The ability to discriminate insect ion channels confers to insecticidal peptides with considerable potential in the development of an efficient bioinsecticide for the control of economically disadvantageous pests or insect vectors of new or re-emerging disease [182,193]. Recombinant baculovirus containing the gene encoding an insecticidal peptide has been studied and tested against many insect pests, such as *Heliothis virescens* (cotton bollworm), *Laspeyresia pomonella*

(codlingmoth) and *Neodiprion sertifer* (European sawfly) [183,194]. This biotechnological development could lead to alternative methods for chemical control, resulting in many benefits to the agricultural sector that will ultimately reduce economic losses.

9. Serine Protease Inhibitors

The control of proteases is normally achieved by the regulation of expression, secretion, activation of proenzymes and degradation. A second level of control is based on specific inhibition of activity. Despite microorganisms that produce non-proteinaceous compounds that block host proteases, the remaining all known natural protease inhibitors are proteins [195–197]. Among these natural protease inhibitors, the most extensively studied and described protein inhibitors of proteases are the group of serine protease inhibitors.

Serine protease inhibitors can be classified into one of three different types, according to their structures and the mechanism of inhibition: the canonical inhibitors, the non-canonical inhibitors and the serpins. The largest group is the canonical inhibitors, which are small proteins (14 to ~200 amino acid residues) represented mainly by the Kazal, BPTI (bovine pancreatic trypsin inhibitor), potato I and STI (soybean trypsin inhibitor) families [198,199]. Non-canonical are usually found in blood-sucking organisms and are responsible for blocking the blood-clotting cascade [196]. Serpins (*ser*ine *p*rotease *in*hibitors) are large proteins (typically 350 to 500 amino acids in size), also widely distributed in nature, and are abundant in human plasma. Similar to the canonical inhibitors, serpins exhibit binding loops and interact with the target enzyme in a substrate-like manner. However, cleavage of the serpin loop by the protease leads to dramatic conformational changes in the global structure of the inhibitor [196,200,201].

In brown spider venom, protease inhibitors were first reported in *L. laeta* [51]. The transcriptome analysis approach, which detected 0.6% of sequences with identity to intracellular coagulation inhibitor from *Tachypleus tridentatus* and sequences with identity to serine (or cysteine) proteinase inhibitors from *Mus musculus*, *Aedes aegypti*, *Branchiostoma lanceolatum*, *Gallus gallus*, and *Boophilus microplus* . Similar results were obtained for *L. intermedia* [27], in which one transcript presented significant similarity with a serine (or cysteine) peptidase inhibitor, clade I, member 1 from *Mus musculus*. In both cases (*L. laeta* and *L. intermedia*), the sequences analyzed were similar to serine proteinase inhibitors belonging to the Serpin superfamily.

Playing roles as potential toxins, serine protease inhibitors have been intensively described in several snake venoms, especially for those of the *Elapidae* and *Viperidae* families [202]. In these venoms, the majority of inhibitors characterized belong to the canonical type, particularly the Kunitz/BPTI inhibitors of trypsin and chymotrypsin. The peptides were typically 6–7 kDa in size and were isolated from crude venoms and studied by different methods [203–210]. The identification of this type of molecule allowed future isolation and further characterization of putative protease inhibitors, suggesting the possibility of a biotechnological application. The best example for this purpose is Textilinin-1, which is a well-known 6.7 kDa Kunitz-type serine protease inhibitor from the venom of the snake *Pseudonaja textilis* which binds and blocks certain proteases, including plasmin and trypsin [211]. The ability to reversibly inhibit plasmin has raised the possibility of using this drug as an alternative to aprotinin (Trasylol®), as a systemic antibleeding agent in cardiac surgery. Like

aprotinin, Textilinin-1 (in equimolar concentrations) almost completely inhibits tissue plasminogenactivator-induced fibrinolysis of whole blood clots. In mouse bleeding models, Textilin-1 shows shorter time of hemostasis compared to aprotinin and appears to be a more specific plasmin inhibitor than aprotinin [210–212].

Despite their presence in the majority of snake venoms, serine protease inhibitors have also been described and characterized in other organisms. Zhao *et al.* [213] isolated and characterized a 60 kDa serpin from skin secretions of *Bufo andrewsi*, which was denoted as Baserpin. This protein was able to irreversibly inhibit trypsin, chymotrypsin and elastase. Serine protease inhibitors are also present in spider venoms, particularly in the venom of tarantulas (*Ornithoctonus huwena* and *Ornithoctonus hainana*). The prototypic molecule in tarantula venom is HWTX-XI, 6.1 kDa peptide from *Ornithoctonus huwena* venom, which belongs to the Kunitz-type family of serine protease inhibitors. Just like Kunitz-type toxins in snake venoms, HWTX-XI is considered to be a bi-functional toxin because it is a strong trypsin inhibitor as well as a weak Kv1.1 potassium channel blocker [214].

Zhao *et al.* [213] isolated and characterized a 60 kDa serpin from skin secretions of *Bufo andrewsi*, which was denoted as Baserpin. This protein was able to irreversibly inhibit trypsin, chymotrypsin and elastase. The considerations above represent just a few insights concerning serine protease inhibitors uses and applications. The great importance of proteases in numerous different biological processes and the large number of protease inhibitors described suggest their strong biotechnological potential.

10. Conclusion

Research in brown spider venom toxins has increased over recent years, but the challenges and opportunities are enormous. To move the field forward, scientists must have access to the biodiversity of spiders within their countries. Different *Loxosceles* genus spider species are reported to inhabit every continent [5,6,8], and bureaucracy related to the capture of spiders should not be a hindrance to researchers on toxinology area. Official collaborations with groups based where brown spiders are endemic will facilitate access to their venom.

Another difficulty in working with *Loxosceles* venoms is the fact that the volume of venom is minute (just microliters, containing a few micrograms of protein, as previously discussed). This makes work difficult for researchers that use crude venom in their experiments. To overcome this difficulty, works can collect venom from hundreds, or even, thousands, of spiders during specific periods of the year when there is an abundance of spiders and store the venom under appropriate conditions (*i.e.*, lyophilized or in solutions at −80 ℃) [18]. Alternatively, brown spiders could be captured from the wild and kept individually (because they kill one another) under laboratory conditions, using insect larvae as food and with periodic hydration via water-soaked cotton balls, with venom collected as necessary.

Another technical solution for venom production is the standardization of long-term primary culture of secretory cells from the venom gland and the production of venom *in vitro*. The culture of secretory cells from different venomous animals has shown promising results, and represents a good system with which to obtain toxins without capturing animals from the wild and without the related ecological disturbances. To date, several groups have reported expertise on this topic, and have established protocols for the primary culture of secretory cells. Examples include those from the venom glands of

Crotalus durissus terrificus and *Bothrops jararaca* snakes [215,216], as well as those from the venom glands of the *Phoneutria nigriventer* spider [217]. Such protocols ensure that sufficient amounts of native toxins are produced and secreted for culture medium and used for technical purposes after purification. Unfortunately, for *Loxosceles* venom gland cells, there are no reports to date of successful primary cultures of secretory cells. This situation represents a rational challenge for the future regarding the acquisition of sufficient amounts of native molecules. Finally, the venom of *Loxosceles* species is commercially available, as is the case for *L. deserta* (Sigma, St. Louis, USA).

The cDNA library construction of *L. intermedia* venom gland [35], transcriptome analysis [27,51] and the cloning and synthesis of several recombinant toxins [29,32–37,39,43,52] is helping to elucidate the biology of *Loxosceles* genus and opening possibilities for biotechnology applications. Recombinant toxins have been expressed in bacteria, simple organisms that are easy to manipulate and cheap to work with; unfortunately these do not generate co- and post-translational modifications such as disulphide bonds and protein glycosylations. Certain recombinant molecules are expressed in their unfolded form, have incorrected conformations, are water insoluble, and have no biological function.

With regard to phospholipase-D family members, these recombinant toxins purified from bacteria have biological functions compatible with those described for native toxins. For native toxins, it was already very well demonstrated that inflammatory response with cytokines release is induced at the bite site, and lipid content might be relevant for tissue damage [218,219]. These recombinant toxins induce dermonecrosis, platelet aggregation, increased vessel permeability, deep inflammatory responses, and phospholipase-D activity [34–37]. On the other hand, a great number of brown spider venom recombinant toxins synthesized by bacteria are water-insoluble and have no biological function. To surpass this technical obstacle, insoluble toxins can be refolded by methods of protein refolding [220], but the final concentration of refolded toxins obtained is generally not enough for biotechnological uses.

Alternatively, toxins can be synthesized using other expression models, such as the yeast *Pichia pastoris* [221], an organism that has subcellular organelles as endoplasmic reticulum and Golgi apparatus. This yeast is able to perform co- and post-translational modifications of proteins. For *Loxosceles* toxins, preliminary experiments are underway [75], but a frequent problem to be overcome is the hyperglycosylation of secreted proteins, which alters the biological functions of the toxins. Expression in systems of insect cells, such as *Drosophila* Schneider cells, is a possible alternative method [222] because it is a eukaryotic expression system, in which proteins undergo post-translational modifications.

For *Loxosceles* toxins, again, experiments are just beginning and results are preliminary [75], but they can provide secreted toxins that are correctly folded and, in the near future, may be used as tools for biological evaluations. Baculovirus vector for protein expression in insect and mammalian system is also feasible [223], but we do not have information on *Loxosceles* molecules produced using this technique. Finally, the mammalian expression system is a rational alternative for expression of correctly folded recombinant proteins. Mammalian cells have the capacity for proper protein folding and assembly, as well as co- and post-translational modifications [224]. Currently, there are no data on *Loxosceles* venom toxins obtained using this system. However, because this model is a viable method for recombinant proteins of therapeutic use, scientists are expected to explore this system in the future.

The advancement of *Loxosceles* venom toxin research will also involve techniques from proteomic analysis. These techniques generally have high sensitivity and accuracy and normally use low venom concentration for analysis. To date, at least two works have been completed addressing this topic. By using proteomics methodologies, such as bi-dimensional electrophoresis, N-terminal amino acid sequencing and mass spectrometry, eleven isoforms for phospholipase-D toxin were identified in *L. gaucho* venom [25]. In addition, through mass spectrometry analysis using *L. intermedia* crude venom, 39 proteins were identified, and putative effects for envenomation were discussed [24]. The use of combinatorial data from proteomic and molecular biology techniques, such as mass spectrometry, transcriptome analysis and cDNA library constructions, will open possibilities for the discovery of novel toxins in complex venoms [225].

Additionally, in the near future, the biotechnological use of *Loxosceles* toxins could provide information related to the tridimensional structure of identified toxins, through crystallography and X-ray diffraction and/or nuclear magnetic resonance for soluble toxins [59]. Findings in these areas will bring insight related to the molecular structure of toxins and will be very important for the discovery of catalytic sites, sites that interact with natural substrates or ligands, and from such data, synthetic ligands, analogs, or inhibitors could be designed for biotechnological purposes.

Regarding *Loxosceles* spider venom toxins, a recombinant phospholipase-D from *L. laeta* was analyzed by crystallography and X-ray diffraction. The data collected allowed description of the amino acid residues involved in catalysis and metal ion coordination important for sphingomyelinase activity [226]. Experiments using other isoforms of phospholipase-D from *L. intermedia* venom (LiRecDT1, LiRecDT2, LiRecDT6, GFP-LiRecDT1, and LiRecDT1H12A, with a mutation on the catalytic site, [46]) are currently being conducted using crystallography and X-ray diffraction. Additionally, other *Loxosceles* recombinant toxins (enzymes and peptides) could be evaluated and represent potential biological tools in a wide range of fields.

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Review

Recent advances in the understanding of brown spider venoms: From the biology of spiders to the molecular mechanisms of toxins

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ABSTRACT

The *Loxosceles* genus spiders (the brown spiders) are encountered in all the continents, and the clinical manifestations following spider bites include skin necrosis with gravitational lesion spreading and occasional systemic manifestations, such as intravascular hemolysis, thrombocytopenia and acute renal failure. Brown spider venoms are complex mixtures of toxins especially enriched in three molecular families: the phospholipases D, astacin-like metalloproteases and Inhibitor Cystine Knot (ICK) peptides. Other toxins with low level of expression also present in the venom include the serine proteases, serine protease inhibitors, hyaluronidases, allergen factors and translationally controlled tumor protein (TCTP). The mechanisms by which the *Loxosceles* venoms act and exert their noxious effects are not fully understood. Except for the brown spider venom phospholipase D, which causes dermonecrosis, hemolysis, thrombocytopenia and renal failure, the pathological activities of the other venom toxins remain unclear. The objective of the present review is to provide insights into the brown spider venoms and loxoscelism based on recent results. These insights include the biology of brown spiders, the clinical features of loxoscelism and the diagnosis and therapy of brown spider bites. Regarding the brown spider venom, this review includes a description of the novel toxins **revealed by molecular biology and proteomics techniques**, the data regarding three-dimensional toxin structures, and the mechanism of action of these molecules. Finally, the biotechnological applications of the

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venom components, especially for those toxins reported as recombinant molecules, and the challenges for future study are discussed.

1. Introduction

Spider bites of the genus *Loxosceles* have been associated with clinical manifestations characterized by dermonecrosis with gravitational spreading near the lesion site and, to a lesser extent, with systemic toxicity, such as the hematological disturbances of intravascular hemolysis, thrombocytopenia, disseminated intravascular coagulation and acute renal failure. The spiders of the genus *Loxosceles* are encountered in all continents and different species have been reported in North America, Central America, South America, Europe, Africa, the Middle East, Oceania and Asia. Five species (*Loxosceles rufescens*, *Loxosceles laeta*, *Loxosceles intermedia*, *Loxosceles gaucho* and *Loxosceles reclusa*) are responsible for most cases of human envenomation by the *Loxosceles* genus, and the pathology and clinical features of these spider bites are termed loxoscelism. Nevertheless, sporadic accidents caused by others *Loxosceles* species (*Loxosceles deserta*, *Loxosceles arizonica*, *Loxosceles anomala*, *Loxosceles similis*, for instance) have been described around the world (da Silva et al., 2004; Hogan et al., 2004; Swanson and Vetter, 2006; Bucaretchi et al., 2010; Isbister and Fan, 2011; Chatzaki et al., 2012).

The venom of the brown spider is a colorless and crystalline liquid, formed by a complex mixture of toxins enriched in proteins, glycoproteins and low molecular mass peptides with a predominance of toxins in the range of 5–40 kDa (Sams et al., 2001; da Silveira et al., 2002; da Silva et al., 2004; Machado et al., 2005; Swanson and Vetter, 2006). Previously published data have described highly expressed molecules, such as phospholipases D, astacin-like metalloproteases and low molecular mass insecticidal peptides (characterized as ICK peptides) (da Silva et al., 2004; de Castro et al., 2004; da Silveira et al., 2007a; Gremski et al., 2010; Matsubara et al., 2013). Together, these three toxin classes comprise the majority of the toxin-encoding transcripts in the venom gland of *L. intermedia* (approximately 95%) (Gremski et al., 2010). Other toxins with low level of expression, such as hyaluronidase, serine proteases, serine protease inhibitors, venom allergens and a TCTP family member, have been identified in the venom (de Castro et al., 2004; Barbaro et al., 2005; Gremski et al., 2010; Sade et al., 2012; Ferrer et al., 2013).

Regarding the hemolymph of brown spiders, no current description of its molecular composition, biological activities or even physical properties exists. Nevertheless, the potential of the hemolymph to contain natural inhibitors, antifungals or antibiotics is significant and is based on the environment in which the spiders live and the toxins that the brown spiders produce.

In recent years, knowledge of brown spider venoms has advanced significantly through the use of molecular biology techniques. The transcriptomes of the *L. laeta* and *L. intermedia* venom glands were analyzed for the first time, and this analysis confirmed the complexity of *Loxosceles* venoms (Fernandes-Pedrosa et al., 2008; Gremski et al., 2010). Additionally, by using recombinant DNA technology, heterologous toxins have been expressed and purified. These advances obtained with the recombinant *Loxosceles* venom toxins helped to overcome the obstacles to studying spider toxins, such as the low venom volumes and the difficulty in the purification of native toxins from crude venom. Moreover, these recent advances have enabled researchers to utilize cell biology, biochemistry, immunology, pharmacology and crystallography to clarify the general characteristics of *Loxosceles* toxins.

By using proteomics approaches, such as **two-dimensional gel** electrophoresis, N-terminal amino acid sequencing and mass spectrometry, the venoms of *L. gaucho and L. intermedia* have been investigated (Machado et al., 2005; dos Santos et al., 2009).

Recent advances in protein purification techniques, the application of different models for the synthesis of recombinant toxins, the modeling of domains, the knowledge of the binding or catalytic sites of the toxins of interest and, finally, the availability of the varied cellular and animal models for assessing the products obtained have created possibilities for a broad putative biotechnological use of brown spider venom toxins as important tools (Senff-Ribeiro et al., 2008; Gremski et al., 2010; Chaim et al., 2011a; Wille et al., 2013).

This review focuses on the most recent literature examining brown spider venom and loxoscelism. It discusses the molecular biology techniques used for the characterization of the molecules in brown spider venom, such as transcriptome projects, as well as the production and evaluation of recombinant toxins. Furthermore, it also describes the recent advances in the molecular complexity of venom toxins, and finally, it lists the putative biotechnological applications of several brown spider venom components.

2. Biology of brown spiders

The spiders of the *Loxosceles* genus belong to the *Sicariidae* family, sub-order *Labidognatha*, order *Araneida*, class *Arachnida*, and phylum *Arthropoda* (Platnick, 2013) (Fig. 1). In North America, this genus is popularly referred as recluse spiders, brown recluse spiders and violin spiders (fiddle back), due to a characteristic violin shape on the dorsal surface of the spider's cephalothorax. In South America, they are called brown spiders (da Silva et al., 2004; Vetter, 2008). The name *Loxosceles* means "*slanted legs*" because of the way the spider positions its legs at rest (Vetter, 2008). **Approximately 130 species** of the *Loxosceles* genus have been described and are extensively distributed worldwide (Platnick, 2013). The majority of these spiders are present in the Americas, West Indies and Africa, and some species

Fig. 1. Adult brown spiders. *Loxosceles gaucho* female (A) and male (E); *Loxosceles intermedia* female (B) and male (F); *Loxosceles laeta* female (C) and male (G). An adult brown spider and an ootheca (arrow) (D). The classic violin pattern (arrow) appears on the dorsal surface of the cephalothorax from *Loxosceles gaucho* adult spider (H). Photos are courtesy of Denise Maria Candido from the Instituto Butantan, São Paulo, Brazil. The colored figure refers to the on-line image. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

have been described in Mediterranean Europe and China (Binford et al., 2008). There is evidence that *Loxosceles* and *Sicarius* originated from a common sicariid ancestor on Western Gondwana, before the separation of the African and South American continents (Binford et al., 2008). Currently, in Brazil, 12 known species of spiders of this genus are present (Bertani et al., 2010; Gonçalves-de-Andrade et al., 2012).

They are small spiders, varying from 1 to 5 cm in length, including the legs. They exhibit sexual dimorphism, with females usually larger than males. The males have palps with modified tarsi with an additional structure specialized for the transfer of sperm, the spermophore (Gilbert and Raunio, 1997). The spiders of the *Loxosceles* genus possess six eyes arranged in non-touching pairs and a U-shaped pattern. This eye positioning has been described as the best means to identify brown spiders (da Silva et al., 2004; Appel et al., 2005; Vetter, 2008; Chaim et al., 2011a).

Brown spiders can survive several months without food or water and can withstand temperatures ranging from 8 to 43 °C (da Silva et al., 2004). The reported longevity for the *L. intermedia* is 1176 ± 478 days for females and 557 ± 87 days for males (Fischer and Vasconcellos-Neto, 2005a). They construct irregular webs that resemble cotton threads (da Silva et al., 2004). Other studies indicated the preference for dead prey (Sandidge, 2004; Fischer and Vasconcellos-Neto, 2005b), although this preference depends on the size and freshness of the live or dead prey (Cramer, 2008; Vetter, 2011a).

The hemolymph of arachnids and insects has many important functions. It participates in homeostatic processes (Ruppert et al., 2004) in the transport of hormones, enzymes and nutrients, as well as metabolic residues for excretion; in the animal's protection; and in the storage of water and lipids (Araújo, 2009).

Although spiders are arachnids and not insects, insecticides are effective in reducing the brown spider population. Many chemicals may not kill the spider but will disrupt the nervous system and other bodily functions (Sandidge and Hopwood, 2005). Lindane and chlordane are insecticides identified as effective, lethal substances to *Loxosceles* spiders, however, these products have carcinogenic effects and, therefore, are no longer used for spider population control (Navarro-Silva et al., 2010). The use of pyrethroids for spider population control has also been evaluated in the field and the laboratory by testing the susceptibility of *L. intermedia* specimens to this class of insecticides. In laboratory tests, microencapsulated lambda-cyhalothrin (ME lambda-cyhalothrin) demonstrated the highest toxicity. A field test confirmed these laboratory results, and the authors concluded that ME lambda-cyhalothrin would be useful in integrated pest management programs for *L. intermedia* (Navarro-Silva et al., 2010). Sandidge (2004) investigated the potential to biologically control *L. reclusa* using the natural arachnid fauna found in most homes. Three common web-building cosmopolitan spiders, *Achaearanea tepidariorum*, *Steatoda triangulosa* and *Pholcus phalangioides*, readily feed on brown recluse spiders and are deemed beneficial in the control of these populations; most importantly, they are relatively harmless to humans. Additionally, vacuum cleaner use in the home has been considered as an effective tool for the integrated management of *L. intermedia* and other spider populations (Ramires et al., 2007). Furthermore, tolerance to the presence of geckos at home, which are considered a natural predator of spiders, has also been considered a promising tool (Ramires and Fraguas, 2004).

3. Clinical features of loxoscelism

The number of loxoscelism cases worldwide is likely underestimated because most cases are not reported (da Silva et al., 2004; Hogan et al., 2004; Dyachenko et al., 2006; Abdulkader et al., 2008; Makris et al., 2009; Pippirs et al., 2009; Pernet et al., 2010; Bajin et al., 2011; Lane et al., 2011; Sanchez-Olivas et al., 2011; Huguet et al., 2012; Ribuffo et al., 2012). Notwithstanding their prevalence as an underreported condition, *Loxosceles* spider bites are considered a public health problem in countries such as Brazil, Chile and Peru because of their frequency and associated morbidity (da Silva et al., 2004; Hogan et al., 2004; Zambrano et al., 2005; Swanson and Vetter, 2006; de Souza et al., 2008; Manríquez and Silva, 2009; Vetter, 2009; Isbister and Fan, 2011; Malaque et al., 2011). As shown in Table 1, since 2001, Brazil has experienced a progressive increase in the number of reported loxoscelism cases. It is currently estimated that approximately 8000 spider bites occur annually, and most of them are reported in the southeastern and southern urban areas of Brazil. The increase in loxoscelism reports in recent years could be a consequence of an ecological imbalance caused by the deforestation and extinction of natural predators, climate change, and pest management practices, which results in the adaptation of spiders to the urban environment (da Silva et al., 2004; Hogan et al., 2004; Swanson and Vetter, 2006; Isbister and Fan, 2011; Saupe et al., 2011; Vetter, 2011a).

Spiders displaced from their natural environment end up inhabiting the breaches, fissures and orifices of human houses. They also seek shelter in storage boxes or in the corners of cupboards and drawers. Accordingly, they can be found inside clothes, towels and bedclothes. As a result,

Table 1
Notifications of *Loxosceles* accidents occurred in Brazil from 2001 to 2012.

Year	Notifications	Deaths	% (deaths/notifications)
2012	7528	2	0.03
2011	8033	6	0.08
2010	7885	2	0.03
2009	8472	2	0.02
2008	7977	3	0.04
2007	9277	12	0.13
2006	7619	5	0.07
2005	7702	3	0.04
2004	8207	1	0.01
2003	7806	3	0.04
2002	6303	3	0.05
2001	5011	5	0.01

http://dtr2004.saude.gov.br/sinanweb/index.php?name=Tnet.
http://dtr2004.saude.gov.br/sinanweb/tabnet/dh?sinan/animaisp/bases/animaisbr.def.
Source: Brazilian Ministry of Health - SINAN/SVS:

most spider bites occur when the victims press the spider against their body; for that reason, victims are most frequently bitten on the trunk, thigh and arm. The fangs of the *Loxosceles* spider are small, and the venom is likely injected by intradermal injection. The spider bites mainly occur during the warmest seasons (spring and summer) (da Silva et al., 2004; Hogan et al., 2004; Isbister and Fan, 2011; Vetter, 2011b; Rader et al., 2012).

Loxosceles bites lead to a mild stinging with clinical signs and symptoms developing only several hours afterward; consequently, the bite is barely noticed, and the spider is rarely captured (~10% of cases) at the time of the bite. Therefore, the diagnosis of loxoscelism is usually presumptive and based on the clinical and epidemiological features presented by the patient at the time of hospital admission, which usually occurs from 12 to 24 h after the bite, when the skin damage is more prominent (da Silva et al., 2004; Hogan et al., 2004; Hubbard and James, 2011; Isbister and Fan, 2011). The classical clinical symptoms caused by *Loxosceles* spider bites are characterized by an intense inflammatory reaction at the bite site followed by local necrosis and can be classified as cutaneous loxoscelism (more than 70% of the cases). The cutaneous loxoscelism is characterized by initial local symptoms, such as burning-stinging, undefined pain, and edema. Most of the time, the patients seek help only several hours after the bite when the signs and symptoms become more severe, such as burning pain, edema, blister formation, erythema, ecchymosis, and paleness (called marble plaques) (Fig. 2 A–C). After several days, a necrotic area forms, which is followed by an ulcer of variable size that scabs over and frequently leaves a sharply defined area surrounded by the raised edges of healthy skin (Fig. 2 D). These necrotic wounds can take several weeks to heal. Other symptoms, such as a scarlatiniform or morbilliform rash, malaise, nausea, vomiting, a low-grade fever or headache, can also occur (da Silva et al., 2004; Hogan et al., 2004; Isbister and Fan, 2011).

Apart from the venom, many other factors associated with the spider (intra- and inter specific variations, the developmental stage and the amount of venom injected) or the patient (the adipose tissue at the bite site, the amount of tissue sphingomyelin, the patient's age and the individual genetic variance) can influence the severity of the bite (da Silva et al., 2004; Hogan et al., 2004; de Oliveira et al., 2005; Tambourgi et al., 2010). For instance, terminal circulation areas or adipose tissue are more sensitive to the venom's action, developing necrosis and severe tissue injury, which may require corrective plastic surgery. Despite the severity of the injury caused by *Loxosceles* venom at the bite site, the development of secondary infections is rare (Hogan et al., 2004; Abdulkader et al., 2008; Isbister and Fan, 2011; Malaque et al., 2011; Huguet et al., 2012; Ribuffo et al., 2012).

Severe cases can be classified as viscerocutaneous or systemic loxoscelism and range from 0.7% to 27% varying geographically or by the *Loxosceles* species responsible for the spider bite (Barbaro and Cardoso, 2003; Hogan et al., 2004; Abdulkader et al., 2008; Isbister and Fan, 2011). For instance, some data have demonstrated that viscerocutaneous loxoscelism has a higher prevalence in several countries, such as Chile (15.7%) and Peru (27.2%), as well as in Santa Catarina state/Brazil (13.1%), where *L. laeta* is found (da Silva et al., 2004; Hogan et al., 2004).

Systemic loxoscelism is characterized by hematuria, hemoglobinuria, jaundice, fever, nausea and disseminated intravascular coagulation (da Silva et al., 2004; Hogan et al., 2004; Isbister and Fan, 2011). Recently, Malaque et al. (2011) found that mild hemolysis is frequent in patients bitten by *L. gaucho* (present in one-third of the cases examined, including those classified as cutaneous loxoscelism). However, acute kidney injury occurred exclusively in patients with extensive hemolysis. Oliguria and dark urine, which can suggest extensive intravascular hemolysis or rhabdomyolysis, can result in acute renal failure, which is the primary cause of death associated with loxoscelism. Nevertheless, the level of mortality (Table 1) caused by *Loxosceles* spider bites is low (França et al., 2002; da Silva et al., 2004; Hogan et al., 2004; Abdulkader et al., 2008; de Souza et al., 2008; Isbister and Fan, 2011; Malaque et al., 2011). Although large case studies report systemic loxoscelism across all age groups, most cases are reported in children (Schenone et al., 2001; Hostetler et al., 2003; da Silva et al., 2004; Hogan et al., 2004; Elbahlawan et al., 2005; Hubbard and James, 2011; Isbister and Fan, 2011; Taskesen et al., 2011; Rosen et al., 2012).

4. Diagnosis and therapy

No consensus treatment for loxoscelism exists, and different therapies are used in different parts of the world. In some countries, the therapy is based on dapsone, antihistamines, analgesics and corticosteroids. However, other treatments, such as the use of steroids, surgical excision, hyperbaric oxygen therapy, and negative pressure wound therapy (vacuum-assisted closure), have been employed in an attempt to remedy the effects of envenomation (da Silva et al., 2004; Hogan et al., 2004; Swanson and Vetter, 2005; Tutrone et al., 2005; Swanson and Vetter, 2006; Abdulkader et al., 2008; Vetter and Isbister, 2008; Wong et al., 2009; Tambourgi et al., 2010; Andersen et al., 2011; Hubbard and James, 2011; Isbister and Fan, 2011). Another treatment option is the administration of antivenoms against the *Loxosceles* venoms, which are available in Brazil, Argentina and Mexico [horse-derived F(ab′)2 antivenoms] and Peru (whole IgG antivenom) (Isbister et al., 2003; da Silva et al., 2004; Hogan et al., 2004; Pauli et al., 2009; Isbister and Fan, 2011). The antivenom is administered intravenously, and the number of vials used varies according to the clinical severity of the envenomation (cutaneous loxoscelism) present at hospital admission and is administered to all patients with viscerocutaneous loxoscelism (Brasil, 2001).

The delay in seeking medical care by patients (approximately 24 h after the spider bite) can further contribute to the extension of local tissue damage at the bite site because the cutaneous necrosis and systemic clinical symptoms induced by the venom are irreversible and begin a few hours after envenomation (da Silva et al., 2004; Hogan et al., 2004). Accordingly, the type and effectiveness of the treatment are influenced by the amount of time between the spider bite and the diagnosis.

Fig. 2. Cutaneous loxoscelism. A – A female patient bitten on the thigh. The lesion (2 days post-bite) is characterized by edema and erythema, paleness and hemorrhagic areas (marble plaque about 14 cm in diameter), and blistering with hemorrhagic content. B – Male patient bitten on the calf. Two days after spider bite, the injury appeared with extensive bruising, serous blisters that progressed rapidly to hemorrhagic content and burning pain with the additional presence of a cutaneous rash, myalgia and dizziness. Twenty days after bite, the patient presented with desquamation at the injury site without ulcer formation. C and D – Male patient bitten on the inner left thigh while wearing clothes. Four days (C) after the bite, local damage is present and characterized by edema and erythema with ecchymotic, paleness and hemorrhagic areas (marble plaque) and the presence of necrosis 20 days after the bite (D). The presumptive cutaneous loxoscelism because the patient did not bring the spider for formal identification. [Photos are courtesy of Dr. Marlene Entres, Centro de Controle de Envenenamentos de Curitiba, Secretaria de Estado da Saúde, Paraná, Brazil (A, B), and Dr. Ceila M. S. Malaque, Hospital Vital Brazil – Instituto Butantan, São Paulo, Brazil (C, D)]. The colored figure refers to the on-line image. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

However, the effectiveness of the treatments described in the international literature has been widely debated, and a definitive treatment has not yet been established (Isbister et al., 2003; da Silva et al., 2004; Hogan et al., 2004; Pauli et al., 2009; Isbister and Fan, 2011). The bite severity, however, can be estimated by laboratory tests and clinical characteristics, such as evidence of hemolysis (Malaque et al., 2011) and the presence of creatine kinase in the serum, which indicates rhabdomyolysis (França et al., 2002), that can determine the presence of a viscerocutaneous manifestation of envenomation. In these cases of systemic loxoscelism, vigorous hydration and urinary alkalinization should be established early to avoid pigment-induced renal failure (Hogan et al., 2004).

Currently, no commercial test is available to confirm loxoscelism, and most patients never see or capture the offending spider; consequently, the diagnosis is usually based on the clinical features presented by the patient. Moreover, the epidemiological information, such as the circumstances of the bite (sleeping, dressing, etc.), the site of the bite, and the timing of the injury progression (because the clinical signs and symptoms of *Loxosceles* envenomation occur slowly), can aid in the diagnosis of loxoscelism. There are many other medical causes of focal skin necrosis (as described previously by literature data) and laboratory tests can be helpful in determining the presence of these other diseases. In addition, an enzyme-linked immunosorbent assay has been used to detect the venom from patient lesions and sera or the circulating antibodies to the venom, but it is not in widespread clinical use (Gomez et al., 2001, 2002; da Silva et al., 2004; Hogan et al., 2004; Stoecker et al., 2006; Akdeniz et al., 2007; Stoecker et al., 2009).

5. Brown spider venom

Loxosceles spider venom is a colorless and crystalline liquid produced from two bulbous glands situated in the cephalothorax of the spider and flows through an inoculator apparatus composed of a pair of chelicerae (dos Santos et al., 2000; da Silveira et al., 2002; da Silva et al., 2004). Histological findings have revealed that these glands are made up of two adjacent layers of striated muscles fibers, one external and the other internal, in contact with an underlying basement membrane that separates the muscle cells from the secretory epithelium and use a holocrine secretion mechanism (dos Santos et al., 2000).

The volume of venom produced by *Loxosceles* spiders is generally on the order of a few microliters, and it contains approximately 20–200 μg of total protein (Binford and

Wells, 2003; da Silva et al., 2004; Senff-Ribeiro et al., 2008). The amount and the content of the venom produced depend on several factors associated with the spider specimen, including species, size, sex, nutritional state and age. Using SDS-PAGE analysis, de Oliveira et al. (2005) showed that significant variations occurred between the content of the *L. intermedia* and *L. laeta* venoms. These variations in the venom content can be enhanced by other differences in their biological activities, such as the more potent dermonecrotic activity (measured by the lesion size) of *L. laeta* venom compared with *L. intermedia* venom observed in rabbits. In addition, the lesions caused by venom from females were larger in area than those lesions caused by venom from males (de Oliveira et al., 2005). Through 2D electrophoresis (IEF and SDS-PAGE), the venom of *L. intermedia* has been found to be enriched mainly in two groups of proteins at 20–40 kDa and 2–5 kDa (Fig. 3).

In vivo experiments using a rabbit model have shown that *Loxosceles* spp. venoms are associated with the development of a characteristic dermonecrotic lesion with gravitational spreading and ecchymosis. Analyses of rabbit skin exposed to *Loxosceles* venoms shown the following characteristics: an initial edema under the dermis, an increased vascular permeability, an intravascular fibrin network deposition, the thrombosis of dermal blood vessels and the degeneration of the blood vessel walls as well as the infiltration and aggregation of inflammatory cells. At longer exposure times, myonecrosis of the myofibrils and leukocyte infiltration in the skeletal muscle occur. Finally, the destruction of epidermis integrity, massive hemorrhage and the necrosis of surrounding collagen near the epidermis are observed (Ospedal et al., 2002; Tavares et al., 2004; Pretel et al., 2005; Silvestre et al., 2005; Chatzaki et al., 2012). The ability of the venom of *Loxosceles* spiders to be lethal to mice has also been described. Mota and Barbaro (1995) reported this lethality in mice injected with *L. intermedia*, *L. gaucho* and *L. laeta* venoms, and the LD_{50}s determined were 0.48, 0.74 and 1.45 mg/kg, respectively. Appel et al. (2008) found 100% mortality of the mice tested at the concentrations of 50 and 100 μg/kg of *L. intermedia* venom after 16 h post-injection. Silvestre et al. (2005) found an LD_{50} of 0.32 mg/kg for *L. similis* venom, and Pretel et al. (2005) indicated an LD_{50} of 0.696 mg/kg for *Loxosceles adelaida* venom.

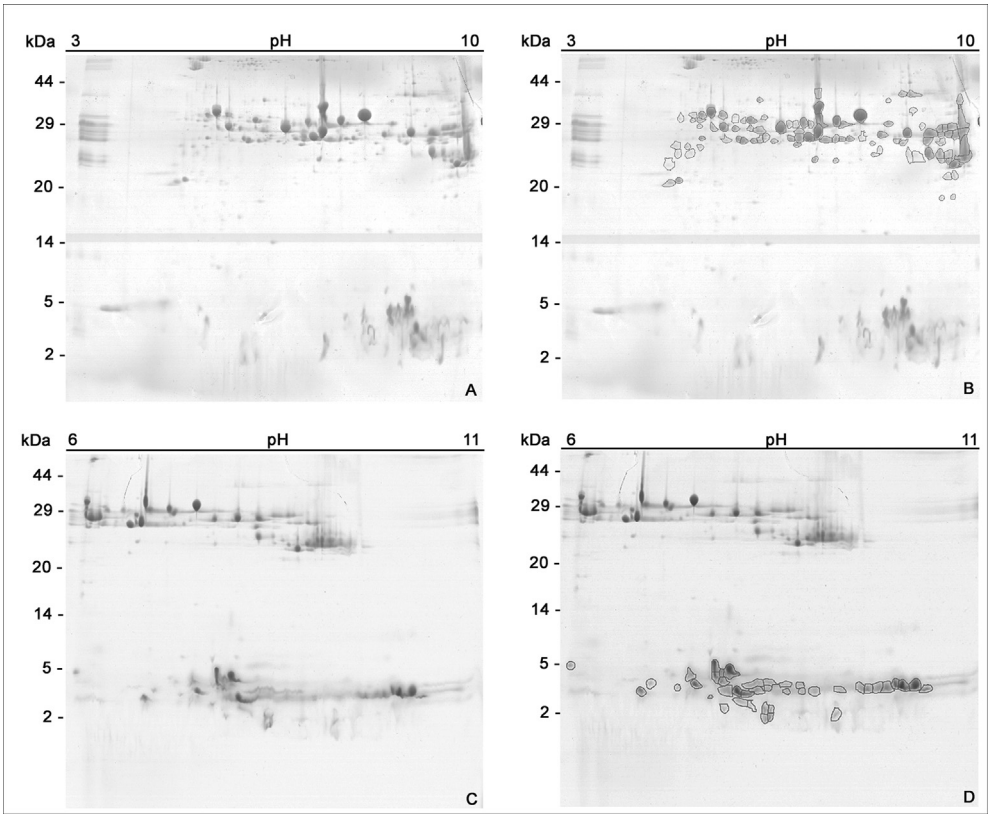

Fig. 3. Two-dimensional (2-DE) protein profile of *Loxosceles intermedia* venom. Samples (150 μg) of *Loxosceles intermedia* crude venom were separated by isoelectric focusing (IEF) on 13-cm immobilized pH gradient (IPG) gel strips with a pH linear range of 3–10 or a pH linear range of 6–11 (GE Healthcare, Piscataway, NJ, EUA) in the first dimension. The second dimension analysis was performed on a 20% polyacrylamide SDS-PAGE gel under reducing conditions. The 2-DE gels were stained with colloidal Coomassie Brilliant Blue. (A) Then, the gels were maintained in a 1% acetic acid solution and scanned using an ImageScanner III LabScan 6.0 (GE Healthcare). The detection of the gel spot and calculation of the isoelectric point (pI) and molecular mass (MM) were obtained using Image Master 2D Platinum software (GE Healthcare). (A) The protein profile of *L. intermedia* venom separated on a pH linear range of 3–10. (B) In total, 97 spots were detected in the 20–40 kDa region, and the spots with pI values from 4.6 to 9.8 are highlighted in circles. (C) The protein profile of *L. intermedia* venom separated on a pH linear range of 6–11. (D) 40 spots were detected in the 2–5 kDa region, and the spots with pI values from 6.1 to 10.4 are highlighted in circles.

The full venom content of the *Loxosceles* spiders is still under investigation; however, many studies have shown that its biochemical composition consists of a complex mixture of biologically active compounds, mainly proteins and peptides with toxic and/or enzymatic action (Veiga et al., 2000a; Gremski et al., 2010). HPLC analysis of whole venom of *L. intermedia* showed the presence of histamine. According results in a sufficient concentration to induce inflammatory responses (Paludo et al., 2009). Additionally, NMR spectroscopy and mass spectrometric analyses of *L. reclusa* crude venom pointed for the presence of sulfated guanosine derivatives as the major small-molecule components of the venom. Nevertheless, results were restricted to chemical analyses and data did not describe for a correlation with biological/pathological activities for these molecules (Schroeder et al., 2008).

In the following sections, the main identified and characterized molecules in the venom of *Loxosceles* spiders will be presented as well as the relevance of these toxins to the understanding of the envenomation process and potential biotechnological applications.

5.1. Proteomic analysis of brown spider venom

Proteomic analyses of brown spider venom are scarce and, in general, are focused on the phospholipase D family members. The first report using mass spectrometry for the identification of the proteins in *Loxosceles* spider venom was in 2003 (Binford and Wells, 2003). The aim of this study was to identify the spider phylogenetic groups with sphingomyelinase and to identify the evolutionary origin of this toxin. Venoms from distinct spiders were analyzed, including *L. laeta* and 9 other *Loxosceles* species from Africa and North America. Sphingomyelinase D (SMD) activity was identified in all surveyed *Loxosceles* species and in two *Sicarius* species (*Loxosceles* sister taxon), and the mass spectrometry analyses found several molecules corresponding to the known SMD size range of 31–35 kDa. The *Loxosceles* venom was first submitted to two-dimensional electrophoresis in 2004 by Luciano and colleagues. They demonstrated that *L. intermedia* venom is enriched in cationic and low molecular mass **proteins** (20–35 kDa). Shortly thereafter, the venoms from *L. adelaida* and *L. gaucho* were subjected to 2D electrophoresis analysis (Pretel et al., 2005). Although the toxins of these venoms displayed a similar distribution with regard to the molecular mass of proteins, only 40% of the components exhibited the same *pI* and molecular mass in the *L. adelaida* and *L. gaucho* venoms. *L. similis* venom was also analyzed by 2D gel electrophoresis and exhibited protein bands ranging from 28 to 112 kDa, and the *pI* values were between 4.0 and 7.0, which matched previous profiles of other *Loxosceles* species (Silvestre et al., 2005).

In 2005, the protein contents of the *L. gaucho*, *L. laeta* and *L. intermedia* venoms were analyzed using 2D electrophoresis. The protein profiles of these three different venoms were similar, possessing the majority of protein bands in the 30–35 kDa range. All *Loxosceles* species presented protein bands of a high molecular mass (45–94 kDa) and exhibited few proteins in the low molecular mass region (14–25 kDa) (Machado et al., 2005).

To identify the dermonecrotic proteins in *L. gaucho* venom, several protein bands present in the 30–35 kDa range after separation by 2D electrophoresis were analyzed using mass spectrometry de novo sequencing combined with N-terminal chemical sequencing. Only eight spots were identified as sphingomyelinase D (Machado et al., 2005). The low abundance of all other protein bands analyzed did not enable their identification. In addition, by LC-MS analysis, 11 distinct proteins were detected in the molecular mass range of the dermonecrotic toxins, suggesting that more isoforms of sphingomyelinase D could be present in *L. gaucho* venom (Machado et al., 2005). The difficulties of protein identification in brown spider venoms using MS approaches are due to the limited amount of *Loxosceles* protein sequences available in online data banks. Thus, sequencing the *Loxosceles* genome is still an ongoing challenge in loxoscelism research that will help guide future studies in this area. More recently, a proteomic analysis was performed using the *L. intermedia* venom by MudPiT (Multidimensional protein identification technology). This approach allowed the identification of 39 proteins; 14 proteins were grouped as toxins generally found in animal venoms and were considered responsible for the tissue damage observed in loxoscelism (dos Santos et al., 2009).

Thus, mass spectrometry and proteomic analysis are underused in the investigation of brown spider toxins. These approaches could be applied in many studies, such as a complete analysis of the protein content of *Loxosceles* venoms to generate a *Loxosceles* protein data bank and the identification of the post-translational modifications of the to toxins. In addition, these techniques could be helpful in analyzing the *in vivo* effects of *Loxosceles* crude venom or a specific recombinant toxin on certain tissues by examining the protein content after treatments.

5.2. Molecular biology approaches for studying brown spider venom

The advent of molecular biology has allowed the development of numerous novel techniques and applications that have enriched the knowledge in many scientific fields. Specifically, molecular biology has introduced new approaches for studying venoms and insights into their mechanisms of action.

One of the most successful tools applied in the study of the *Loxosceles* venoms is undoubtedly the cloning and heterologous expression of recombinant toxins, which circumvent the difficulties presented by the low amounts of venom that can be collected from these spiders (Senff-Ribeiro et al., 2008; Catalan et al., 2011). Fernandes-Pedrosa et al. (2002) published the first report of the cDNA cloning and expression of a *Loxosceles* toxin, in which a functional phospholipase D (SMase I) obtained from a cDNA library of *L. laeta* venom glands was expressed. Thereafter, several recombinant phospholipases D of other *Loxosceles* species were produced and allowed for the complex biological and biochemical characterization of these toxins (Kalapothakis et al., 2002; Araújo et al., 2003; Lee and Lynch, 2005; Tambourgi et al., 2005; Chaim et al., 2006; da Silveira et al., 2006, 2007b; Olvera et al., 2006;

Ribeiro et al., 2007; Appel et al., 2008; Christoff et al., 2008; de Almeida et al., 2008; Kusma et al., 2008; Chaves-Moreira et al., 2009; de Santi Ferrara et al., 2009; Paludo et al., 2009; Catalan et al., 2011; Chaim et al., 2011b; Chaves-Moreira et al., 2011; Zobel-Thropp et al., 2012; Vuitika et al., 2013; Wille et al., 2013). Recently, two recombinant phospholipases D (recLiD1 and LiRecDT1) were developed as tools to assay the sphingomyelinase D activity in crude venoms or recombinant enzymes (Gomes et al., 2011). Concurrently, these techniques also revealed that phospholipases D comprise a family of toxins in *L. intermedia* venom, as several isoforms were described (Chaim et al., 2006; da Silveira et al., 2006; da Silveira et al., 2007b; Appel et al., 2008; Vuitika et al., 2013; Wille et al., 2013).

Site-directed mutagenesis of recombinant *Loxosceles* phospholipases D allowed for the production of recombinant molecules with drastically decreased enzymatic activity used as tools to elucidate the major role of the catalytic activity of this enzyme's toxicity (Lee and Lynch, 2005; Kusma et al., 2008; Chaim et al., 2011b; Chaves-Moreira et al., 2009).

In addition, the recombinant toxins allowed for the resolution of the crystal structures of two *Loxosceles* phospholipases D, SMase I from *L. laeta* (class I PLD) and LiRecDT1 from *L. intermedia* (class II PLD) (Murakami et al., 2005; de Giuseppe et al., 2011; Ullah et al., 2011). These studies were fundamental in the understanding of the toxins' catalytic mechanisms.

Recombinant toxins could be used in animal immunization, thus eliminating the need of spiders as a source of venom (Gutiérrez et al., 2011). Recombinant phospholipases D have already been tested as antigens for the development of a polyvalent antivenom, which is effective in the neutralization of the crude venom and for understanding of antigenicity of toxins (Alvarenga et al., 2003; Olvera et al., 2006; de Moura et al., 2011; Mendes et al., 2013).

Although the vast majority of studies focus on producing phospholipases D as recombinant molecules, more recent studies set out to produce other recombinant *Loxosceles* toxins. For instance, a recombinant metalloprotease from *L. intermedia* venom has been produced and characterized (da Silveira et al., 2007a). Additionally, a recombinant hyaluronidase from *L. intermedia* venom was recently produced and used to demonstrate the role of this toxin in the venom (Ferrer et al., 2013). Moreover, a TCTP member-family toxin (Sade et al., 2012) and an Inhibitor Cystine Knot peptide (Matsubara et al., 2013) have also been cloned and expressed and will enable functional and structural studies to further characterize these poorly studied brown spider venom toxins. As will be further discussed below, these recombinant toxins enabled additional insights into loxoscelism and will also be putatively useful as tools for a variety of biotechnological applications.

The evolutionary aspects concerning both *Loxosceles* specimens and phospholipase D toxins have been thoroughly investigated by employing molecular biology techniques, such as cDNA, rRNA and genomic sequencing, analyses of positive selection, structural modeling of amino acid conservation and phylogenetic analyses (Binford et al., 2005, 2008; 2009; Cordes and Binford, 2006; Duncan et al., 2010).

Thus, these molecular biology techniques underlie the recent advances in the understanding of the *Loxosceles* toxins that occurred in the last few decades. Novel technologies not yet applied specifically to the study of *Loxosceles* toxins are promising, such as quantitative PCR, RNA interference and the expression of recombinant toxins in eukaryotic cells. Molecular biology approaches not only expand the knowledge of spider biology and the pathophysiology of loxoscelism but also reveal novel molecules for biotechnological approaches.

6. Highly expressed toxin families

Over the last few years, several studies focusing on the expression profiles of venomous glands of various organisms, such as snakes, scorpions and spiders, have been conducted. As expected, most of the profiles showed the prevalence of the toxin families that have a direct role in the main signs and symptoms observed in envenomation with these animals. In addition, some profiles showed that the highly expressed toxins are mainly involved in the mechanisms of prey capture for feeding purposes (Zhang et al., 2010; Rokyta et al., 2011; Ma et al., 2012).

In *Loxosceles* spider venom, the transcriptome analysis expression profiles of the venomous glands of different species showed different profiles of the highly expressed toxin families. On one hand, Fernandes-Pedrosa et al. (2008) reported the prevalence of transcripts coding for phospholipase D toxins in the *L. laeta* venomous gland. Subsequently, Gremski et al. (2010) affirmed that in the *L. intermedia* venomous gland, transcripts coding for ICK peptides were prevalent. This observation is consistent with the fact that the primary role of brown spider venom, as in all arachnids, is to paralyze or kill envenomed prey. Hypotheses supporting the differences in the expression levels of these toxins have been discussed previously (Gremski et al., 2010).

Both the *L. laeta* and *L. intermedia* transcriptomes exhibit high expression of the phospholipase D (PLDs) and metalloprotease toxin families in the venomous glands. In fact, PLDs (referred to as sphingomyelinases D by Fernandes-Pedrosa et al., 2008) are able to reproduce the major symptoms of loxoscelism. Moreover, a recent study demonstrated that PLDs also possess a potent insecticidal activity (Zobel-Thropp et al., 2012). Thus, based on the known activities of *Loxosceles* PLDs in vertebrates and arthropods, it is not surprising that this toxin family is highly expressed in brown spider venom glands.

Metalloproteases are also highly expressed toxins in *L. laeta* and *L. intermedia* venom glands (Fernandes-Pedrosa et al., 2008; Gremski et al., 2010). They comprise a family of venom enzymes and may be involved in the initial digestion of prey. These toxins may also have a role in the hemorrhaging observed in loxoscelism and in the systemic spreading of other toxins in victims (da Silveira et al., 2007a; Trevisan-Silva et al., 2010).

Analyses of venom gland expression profiles reveal a consistent redundancy of transcripts coding for the toxins that are highly expressed (Cidade et al., 2006; Neiva et al., 2009). Functional redundancy in proteins is a rare phenomenon; venoms represent a rare case of this

phenomenon (Morgenstern and King, 2013). Some authors argue that to maintain effective toxins against prey and predators, the genes encoding venom peptides and proteins underwent multiple duplication events. In turn, the duplicated genes acquired related or even novel functions through adaptive evolution (Ma et al., 2012). In fact, because these highly expressed toxins are often related to the venom's main actions, the genes encoding these toxins can be assumed to more likely undergo multiple duplication events, generating redundancy.

6.1. Phospholipases D

The phospholipase D (PLD) family of toxins is the most studied and well-characterized component in the *Loxosceles* species venoms. These molecules have been reported to play an important role in the development of clinical sign and symptoms in loxoscelism. Due to their ability to trigger dermonecrosis *in vivo*, the brown spider PLDs are also known as dermonecrotic toxins (da Silva et al., 2004; Appel et al., 2005; Swanson and Vetter, 2006).

Dermonecrotic toxins are soluble in water or physiological buffers and active enzymes are secreted by the brown spider venom glands. These molecules catalyze the hydrolysis of phospholipids, such as sphingomyelin, at a terminal phosphodiester bond to release choline and produce ceramide 1-phosphate (C1P) (da Silva et al., 2004; Chaim et al., 2011b; Wille et al., 2013). PLDs are also able to hydrolyze lysophosphatydilcholine in a Mg^{+2} dependent-manner (van Meeteren et al., 2004; Chaim et al., 2011b; Horta et al., 2013; Wille et al., 2013). These toxins are proteins which vary in molecular mass from 30 to 35 kDa, and include a signal peptide followed by a propeptide. The amino acid sequences of PLDs are highly conserved (55–99%), especially in the residues around the catalytic cleft. Based on phylogenetic studies, PLDs have been distributed in six different groups of the Loxtox family (*Loxosceles* toxin) (Chaim et al., 2006; Kalapothakis et al., 2007). In the same vein, Binford et al. (2009) have proposed a new nomenclature based on the evolution and phylogenetics of the PLD genes, termed the SicTox family (Sicariidae Toxin).

Gremski et al. (2010) revealed that 9% of the analyzed transcripts from the *L. intermedia* venom gland corresponded to PLDs, comprising 20.2% of all the toxin-encoding ESTs (**Expressed Sequence Tags**), which is a very significant proportion of the toxins. For *L. laeta*, the content of the PLD-encoding transcripts was present at higher levels (16.3% of all ESTs present in database hits). However, the transcriptome analysis for *L. laeta* had methodological limitations due to using only female specimens, which are already known to produce a greater quantity of venom when compared with male spiders (Fernandes-Pedrosa et al., 2008; Gremski et al., 2010). Moreover, the *L. intermedia* transcriptome analysis criteria for bioinformatics screening provided new putative isoforms of PLD (Vuitika et al., 2013), which can be included as novel groups in the LoxTox family (Kalapothakis et al., 2007). These data corroborate the findings of Machado et al. (2005), who identified at least 11 PLD isoforms in the venom of *L. gaucho*, termed Loxnecrogin, or data reported by Wille et al. (2013), which showed by 2D electrophoresis at least 25 spots immunologically related to PLD toxins in the *L. intermedia* crude venom.

Several PLD isoforms were also characterized in the venom of other *Loxosceles* species. In *L. reclusa* venom, the native PLDs were present at molecular mass of approximately 32 kDa, and the four active isoforms were characterized as able to induce dermonecrotic lesions, hemolysis, and platelet aggregation (da Silva et al., 2004; Vetter, 2011a, 2011b). Two PLD isoforms, SMase I (32 kDa) and SMase II (35 kDa), were also described in *L. laeta* venom, which experimentally demonstrated complement-dependent hemolysis, dermonecrosis and hydrolysis of sphingomyelin (Fernandes-Pedrosa et al., 2002; de Santi Ferrara et al., 2009). Catalan et al. (2011) reported two new PLD isoforms in *L. laeta*, rLIPLD1 was dermonecrotic and active on sphingomyelin while rLIPLD2 seemed to be inactive; but rLIPLD2 was cloned and expressed and was missing a large portion of the PLD region, i.e., it did not include the initial amino acids of the catalytic site, such as His^{12}. From *L. intermedia* venom, many PLD isoforms have been described, and nine isoforms have already been expressed as recombinant proteins. It has been shown that recombinant isoforms of PLD are able to reproduce most of the toxic effects observed in loxoscelism and antigenic properties of the venom (Kalapothakis et al., 2002; Fernandes-Pedrosa et al., 2002; Chaim et al., 2006; da Silveira et al., 2006; da Silveira et al., 2007b; Appel et al., 2008; Vuitika et al., 2013). Several isoforms of PLD were also very well characterized and cloned from the venom of other *Loxosceles* species (Ramos-Cerrillo et al., 2004; Barbaro et al., 2005; Magalhães et al., 2013).

The PLDs are responsible for a large variety of disturbances in loxoscelism. Both native and recombinant forms of PLDs have been reported to trigger dermonecrotic lesions, an increase in vascular permeability, an intense inflammatory response at the inoculation site and at a systemic level, platelet aggregation, hemolysis, nephrotoxicity, and even lethality in controlled experiments (Cunha et al., 2003; Appel et al., 2005; da Silveira et al., 2006, 2007b; Swanson and Vetter, 2006; Kusma et al., 2008; Senff-Ribeiro et al., 2008; Chaves-Moreira et al., 2009; Tambourgi et al., 2010; Chaim et al., 2011b).

Toxicity to a variety of cell types and structures is often enzyme dependent. At the beginning of the Loxtox protein family characterization, it was thought that these toxins were exclusively able to cleave the head-groups of sphingomyelin, the so-called sphingomyelinases. Further studies have described other substrates to be included as susceptible to catalysis by the PLDs, such as glycerophospholipids and lysophospholipids. Thus, the term phospholipase D for brown spider dermonecrotic toxins is more suitable (Lee and Lynch, 2005; Chaim et al., 2011a; Chaves-Moreira et al., 2011; Wille et al., 2013).

Studies comparing recombinant isoforms with distinct capacities of degrading substrates have shown differences in the intensity of their effects (Gomez et al., 2002; Chaim et al., 2011b; Stock et al., 2012). Several recombinant PLD isoforms from the *Loxosceles* genus were heterologously produced in *Escherichia coli*. These recombinant PLDs are easily obtained in their soluble and active enzyme forms in

large amounts, which provided interesting results concerning the structural and functional properties of the PLDs. For example, critical data examining the putative enzyme mechanism and three-dimensional scaffold were obtained by X-ray crystallography (more details, see Sections 10 and 11). In summary, research into the catalytic site revealed important insights into the enzymatic capabilities of each isoform (Murakami et al., 2005, 2006; de Giuseppe et al., 2011; Ullah et al., 2011). Recently, de Giuseppe et al. (2011) published the crystal structure of LiRecDT1 from *L. intermedia*, indicating that it contained an additional disulfide bond in the PLD structure catalytic loop compared with the previously described PLD from *L. laeta*. These details of PLD molecules can explain the distinct enzymatic behaviors of the venom from different species. PLDs with different structures could have different substrate affinities or enzymatic activities; therefore, these differences could explain the clinical symptoms or severity observed at the local bite site or the systemic effects during envenomation by different species of the *Loxosceles* genus.

Furthermore, there are clear differences in the hydrolytic ability of PLD isoforms within the *Loxosceles* genus (Gomez et al., 2002; Chaim et al., 2011a; Stock et al., 2012). All studies with the named LiRecDTs (isoforms 1–7) showed dermonecrosis at different levels in rabbit skin, consistent with the results of the spectrofluorimetric analysis of sphingomyelin hydrolysis (Appel et al., 2008; Chaves-Moreira et al., 2011; Vuitika et al., 2013). Ribeiro et al. (2007) reported that LiRecDT1 and LiRecDT2 were similar in all functional tests, such as *in vivo* edema or cytotoxicity, while the LiRecDT3 effect was significantly less intense. The amino acid alignment observed paralleled these results: LiRecDT1 and LiRecDT2 were very similar, but LiRecDT3 slightly was different. LiRecDT3 showed some important differences in hydrophobicity at the boundaries of the catalytic site, which can explain its differential performance.

Furthermore, site-directed mutagenesis of His^{12} of LiRecDT1, predicted to play a central role during catalysis, was not sufficient to completely abolish its catalytic activity. Moreover, the LiRecDT1H12A mutant isoform has a drastic reduction in its enzymatic activity, but with no change in the secondary structure, compared with LiRecDT1. Interestingly, the mutant isoform was unable to induce the same level of any activity examined, but the attachment to the cell surface or to mobilized lipids was unaltered (Kusma et al., 2008; Paludo et al., 2009; Chaim et al., 2011b; Wille et al., 2013). Most likely, the other protein domains besides the catalytic cleft were preserved, as they might be relevant for the interaction of the toxin with the cell membrane or lipid substrates. In general, the main value of PLD catalysis can be related to the release of lipid metabolites, which could modulate a wide range of biological events, such as the cell cycle, cell proliferation, cell differentiation processes and cell death (Marchesini and Hannun, 2004; Tani et al., 2007).

Studies have shown the upregulation of the expression of proinflammatory cytokines/chemokines after the exposure of human fibroblasts to the *L. reclusa* PLD (Dragulev et al., 2007), which hydrolyzes the cell membrane sphingomyelin to ceramide 1-phosphate (C1P) and would lead to a receptor-dependent inflammatory response. This idea challenged the hypothesis that lysophosphatidic acid (LPA) was a preferential product and bioactive metabolite instead of C1P, due to the relative LPC abundance in the plasma as a substrate (van Meeteren et al., 2004, 2007). Recently, Horta et al. (2013) showed that cell death was induced by *L. similis* whole venom (LsV) and especially with a recombinant isoform of *L. intermedia* PLD, recLiD1 (Kalapothakis et al., 2002; Felicori et al., 2006). LPA released by the PLD activity of LsV and recLiD1 was unable to activate LPA receptors in the presence of an LPA1/LPA3 antagonist. This effect was indirectly observed by ELISA assays for IL-6, IL-8, CXCL1, and CXCL2. Moreover, the authors did not find that LPA played a role in the apoptosis induced by LsV or recLiD1 in fibroblast and endothelial cells *in vitro*, which may be related to other LPA-independent stimuli or to C1P acting on the cell membrane receptors, as has been previously described (Horta et al., 2013). The variety of molecular mechanisms triggered by *Loxosceles* PLDs and their lipid metabolites remains open to further investigation as a complex event dependent on the cell types involved, lipid substrate abundance and availability and intracellular signaling cascades. PLDs can serve as biotools for the study of cell–cell communication via cell membranes in the context of inflammation. PLD isoforms have been proposed as potential models for designer drugs or other biotechnological applications (Senff-Ribeiro et al., 2008; Tambourgi et al., 2010; Chaim et al., 2011a). The production of more stable PLD isoforms with enhanced enzymatic activity would greatly contribute to many areas of toxinology and to the complete understanding of the biochemical features of PLDs, their many biological implications and their related molecular mechanisms.

6.1.1. Phospholipase D topology and structure

The amino acid sequence comparisons of spider venom phospholipases D indicate that they contain either 284 or 285 amino acids and display a significant degree of homology (de Santi Ferrara et al., 2009). This single polypeptide chain folds to form a distorted barrel where the inner barrel surface is lined with eight parallel β-strands (termed A–H) linked by short flexible loops to eight α-helices (termed helices 1–8) that form the outer surface of the barrel (Murakami et al., 2005) (Figs. 4 and 5). This structural motif was first observed in the structure of the triose phosphate isomerase (TIM) and is referred to as a TIM barrel or as an $(\alpha/\beta)_8$ barrel. The topology diagram (Fig. 5) presents a structural schematic where the α-helices and β-strands are depicted as cylinders and arrows, respectively, and the central region forms the $(\alpha/\beta)_8$ barrel. The interconnecting loops are primarily hydrophilic and hydrophobic in the upper and lower sections, respectively (Fig. 5). A short β strand (B′) is inserted between strand B and helix 2, and two short helices (3′ and 4′) are inserted between helix 3 and strand D, and helix 4 and strand E. The catalytic loop is stabilized by a disulfide bridge (Cys^{51} and Cys^{57}) and a second disulfide bridge (Cys^{53} and Cys^{201}) is present only in the class II enzymes (de Giuseppe et al., 2011), which links the catalytic loop to the flexible loop to significantly reduce the flexibility of the latter loop, as evidenced by the mean temperature factors (Figs. 4 and 6).

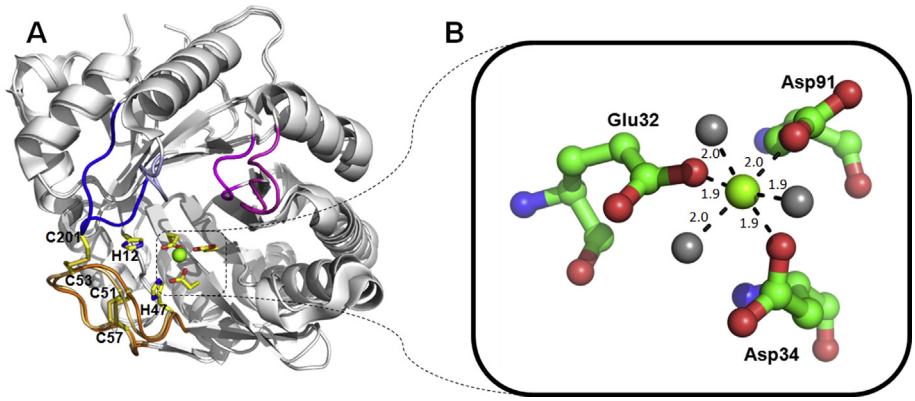

Fig. 4. Structural alignment between the class I and class II phospholipases D. (A) The residues involved in the metal–ion binding and catalysis are highlighted. The catalytic, flexible and variable loops are colored in orange, blue and magenta, respectively. The dark and light colors refer to phospholipase D II and phospholipase D I, respectively. The Mg^{+2} ion is represented by a green sphere. The disulfide bridges are represented as yellow-colored sticks. (B) The coordination sphere of the Mg^{2+} ion. The figure color codes refer to the on-line images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Because the α-helices, β-strands and loops vary in length and character, the barrel with a surface area of 11,254 $Å^2$ is significantly distorted (Fig. 4). The interior of the barrel is densely packed with hydrophobic amino acids, and the short N-terminal section and the C-terminal extension, which contains a short helix (8′), a β-strand (H′) and a random coiled region, serve to cap the torus of the far side of the barrel. The surface loops forming the near side of the barrel are mainly hydrophobic, and a narrow cavity provides access to the catalytic site, which is characterized by a ring of negatively charged amino acids (Murakami et al., 2005; de Giuseppe et al., 2011). The catalytic, variable and flexible loops are located on the same face of the barrel and are colored orange, magenta and blue, respectively (Fig. 4). The catalytic and Mg^{2+} binding sites are located in a shallow depression and contain His^{12}, Glu^{32}, Asp^{34}, Asp^{91}, His^{47}, Asp^{52}, Trp^{230}, Asp^{233}, and Asn^{252}, which are fully conserved in *Loxosceles* PLD isoforms (Figs. 4 and 7) (Murakami et al., 2005; de Giuseppe et al., 2011).

Mutagenesis studies of PLDs (de Giuseppe et al., 2011; Ullah et al., 2011) and its crystal structure (Murakami et al., 2005) indicate the involvement of two histidines that are in close proximity to the metal ion-binding site in the acid-base catalytic mechanism. Based on the structural results, His^{12} and His^{47} of PLD have been identified as the key residues for catalysis and are assisted by a hydrogen bond network that involves Asp^{52}, Asn^{252}, and Asp^{233}. The metal ion is coordinated by Glu^{32}, Asp^{34}, Asp^{91}, and solvent molecules (Fig. 4B).

6.1.2. *Involvement of the Mg^{2+} ion in the phospholipase D catalytic mechanism*

The Mg^{2+} ion is essential for catalysis, and its binding site is completely conserved in all spider venom phospholipases D. Mg^{2+} ion is octahedrally coordinated (with a mean Mg^{2+}–O distance of 1.98 Å) (Fig. 4) equatorially by the carboxylate oxygens of the side chains of Glu^{32} and Asp^{34} and by two tightly bound water molecules and

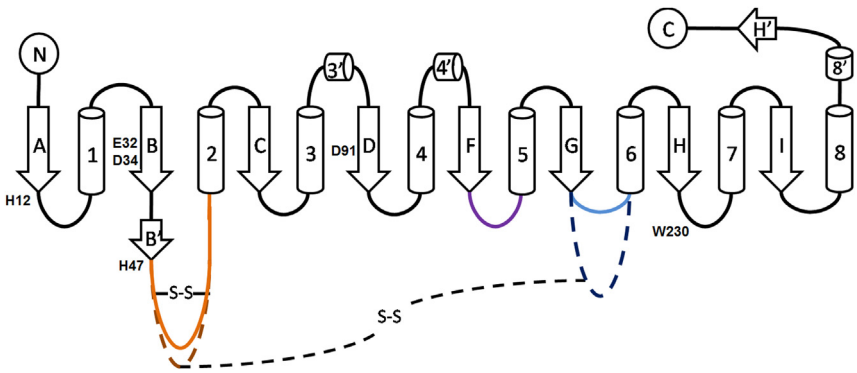

Fig. 5. Structural topology of *L. laeta* phospholipase D I and *L. intermedia* phospholipase D II. The β-strands (arrows) and α-helices (cylinders) forming the $(\alpha/\beta)_8$ barrel are labeled A–H and *1–8*, respectively. The β-strands and α-helices not belonging to the core are designated with a prime. The positions of the catalytic loop B (orange), variable loop E (magenta), flexible loop F (blue), and the disulfide bridge (*S–S*) are indicated. The approximate relative positions of the amino acids involved in the catalysis and Mg^{2+} ion binding are indicated. The dashed line represents the additional disulfide bond in phospholipase D II. Letters N and C represents the positions of amino- and carboxi-terminal domains of proteins. Figure color codes refer to the on-line images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

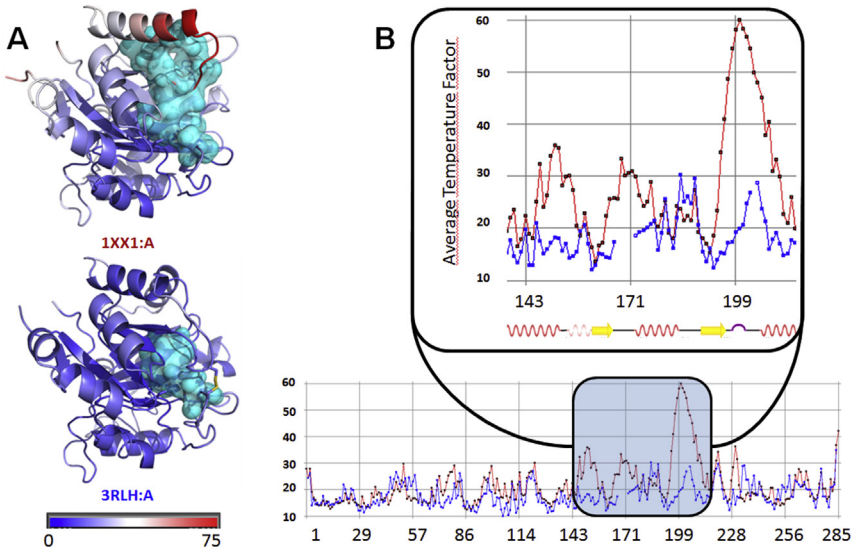

Fig. 6. Protein flexibility by B factor analysis. (A) Ribbon representations of the phospholipase D structures colored to indicate the mean temperature factors as indicated by the bar graph. A light blue surface indicates the cavity volume of the active site. (B) The mean temperature factors (blue, main chain; red, side chain) as a function of the amino acid residues. The inset highlights the flexible region as discussed in the text. 1XX1 and 3RLH are the protein data bank codes for the Class I and Class II enzymes, respectively. Plot performed by MSSP module of BlueStar STING (Neshich et al., 2005). Figure color codes refer to the on-line images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

apically by the side-chain carboxylate oxygen atoms of the Asp^{91} and by a water molecule, which is also hydrogen bonded to the $Glu^{32}O^{\varepsilon 1}$ atom. The enzyme structure determined in the presence of a bound sulfate ion (Murakami et al., 2005), which is considered to occupy the position of the substrate phosphate moiety, is coordinated by three solvent molecules, two of which also coordinate the Mg^{2+} ion. The indole ring of Trp^{230} is partially disordered and likely plays a role in stabilizing the choline head group of the substrate.

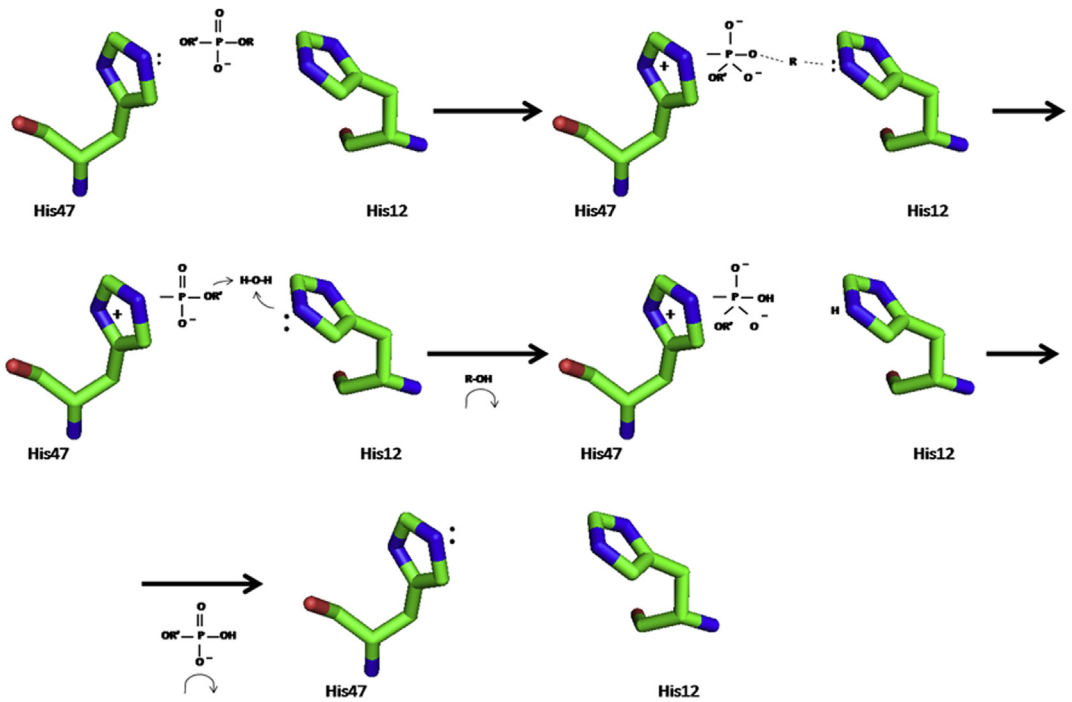

Fig. 7. The reaction mechanism of phospholipase D. The acid-base catalytic mechanism involves His^{12} and His^{47}. R and R′ indicate ceramide 1′-phosphate and choline, respectively.

Based on the crystal structures of phospholipase D, a two-step catalytic mechanism has been suggested where His^{12} and His^{47} play important roles (Fig. 4). In the first step of this mechanism, **His^{47}** plays a role as a nucleophile that initiates the attack on the substrate scissile phosphodiesterase bond, which is followed by the formation of a penta-coordinated intermediate that is subsequently destabilized by the donation of a hydrogen atom by **His^{12}**, leading to the formation of a choline molecule. In the second reaction step, **His^{12}** abstracts a proton from a solvent molecule that then initiates a nucleophilic attack on the stable covalent histidine intermediate, resulting in the formation of the second product, ceramide 1-phosphate, and a return to the initial state. The Mg^{2+} ion is important for the substrate recognition and binding and for further stabilization of the intermediate state in the two-step catalytic mechanism.

6.2. Astacins

The astacins are a family of proteases belonging to the metzincin super family, which are grouped with the zinc-dependent metalloproteases (Gomis-Rüth, 2003; Sterchi et al., 2008). Many metalloproteases are characterized by a conserved pentapeptide HEXXH in the active site, which is essential for metal ion coordination and catalysis (Sterchi et al., 2008).

The first report of proteases in *Loxosceles* venom was described in *L. reclusa* venom, which showed protease activity against *Heliothis virescens* and *Musca domestica* larvae, as observed by histochemical techniques (Eskafi and Norment, 1976). In addition, the *L. reclusa* venom protease activity was assayed on L-aminoacyl-β-naphthylamide derivatives and was shown to act more efficiently on L-Leucyl-β-naphthylamide, although other derivatives were also susceptible (Jong et al., 1979).

In *L. intermedia* venom, two metalloproteases were identified, Loxolysin A, a 20–28 kDa protease that degrades the Aα and Bβ chains of fibronectin and fibrinogen, and Loxolysin B, a 30–32 kDa protease with gelatinolytic activity (Feitosa et al., 1998). Similarly, proteolytic enzymes were identified in *L. rufescens* venom: a 23 kDa fibrinogenolytic protease and a 27.5 kDa gelatinolytic protease, which were both inhibited by 1,10-phenantroline (Young and Pincus, 2001). The fibrinogenolytic activity was reported in both *L. reclusa* venom and *L. laeta* venom, which showed the same partial effects that were observed in *L. intermedia* venom (i.e., the degradation of the Aα and Bβ fibrinogen chains) (Zanetti et al., 2002). *L. deserta*, *L. gaucho* and *L. reclusa* venoms were also shown to include metalloproteases (Barbaro et al., 2005). Other extracellular matrix components were also demonstrated as targets for the *Loxosceles* metalloproteases, such as entactin and heparan sulfate proteoglycans (Veiga et al., 2000b, 2001a). Although with the identification of these *Loxosceles* proteases, the proof that proteases are venom components and not contamination derived from gastric contents during venom extraction was reported in two crucial studies of *L. rufescens* venom and *L. intermedia* venom (Young and Pincus, 2001; da Silveira et al., 2002). The proteolytic effect of *L. rufescens* venom discussed above was observed in venom obtained by micro-dissection of the venom glands, a procedure that ensures the absence of gastric contaminants (Young and Pincus, 2001). For *L. intermedia* venom, the protein profile and proteolytic activity were very similar between the venom collected by electrostimulation (possible contamination) and macerated venom glands (free from gastric contaminants) (da Silveira et al., 2002).

A sequence encoding an astacin-like metalloprotease was first identified in a cDNA library of *L. intermedia* venom glands (da Silveira et al., 2007a). Astacin-like proteases (Merops M12A family) have a consensus sequence of 18 amino acids forming the catalytic domain –HEXXHXXGXXHEXXRXDR – in which the three histidines are involved in zinc binding, which is necessary for the catalytic activity. In addition, they have a conserved methionine residue involved in a sequence turn, termed a met-turn (MXY) (Gomis-Rüth, 2003; Sterchi et al., 2008; Gomis-Rüth et al., 2012). The identified *L. intermedia* astacin sequence was named LALP (*Loxosceles* astacin-like protease) and possesses astacin family signatures (catalytic domain and met-turn). LALP was shown to be cytotoxic upon rabbit subendothelial cells and able to hydrolyze fibrinogen and fibronectin (da Silveira et al., 2007a). Astacin family members have been described in prokaryotes and eukaryotes and possess diverse and distinct biological functions. In general, they are expressed in specific tissues of mature organisms, and during embryo development, they are temporally and spatially regulated (Gomis-Rüth, 2003; Mörhlen et al., 2003, 2006). The presence of astacin proteases in animal venoms is rare because LALP was the first report in the literature of an astacin molecule as a constituent of animal venom (da Silveira et al., 2007a).

Recently, two new isoforms of astacin-like proteases were identified in *L. intermedia* venom (named LALP2 and LALP3) and in *L. laeta* venom (LALP4) and *L. gaucho* venom (LALP5). These findings demonstrate that astacin proteases are a family of toxins present in *Loxosceles* venoms and that they are important components of these venoms (Trevisan-Silva et al., 2010, 2013). Corroborating the biological importance of the *Loxosceles* astacin-like proteases, transcriptome analyses showed that astacins are high expressed toxins in *L. laeta* and *L. intermedia* venoms (Fernandes-Pedrosa et al., 2008; Gremski et al., 2010). In *L. laeta* venom, astacin's transcripts represents 8% of the total transcripts, and in *L. intermedia* venom, they represent 9.8% of the toxin transcripts, representing the second most expressed toxin in both species (Gremski et al., 2010). Studies concerning the biological activities of *Loxosceles* astacins are essential to improve the knowledge of loxoscelism and to apply these toxins in biotechnology.

6.3. Brown spider venom Inhibitor Cystine Knot (ICKs)

In early the 1990s, many venom peptides from spiders, scorpions, cone snails and sea anemones had their structures solved using various techniques. A great number of cysteine-rich peptides were described, including a common structural motif called the "Inhibitor Cystine Knot" (ICK) (Daly and Craik, 2011). These peptides share a common structure with three disulfide bonds and are composed of three antiparallel β-sheets (Craik et al., 2001; Zhu et al., 2003; Daly and Craik, 2011). Two disulfide bonds

form a ring that is crossed by the third bond, which forms the structural motif ICK. This same structural motif is found in two other families: the Cyclic Cystine Knot (CCK) or cyclotide family and Growth Factor Cystine Knot (GFCK) family (Vitt et al., 2001; Craik et al., 2010; Iyer and Acharya, 2011). This molecular scaffold renders ICK peptides highly resistant to protease action, making them good targets for drug design (Daly and Craik, 2011; King, 2011; Moore et al., 2011). The homology between ICK peptides is usually low, but the distribution of cysteine residues is often conserved. Further studies showed that ICK peptides have an amino acid consensus sequence of $CX_{3-7}CX_{3-6}CX_{0-5}CX_{1-4}CX_{4-13}C$, where X can be any amino acid (Craik et al., 2001). ICK toxins are quite diverse in their biological activity because they can act in voltage-gated sodium, potassium or calcium channels; mechanosensitive channels; nicotinic acetylcholine receptors or ryanodine receptors (Nicholson et al., 2004; Dutertre and Lewis, 2010).

Among spiders, many ICK toxins have been described. One of the most well-studied families is the δ-Atracotoxins (δ-ACTX) family isolated from the venom of Australian funnel-web spiders. δ-ACTX show a similar action to the sea anemone and scorpion ICK toxins, binding at site 3 of the sodium ion channel, which causes neurotransmitter release in the nerve endings and results in the disturbance of the autonomic and somatic nervous systems (Nicholson et al., 2004).

From *Loxosceles* venom, three insecticide peptides named LiTx 1-3 have already been purified (de Castro et al., 2004). These peptides were isolated from *L. intermedia* venom using a combination of chromatography techniques, and their activities were assessed in *Lepidoptera* larvae, resulting in an LD_{50} of 0.90–1.92 μg/g insect. These authors proposed that LiTx 3 may act on **NaV** channels as with other toxins and that LiTx 2 and 3 may act on **NaV** or **CaV** channels. Furthermore, Fernandes-Pedrosa et al. (2008) analyzed the *L. laeta* transcriptome and found that 0.2% of all toxin transcripts matched with the ICK neurotoxin Magi 3 from *Macrothele gigas* (Corzo et al., 2003), which bind at site 3 of **NaV** channels. The transcriptome analysis of the *L. intermedia* venom gland showed that 55.5% of all transcripts putatively encode for toxins that potentially represent insecticide peptides and can be classified as ICK peptides. From the 55.5% of transcripts, 2.3% represent transcripts similar to Magi 3, such as those transcripts described for *L. laeta*. The most abundant venom transcripts found were transcripts similar to LiTx 3 (32%), LiTx 2 (11.4%) and LiTx 1 (6.2%). This transcriptome analysis also described transcripts encoding LiTx 4, another ICK peptide not yet characterized, which represent 3.7% of the toxin transcripts. Recently, it was described the cloning, recombinant peptide production, polyclonal antibody obtention and evaluation of the cross-reactivity of a novel toxin with a great similarity to the ICK family of peptides from *L. intermedia* venom. This peptide was named U2-sicaritoxin-Li1b (U2- SCRTX-Li1b) according to the nomenclature proposed by King et al. (2008) (Matsubara et al., 2013).

The interest in ICK toxin class is due to their targeting of ion channels, which are transmembrane protein complexes regulating ion flux and membrane potential. This ability of ICK peptides to specifically bind to some ion channels provides a great tool not only for electrophysiology and cell biology studies but also for drug design. In addition, the high specificity of spider peptides for insect receptors leads to the proposal of using these peptides for developing novel insecticidal targets or for the development of new biopesticides (Estrada et al., 2007; Dutertre and Lewis, 2010; Klint et al., 2012). Currently, there is no evidence of the involvement of brown spider ICKs in the pathogenesis of spider bites.

7. Low level of expression toxin families

Loxosceles venoms have demonstrated little variation in overall toxin composition (Ramos-Cerrillo et al., 2004; Barbaro et al., 2005; Fernandes-Pedrosa et al., 2008; Gremski et al., 2010; Trevisan-Silva et al., 2010). The high degree of intragenus toxin preservation is evidence of the evolutionary success of the venom formulation and is suggestive of the important functions of some types of toxins (Trevisan-Silva et al., 2010; Corrêa-Netto et al., 2011). However, transcriptome analyses of *L. intermedia* and *L. laeta* venoms indicate some differences in the level of toxin expression in this genus. In the analyses of the *L. laeta* venom gland expression profile, relatively low numbers of transcripts of serine proteases, enzymatic inhibitors, C-type lectin, hyaluronidases, 5′-nucleotidases, chitinases and venom allergens were found (Fernandes-Pedrosa et al., 2008). On the other hand, Gremski and co-workers showed low numbers of transcripts that coded for serine proteases, venom allergen, TCTP (**T**ranslationally **C**ontrolled **T**umor **P**rotein), hyaluronidases and serine proteases inhibitors (Gremski et al., 2010). The hypotheses that may explain these differences in the profile of toxins with low level expression include the different approaches and methodologies employed in the analyses. Additionally, these differences apply to different species, which reinforce the previous data that showed distinct behaviors among the venoms from distinct *Loxosceles* species.

Venom variations occur at all taxonomical levels and can significantly impact the clinical manifestations and efficacy of anti-venom therapies following a spider bite. Cases of incomplete intrageneric antivenom efficacy have been documented, implying a high interspecies venom variation (Casewell et al., 2009). Abundant differences can be observed between the venom compositions of different genera, the venom compositions of different species within a genus and the venom compositions of different individuals within a species (e.g., individuals from different geographical regions). Apparently, the venom composition is subject to strong natural selection pressure as a result of adaptation to specific diets because the primary role of venom is to aid in prey capture (Ruiming et al., 2010). Additionally, toxins with low level of expression do not necessarily possess a low activity. On the contrary, some types of these toxins have been postulated to have a high activity and high stability (Morey et al., 2006; Reitinger et al., 2008; Menaldo et al., 2012; Valeriano-Zapana et al., 2012), and therefore, these molecules would not be synthesized in large amounts. The below sub-items further discuss some of these low level of expression toxin family-members.

7.1. TCTP

The *L. intermedia* venom gland transcriptome analysis described the sequence of a protein identified as a member of the TCTP family and revealed that this TCTP is present at relatively low levels in the venom, only 0.4% of the transcripts that encoded toxins (Gremski et al., 2010). The name TCTP stands for ***T**ranslationally **C**ontrolled **T**umor **P**rotein*, as this protein was described by scientists studying proteins that were regulated at the translational level. The *tumor* is derived from the first TCTP cDNA sequence described, which was obtained from a human mammary tumor (Bommer, 2012). This protein was also shown to be a histamine-releasing factor (HRF) (McDonald et al., 1995) and a fortilin (Li et al., 2001). The *L. intermedia* TCTP was cloned and expressed as a heterologous protein in an *E. coli* expression system. The functional characterization of the recombinant protein, LiTCTP, showed that this toxin caused edema and enhanced vascular permeability (Sade et al., 2012). The cutaneous symptoms of envenomation with *Loxosceles* venoms include erythema, itching and pain. In some cases, *Loxosceles* spider bites can cause hypersensitivity or even allergic reactions. These responses could be associated with histaminergic events, such as an increase in the vascular permeability and vasodilatation. LiTCTP could be related to these deleterious venom actions as it was identified in *L. intermedia* venom (Sade et al., 2012). Another *Loxosceles* TCTP has been described in the venom gland of *L. laeta* by transcriptome analysis (Fernandes-Pedrosa et al., 2008). Recently, a transcriptome analysis revealed a TCTP protein (named GTx-TCTP) in the venom gland and the pereopodal muscle of the tarantula *Grammostola rosea* (Kimura et al., 2012).

Proteins of the TCTP super family have already been described in the gland secretions of many arthropods, such as ixodid ticks, and in the venom gland of the wolf spider (*Lycosa godeffroyi*), where it was described as the principal pharmacological toxin (Mulenga and Azad, 2005; Rattmann et al., 2008). TCTP family members are described as extracellular HRFs and are associated with the allergic reactions of parasites. Among species from the same genus, the TCTPs are completely conserved (Bommer and Thiele, 2004). A LiTCTP phylogeny tree demonstrates the similarities with the TCTPs from ixodid ticks, which were also characterized as HRFs (Mulenga and Azad, 2005; Sade et al., 2012). In the case of the *Loxosceles* venom gland, the TCTP and other constituents of the whole venom are secreted via a holocrine secretion as determined by ultrastructural studies of the *L. intermedia* venom gland (dos Santos et al., 2000; Gremski et al., 2010). TCTP secretion from cells proceeds via an ER/Golgi-independent or non-classical pathway, most likely mediated by secreted vesicles called exosomes (Amzallag et al., 2004; Hinojosa-Moya et al., 2008). TCTP mRNAs do not encode a signal sequence, and no precursor protein has been described; however, a TCTP protein was found in the biological fluid of asthmatic or parasitized patients, in the saliva of ticks (Bommer and Thiele, 2004; Hinojosa-Moya et al., 2008) and in the crude venom of *L. intermedia*. TCTPs represent a large protein family that is highly conserved and ubiquitous in eukaryotes, and they members are widely expressed in various tissues and cell types. TCTP protein levels are highly regulated in response to a wide range of extracellular signals and cellular conditions, which points to an involvement in various participating biological functions at diverse biochemical and signaling pathways. In fact, a wide range of functions and different biochemical roles have already been examined in the TCTP family (Bommer and Thiele, 2004; Choi and Hsu, 2007; Bommer, 2012).

TCTP proteins have already been described as calcium-binding proteins (Graidist et al., 2007) and as proteins that interact with the cytoskeleton by binding to and stabilizing microtubules (Bazile et al., 2009). The involvement of TCTP in the mitotic spindle has also been shown, and TCTP is now considered a regulator of mitosis (Burgess et al., 2008). The crucial role of TCTP has also been described in early development. The loss of TCTP expression in mice leads to increased spontaneous apoptosis during embryogenesis and causes lethality (Chen et al., 2007; Susini et al., 2008). TCTP can be described as a multifunctional protein due to the high number of protein partners and the several areas/pathways of cell metabolism where it is involved (Amson et al., 2013a).

The downregulation of TCTP has been implicated in biological models of tumor reversion (Tuynder et al., 2002, 2004), and the protein is the target of various anticancer drugs (Efferth, 2005; Telerman and Amson, 2009; Amson et al., 2013b).

Studying LiTCTPs can elucidate the biological aspects of loxoscelism, especially those aspects related to the histaminergic symptoms. Moreover, LiTCTP investigation can provide new insights regarding the TCTP family and its different functions. LiTCTP is a promising subject for study in toxinology and in immunological, allergenic and experimental oncology.

7.2. Hyaluronidases

Hyaluronidases are a group of enzymes that degrade hyaluronic acid (HA) and, to a limited extent, chondroitin, chondroitin sulfate (CS) and dermatan sulfate (DS). HA is a ubiquitous component of the vertebrate extracellular matrix where it fills the space between cells and acts as a lubricant and a barrier to the penetration of foreign particles (Markovic-Housley et al., 2000).

This type of matrix-degrading enzyme are found in many animal venoms, such as lizards, scorpions, spiders, bees, wasps, snakes and stingrays (Girish and Kemparaju, 2005; Kemparaju and Girish, 2006; Magalhães et al., 2008). These enzymes are always reported as “spreading factors” in the venoms due to their ability to degrade extracellular matrix components and to increase the diffusion of other toxins from the inoculation site (Kemparaju and Girish, 2006).

Wright et al. (1973) were the first to describe hyaluronidase activity in the *Loxosceles* genus. This work was performed with *L. reclusa* venom, and the purified enzymes were estimated to have molecular mass of 33 and 63 kDa (the 63 kDa protein is thought to be a 33 kDa-dimer) by SDS-PAGE. These enzymes exhibited activity against HA

and CS types A, B, and C. (Wright et al., 1973). Although *Loxosceles*-derived hyaluronidases alone are not able to produce necrosis, they are thought to be an important factor in the spread of these venoms. The detection by HA-substrate SDS-PAGE of a hyaluronidase of 32.5 kDa from *L. rufescens* venom has been reported (Young and Pincus, 2001). In *Loxosceles* envenomation, the presence of edema, erythema and necrosis is common, which indicates extracellular matrix disturbances.

Barbaro et al. (2005) found hyaluronidase activity on hyaluronic acid in a 44 kDa protein in *L. deserta*, *L. gaucho*, *L. intermedia*, *L. laeta and L. reclusa* venoms. Shortly after, da Silveira et al. (2007c) showed that *L. intermedia* venom contained at least two hyaluronidase isoforms. This venom demonstrated lysis of both HA and CS substrates at 41 and 43 kDa. These authors also showed, using biochemical assays, that the hyaluronidases from this venom are pH-dependent endo-β-N-acetyl-D-hexosaminidase hydrolases. *L. intermedia* venom was also able to degrade HA in rabbit skin (da Silveira et al., 2007c). A proteomic study also corroborated the presence of hyaluronidases in *Loxosceles* venoms (dos Santos et al., 2009).

Analyzing the transcriptome of *L. laeta*, Fernandes-Pedrosa et al. (2008) found 4 clones within 1 cluster with similarity to the hyaluronidase from *Bos taurus* (gb|AAP55713.1), which represented 0.13% of the total transcriptome. In addition, Gremski et al. (2010) demonstrated a unique partial sequence in the *L. intermedia* transcriptome with similarity to hyaluronoglucosaminidase 1 from *Rattus norvegicus* (gb|EDL77243.1). Recently, the first recombinant hyaluronidase from the *Loxosceles* venom was produced from *L. intermedia* venom gland cDNA (Dietrich's Hyaluronidase). The recombinant toxin was expressed in *E. coli* and had a molecular mass of approximately 45 kDa. Hyaluronidase activity of this recombinant toxin was detected on HA and CS after refolding *in vitro*. An assessment of dermonecrosis *in vivo* showed that Dietrich's Hyaluronidase increased the macroscopic erythema, ecchymosis and dermonecrotic effect induced by the recombinant dermonecrotic toxin (LiRecDT1) a phospholipase D homologue in rabbit skin. This work confirmed the hypothesis that hyaluronidase acts as a spreading factor in *Loxosceles* venoms (Ferrer et al., 2013).

HA levels are markedly increased during embryogenesis, inflammation, malignant transformation, and wound healing and whenever fast tissue turnover and remodeling is required. The occurrence of various diseases related to HA metabolism suggest that the level of HA must be tightly controlled (Markovic-Housley et al., 2000; Girish and Kemparaju, 2007). The process of degradation of glycosaminoglycans from connective tissues is related to bacterial pathogenesis, the spread of toxins and venoms, fertilization processes, and cancer progression (Hynes and Walton, 2000; Girish et al., 2004; Girish and Kemparaju, 2007; Lokeshwar and Selzer, 2008). Therefore, the identification and characterization of hyaluronidase inhibitors could be important in the development of new drugs and biotechnological tools to be applied in the above-mentioned fields (Botzki et al., 2004; Barla et al., 2009).

7.3. Serine proteases

Serine proteases were first identified in *Loxosceles* venom as zymogens activated by trypsin (Veiga et al., 2000a). In Veiga et al. (2000a), using zymography assays **with venom previously incubated with exogenous proteases**, trypsin was shown to activate two gelatinolytic molecules of 85 and 95 kDa in *L. intermedia* venom. Among the various protease inhibitors assayed, only serine protease inhibitors were able to inactivate these enzymes. The activity of the assayed *L. intermedia* serine proteases were optimal in a pH range of 7.0–8.0, and no enzymatic activity was observed on hemoglobin, immunoglobulin, albumin, fibrinogen or laminin, suggesting the specificity of their proteolytic actions.

At the time, no previous descriptions of proteases that behaved as zymogens had been described for spider venoms. However, as this feature had already been reported for several snake venom proteases, the authors suggested that trypsin treatment could specifically degrade the pro-peptide domains of the zymogen molecules and release the active proteases. As this activation was only observed after treatment with trypsin, even though various proteases were assayed, it was suggested that the hydrolysis of zymogen molecules of *L. intermedia* serine proteases was specific because trypsin hydrolyzes peptide bonds immediately after a lysine or arginine (Veiga et al., 2000a).

Consistent with the results of the Veiga et al. (2000a) study, Machado et al. (2005) also found high molecular mass proteins at 85–95 kDa in 2-DE gels. These protein spots were also detected in *L. laeta* and *L. gaucho* venoms.

The transcriptome analysis of the *L. laeta* venom gland revealed twelve clusters that grouped fourteen ESTs putatively assigned as serine proteases coding sequences (Fernandes-Pedrosa et al., 2008). All clusters are similar to serine proteases described in arthropods, such as ticks, spiders and crabs. Shortly thereafter, a proteome study of *L. intermedia* venom described five peptide sequences similar to snake venom serine proteases (dos Santos et al., 2009).

A transcriptome analysis of the *L. intermedia* venom gland putatively assigned five transcripts as serine proteases (Gremski et al., 2010). The ESTs were grouped into two clusters with no sequence similarity with each other. One of the sequences significantly aligned with an arthropod serine protease that was most likely synthesized as an inactive precursor (Nene et al., 2007). The other cluster was similar to a serine protease sequence of the spider *Lycosa sigoriensis* venom gland (Gremski et al., 2010; Zhang et al., 2010). As previously mentioned, *L. intermedia* venom demonstrated serine proteolytic activity at two high molecular mass proteins, suggesting that two or more molecules in the venom exhibit these particular characteristics (Veiga et al., 2000a). Thus, it is not surprising that both transcriptome and proteome studies described distinct sequences coding for serine proteases (Fernandes-Pedrosa et al., 2008; dos Santos et al., 2009; Gremski et al., 2010). The latter *Loxosceles* venom proteome study noted that the serine proteases in venoms have also been related to complement activation (dos Santos et al., 2009). In fact, various studies have related the involvement of complement system factors in the pathological events triggered by

Loxosceles venom, such as hemolysis and dermonecrosis (Lane and Youse, 2004; Tambourgi et al., 2005). However, this feature is currently associated specifically with venom phospholipases D.

Venom serine proteases, in addition to their contribution to prey digestion, can play an important role in local tissue destruction and interfere in blood coagulation and fibrinolysis (Veiga et al., 2000a; Kini, 2005; Devaraja et al., 2010). In fact, venom serine proteases may possess thrombin-like, fibrinogenase and plasminogen-activating activities, and they are molecules with the potential to be novel diagnostic or anti-thrombotic agents (Muanpasitporn and Rojnuckarin, 2007). Snake venom serine proteases, in turn, have been used to determine fibrinogen levels in the presence of heparin (Reptilase® time, Funk et al., 1971) and to remove fibrinogen in samples for thrombin-dependent tests (Mullin et al., 2000). In addition, recombinant Ancrod®, a thrombin-like serine protease from the *Agkistrodon rhodostoma* viper, improves the outcomes after cerebral stroke in humans (Liu et al., 2011), and Defibrase®, from the *Bothrops* spp., is clinically beneficial in ischemic stroke (Guo et al., 2006). Thus, further studies concerning *Loxosceles* serine proteases are imperative for the development of potential novel therapeutic agents.

7.4. Serine protease inhibitors

Proteinaceous inhibitors of proteolytic enzymes comprise the largest group of naturally occurring enzyme inhibitors. Their vast structural diversity is detailed in the MEROPS database of peptidase inhibitors (available in http://merops.sanger.ac.uk/inhibitors/). Recent work comprehensively listed 91 families of protease inhibitors grouped based on their homology. Some families of the serine and cystein protease inhibitors stand out for their high frequency, such as the Kazal and Kunitz-type inhibitor families (e.g., I1 – I3 **peptidase inhibitor families**), serpins (e.g., I4 **peptidase inhibitor family**) and cystatins (e.g., I25 **peptidase inhibitor family**) (Rawlings et al., 2012).

The first report of the presence of protease inhibitors in *Loxosceles* venom glands was made in 2008 by Fernandes-Pedrosa and colleagues, who performed a transcriptome analysis of venom glands of female *L. laeta* spiders. The sequences that matched these molecules were described as “enzymatic inhibitors” and represented 0.6% of the total number of sequences analyzed.

Some transcripts of the *L. laeta* cDNA library are related to serine (or cysteine) protease inhibitors of diverse species, which have been characterized and have been shown to have different functions and activities, such as an *in vitro* anticlotting activity and *in vivo* antithrombotic and anticoagulant activities related to the inhibition of Factor-Xa. A proprotein-convertase (PC) inhibitor sequence of *Branchiostoma lanceolatum* (emb|CAD68157.1) was also listed as similar to some of the *L. laeta* ESTs (Bentele et al., 2006; Fernandes-Pedrosa et al., 2008). This serpin, termed Bl-Spn1, inhibits the proprotein processing proteases PC1/3 and furin (Bentele et al., 2006). Analyses of some of the ESTs from the *L. laeta* cDNA library revealed a similarity with a cystatin sequence from the tick *Boophilus microplus* (gb|ABG36931.1) that was found to inhibit the human cathepsin L and vitellin degrading cysteine endopeptidase (VTDCE). More recently, the venom of the brown spider *L. intermedia* was subjected to proteomic analysis through the MudPIT proteomic strategy, and approximately a dozen peptides were found to be similar to protease inhibitors (dos Santos et al., 2009). Three sequences showed similarity with an inhibitor of *Oryza sativa* from the cystatin super family (P20907) (Kondo et al., 1990; dos Santos et al., 2009). On the other hand, other peptides sequenced in this proteomic analysis are related to the Kunitz-type inhibitors (dos Santos et al., 2009). Finally, the *L. intermedia* proteome revealed some peptides related to an inhibitor from the serpin super family of protease inhibitors (P07385) (dos Santos et al., 2009). Thus, it is possible that *L. intermedia* venom contains proteases inhibitors belonging to different groups (i.e., the serpins, Kunitz-type and cystatin super families).

A transcriptome analysis of the venom glands of *L. intermedia* identified an EST similar to protease inhibitors from the serpin family (Gremski et al., 2010). This EST sequence is related to mammalian and arthropod serpins, such as the human neuroserpin and the *Ambliomma americanum* tick and *Tachypleus tridentatus* horseshoe crab serpins (Gremski et al., 2010).

The function of protease inhibitors in *L. intermedia* venom has been suggested to be related to the protection of the toxin integrity (dos Santos et al., 2009). Some authors that have described serine protease inhibitors in different venoms (snakes, spiders and scorpions) propose that one of the physiological roles of these molecules is to resist prey proteases to protect their venom protein toxins (Zupunski et al., 2003; Yuan et al., 2008; Zhao et al., 2011). In addition, these inhibitors may generate a synergistic effect with other neurotoxins, as suggested by other authors (Yuan et al., 2008; Zhao et al., 2011). Because the proteases are involved in several physiological processes, they represent excellent therapeutic targets. Thus, the protease inhibitors arising from venoms are potential candidates to mediate certain biological processes. The Kunitz-type protease inhibitor isolated from ***Pseudonaja textilis*** venom, **textilinin-1**, was submitted to a preclinical developmental program and has been shown to be equally effective as aprotinin, an anti-fibrinolytic agent that reduces the blood loss associated with cardiac surgery, but with an enhanced safety profile (Flight et al., 2005).

Certain serpins are able to reduce the excess protease activity and consequent damage associated with inflammatory diseases. SERP1 from the myxoma poxvirus, for example, inhibits human coagulation and fibrinolytic proteases and has been shown to have potent anti-inflammatory effects in the treatment of human inflammatory diseases induced by vascular injuries. Therefore, *Loxosceles* protease inhibitors emerge as compounds with potential therapeutic and biotechnological applications, which, in turn, depend on the further characterization of their biochemical and biological features.

7.5. Venom allergen

Hypersensitivity reactions from arthropod stings include immediate reactions, such as local swelling,

generalized urticaria and anaphylaxis. The pathogenesis is, in many cases, most likely an IgE-mediated reaction. Delayed reactions are also possible, for example, local papules or bullous, hemorrhagic reactions, disseminated papules, generalized papular urticaria and general systemic symptoms, such as fever, myalgia and lymphadenopathy. In some studies, deposits of complement components and immunoglobulins have also been found (Arlian, 2002; Bircher, 2005).

With the exception of bee and wasp venom allergies, the immediate type allergic reactions to arthropod stings and bites, such as mosquitoes, flies, ticks, moths, caterpillars and spiders, are rare (Bircher, 2005). Indeed, allergic reactions to the *Loxosceles* genus have been postulated in only a few cases (Donepudi et al., 2005; Robb et al., 2007; Makris et al., 2009; Lane et al., 2011). In approximately 25% of the published loxoscelism cases, a fine macular or papular eruption develops over the entire body (Pippirs et al., 2009). Cases of AGEP (Acute Generalized Exanthematous Pustulosis) following bites by *L. reclusa* and *L. rufescens* have also been reported (Makris et al., 2009; Lane et al., 2011).

The pathogenesis of AGEP is not clear, but it is a rare and severe cutaneous reaction usually triggered by drugs and viruses (Makris et al., 2009). Of note, several studies have shown that *Loxosceles* venom stimulates the release of large amounts of IL-8 and GM-CSF, in addition to other cytokines, such as the growth-related oncogene and protein-1 (Gomez et al., 1999). This release of IL-8 and GM-CSF could contribute to the development of AGEP following *Loxosceles* envenomation (Lane et al., 2011). In addition, the ability of this venom to evoke inflammatory events was partially reduced in compound 48/80 –pretreated animals, suggesting that mast cells may be involved in these responses. Pre-treating mice with receptor antagonists of histamine (prometazine and cetirizine) and of serotonin (methysergide) significantly attenuated the edema and vascular permeability induced by toxins (Paludo et al., 2009).

Corroborating the hypothesis that *Loxosceles* venom may cause allergic reactions, two transcriptome studies on *Loxosceles* venom glands found sequences similar to allergen-like toxins from other venoms. In the case of *L. laeta*, transcripts similar to venom allergen III (sp|P35779|VA3_SOLRI) represented 0.6% of the total sequences. The similarity of the putative amino acid sequence of an allergen from *L. laeta* with known venom allergen III sequences includes the presence of conserved cysteine residues (Fernandes-Pedrosa et al., 2008). Data from work with the cDNAs of the *L. intermedia* venom gland showed that some messages encode for venom allergens that are cysteine-rich molecules. These RNA messages are poorly expressed: two ESTs are grouped in one cluster representing 0.2% of the toxin-encoding transcripts. These transcripts putatively encode for allergens that show significant similarity to allergens from another spider genus (*Lycosa sigoriensis*), scorpion species (*Opisthacanthus cayaporum*) and some mite allergens (*Ixodes scapularis* and *Argas monolakensis*) (Gremski et al., 2010). In addition, an *L. intermedia* venom proteomic study also reported the presence of a putative allergenic protein similar to a mite allergen (dos Santos et al., 2009).

Some of the allergens have been characterized, and a few of them have been synthesized through recombinant techniques (Bircher, 2005). An isoform of a recombinant allergen from *L. intermedia* venom was cloned and had a calculated molecular mass of approximately 46.2 kDa and a predicted hydrophobic import signal (24 residues) to the endoplasmic reticulum (Ferrer, V.P. and de Mari, T.L. personal communication, 2013). With the availability of allergen sequences and purified recombinant allergens, allergen-specific cellular immune responses were investigated, and *in vivo* animal models based on defined and clinically relevant allergens were established (Valenta et al., 2011). In this context, the crystal structures from some recombinant allergens derived from insect venoms (wasps, bees, and fire ants) have been important in the search for specific or cross-reacted epitopes (Henriksen et al., 2001; Hoffman, 2008; Padavattan et al., 2008; Borer et al., 2012). Additionally, recombinant allergens were applied for *in vivo* provocation testing in allergic patients with the aim of comparing their biological activity to natural allergens and to explore their usefulness for *in vivo* diagnosis (Schmid-Grendelmeier and Crameri, 2001; van Hage-Hamsten and Pauli, 2004). These studies confirmed the biological equivalence of most of the recombinant allergen preparations with the corresponding natural allergens, indicating that the recombinant allergens can substitute for natural allergen extracts for *in vivo* applications (Valenta et al., 2011). In this way, the allergen-like toxin from *L. intermedia* venom may be a useful tool for investigating the underling mechanisms of allergic responses following spider bites involving this venom and might serve biomedical purposes in this area.

8. Modulation of cell and tissue structures by brown spider venom toxins

Brown spider venom toxins have been implicated in a number of histological changes following spider bites or experimental envenomation under laboratory conditions (Ospedal et al., 2002; da Silva et al., 2004; Hogan et al., 2004; Swanson and Vetter, 2006). The first and most characteristic tissue/cellular changes observed after brown spider venom exposure is the massive infiltration of inflammatory cells into the dermis and the generation of inflammatory mediators near the bite site or toxin injection (Ospedal et al., 2002; Domingos et al., 2003; Barbaro et al., 2010). Recombinant brown spider phospholipases D reproduce the above-mentioned histological changes (Chaim et al., 2006; da Silveira et al., 2006, 2007b; Ribeiro et al., 2007; Appel et al., 2008).

Although the modulation of leukocyte activity is demonstrated by the massive infiltration of skin structures, *Loxosceles* venom apparently has no direct stimulatory effects on leukocytes in culture, and leukocyte activation represents an indirect effect triggered by the endothelial cells of blood vessels exposed to the venom toxins. This hypothesis is supported by data from cell culture assays using human umbilical vein endothelial cells (HUVEC) treated with *L. reclusa* crude venom. The results pointed to a potent endothelial cell agonist activity of the venom, which stimulated the endothelial cell expression of E-

selectin and the secretion of the granulocyte macrophage colony-stimulating factor and interleukin-8, which resulted in a dysregulated inflammatory response (Patel et al., 1994). The treatment of HUVEC with *L. deserta* crude venom leads to the expression of a growth-related oncogene and to the synthesis and secretion of the monocyte chemoattractant protein-1 and interleukin-8 (Desai et al., 1999; Gomez et al., 1999). In addition, *L. deserta* venom evokes the expression of vascular endothelial growth factor (VEGF) in human keratinocytes (Desai et al., 2000), suggesting that VEGF may contribute to the endothelial activation observed after brown spider envenomation. Additional data from histopathological findings revealed that *L. intermedia* venom acts *in vivo* (intradermally injected) on rabbit vessel endothelial cells, which causes an endothelial-leukocyte adhesion, a massive transmigration of leukocytes across the endothelium, vessel instability, the degeneration of blood vessels and vascular leakage (Veiga et al., 2001b; Ospedal et al., 2002; Zanetti et al., 2002). Exposing cultured rabbit aorta endothelial cells (RAEC) to *L. intermedia* crude venom evokes the disadhesion of the cells and the degradation of heparan-sulfate proteoglycans, nidogen/entactin and fibronectin (Veiga et al., 2001a; Paludo et al., 2006). Moreover, the direct binding of the venom toxins on the endothelial cell surface has also been reported, which induces drastic morphological changes (Paludo et al., 2006). These data are supported by the internalization of the toxins following endothelial cell treatment with the *L. intermedia* crude venom, the involvement of endocytic vesicles and the final homing of toxins to lysosomes, culminating in cell death by anoikis (Nowatzki et al., 2010).

The direct binding of a recombinant *L. intermedia* phospholipase D on the surface of RAEC has also been reported, as well as the catalytic activity of this toxin to degrade RAEC membrane detergent-extracts, which generates important bioactive lipids and cell morphological changes (Chaim et al., 2011b). Additionally, by using cultured human fibroblasts exposed to a recombinant phospholipase D isoform from the *L. reclusa* venom, an upregulation of the human cytokines genes IL-6, IL-8, CXCL1, CXCL2 that are important inflammatory activators has been demonstrated (Dragulev et al., 2007). The authors postulated that together with the endothelia, the fibroblasts in the dermis also mediate the dysregulated leukocyte activation involved in dermonecrosis and are an additional cellular target for the venom toxins.

Other cells targeted by *Loxosceles* venom toxins are erythrocytes. The hemolytic activity evoked by *Loxosceles* venom was first demonstrated using clinical and laboratory observations from spider bite victims, some of which had a lethal outcome. These observations included elevated creatine kinase levels, hemoglobinuria, bilirubinuria, proteinuria, jaundice, acute hemolytic anemia, reticulocytosis, and shock (Lung and Mallory, 2000; França et al., 2002; Zambrano et al., 2005; de Souza et al., 2008; McDade et al., 2010; Malaque et al., 2011). The hemolytic activity is a conserved event because it has also been reported for the *L. similis*, *L. gaucho*, *L. laeta*, *L. reclusa* and *L. intermedia* venoms (da Silva et al., 2004; Silvestre et al., 2005; Zambrano et al., 2005; McDade et al., 2010; Malaque et al., 2011). The lysis of erythrocytes is associated with two distinct mechanisms, which are defined as direct (Chaves-Moreira et al., 2009, 2011) and complement-dependent hemolysis (Tambourgi et al., 2002, 2005, 2007; Pretel et al., 2005). Various studies have already reported that spider envenomation induces the activation of the alternative complement pathway facilitating complement-mediated hemolysis (da Silva et al., 2004; Swanson and Vetter, 2006). Tambourgi et al. (2002, 2005) evaluated the mechanism of hemolysis caused by *L. intermedia* venom and proposed the involvement of phospholipases D in the activation of an endogenous metalloprotease, which then cleaves glycophorins and causes erythrocyte susceptibility to lysis by human complement. In another study, the functional changes of rabbit erythrocytes were described following exposure to *L. gaucho* crude venom. The results pointed to an alteration of red cell function using an osmotic fragility test and greater deformability after venom exposure (Barretto et al., 2007).

Chaves-Moreira et al. (2009) compared the hemolytic activities of a recombinant active phospholipase D from *L. intermedia* venom (LiRecDT1) (Chaim et al., 2006) and its mutated version (LiRecDT1H12A) (Kusma et al., 2008). They demonstrated the involvement of a direct molecular mechanism dependent on the catalytic activity of phospholipase D in hemolysis, strengthening previous data that reported the participation of dermonecrotic toxins in red blood cell lysis. Furthermore, the metabolism of membrane phospholipids, such as sphingomyelin and lysophosphatidylcholine, and the influx of calcium mediated by an L-type channel in human erythrocytes have been shown to be involved in hemolysis (Chaves-Moreira et al., 2011).

Along with erythrocytes, platelets represent another target of brown spider venom. Data indicating platelets as a target were first described in biopsies of animal models exposed to crude venom, which reported the intravascular coagulation and thrombosis inside the dermal blood vessels. The hypothesis of platelet involvement is also strengthened by the findings of the infiltration and aggregation of inflammatory cells in the generated thrombus. In addition, clinical laboratory analysis of envenomed victims often reveals thrombocytopenia (Ospedal et al., 2002; da Silva et al., 2004; Hogan et al., 2004; Swanson and Vetter, 2006). Cellular changes in the bone marrow and peripheral blood of rabbits caused by *L. intermedia* venom include the marrow depression of megakaryocytes correlated with the thrombocytopenia in the peripheral blood observed in the early envenomation (da Silva et al., 2003). Similar results were reported by using *L. gaucho* venom, where a decrease in the platelet count in rabbit peripheral blood after venom and/or purified phospholipase D exposure, without platelet aggregation and no signs of platelet lysis, was observed. The activation of platelets after venom exposure is supported by the increased expression of ligand-induced binding site 1 and P-selectin (Tavares et al., 2004, 2011).

By studying recombinant venom phospholipases D and using human platelet-rich plasma, *in vitro* platelet aggregation activity has been reported (da Silveira et al., 2006, 2007b; Appel et al., 2008), strengthening the hypothesis of venom activity on platelets. However, the molecular pathway by which the toxins cause platelet aggregation is

not fully understood. In the case of phospholipases D, catalysis involvement is expected, such that it would generate a broad range of bioactive lipids on the platelet membrane and finally inducing aggregation.

Renal structures and kidney cells represent other targets for brown spider venom toxins. Renal injury was previously described in clinical and laboratory data from injured patients (da Silva et al., 2004; Hogan et al., 2004; Swanson and Vetter, 2006). Clinical case reports have indicated a direct correlation of renal damage and hemolysis induced by different *Loxosceles* venoms (Zambrano et al., 2005; Abdulkader et al., 2008; de Souza et al., 2008; Hubbard and James, 2011; Malaque et al., 2011). By studying experimental-induced kidney injury in rats exposed to *L. gaucho* crude venom, Lucato et al. (2011) concluded that this acute injury was a consequence of the impaired renal blood flow associated with the systemic rhabdomyolysis. Nevertheless, animal models or cultured MDCK epithelial cells exposed to *L. intermedia* crude venom or a recombinant phospholipase D have also provided evidence for a direct renal cytotoxicity (Luciano et al., 2004; Chaim et al., 2006; Kusma et al., 2008). Using mice exposed to a recombinant phospholipase D and a mutated isoform exhibiting decreased phospholipase activity, is has been shown that *in vivo* nephrotoxicity and *in vitro* MDCK cytotoxicity depends on the catalytic activity of the phospholipases (Kusma et al., 2008).

The activities of *Loxosceles* venom have also been demonstrated in other tissues and organs, such as the heart and liver. Dias-Lopes et al. (2010) showed cardiotoxic effects in mice administered *L. intermedia* venom. These effects were observed through the increase of the levels of markers associated with heart lesions, indicating that the venom antigens can reach the heart tissue and, thus, lead to cardiac dysfunction. Christoff et al. (2008) reported changes in histological and biochemical aspects of the liver in rats injected with *L. intermedia* crude venom. In the venom-treated group, the plasma levels of enzymes, such as alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl-transferase and lactate dehydrogenase, increased. Furthermore, histopathological changes indicating hepatic lesions were also observed.

Finally, the extracellular matrix and associated constituents represent a key histological structure targeted by *Loxosceles* venom toxins, as observed by the histopathological findings of hemorrhage into the dermis at the bite site, fibrin deposition inside the blood vessels, defective wound healing after bites, gravitational lesion spreading and the systemic dissemination of toxins (da Silva et al., 2004; Swanson and Vetter, 2006). Previous work also described the proteolytic activities of *L. intermedia* venom on gelatin, fibronectin and fibrinogen (Feitosa et al., 1998; Veiga et al., 2001a; Zanetti et al., 2002; Paludo et al., 2006), the disruption of EHS basement structures, the degradation of entactin/nidogen and the hydrolysis of heparan sulfate proteoglycan from endothelial cells (Veiga et al., 2001a, b) and more recently the involvement of a hyaluronidase in the noxius activity of venom (Ferrer et al., 2013) (See **topic** 7.2).

9. *Loxosceles* spider toxin immunology and perspectives for development of a new generation of antivenoms

Spiders and other venomous animals contain a complex mixture of biologically active substances developed to block the vital physiological and biochemical functions of the victims. Antidotes prepared from animal anti-sera are effective against all species of *Loxosceles* spiders; these antivenoms are less complex than other spider or snake antivenoms because the major toxic components of these spider venoms are proteins known as dermonecrotic toxins, sphingomyelinases D (SMases D), the phospholipase D family and Loxtox proteins (Kalapothakis et al., 2007; Binford et al., 2009; Wood et al., 2009). Although significant progress has been made in immunological studies of these groups of toxins, few medical and university centers are dedicated to this subject of research. Novel approaches based on epitopes and mimotopes selected from microarray peptides (Spot-synthesis) or from phage-displayed random peptide libraries have generated information sufficient to develop a new generation of antivenoms for therapeutic or vaccines purposes. The immunological investigation of the phospholipase D of *Loxosceles* spiders was mainly performed with LiD1, a dermonecrotic toxin from the venom of the *L. intermedia*, by Chávez-Olórtegui and his collaborators (Kalapothakis et al., 2002; Araújo et al., 2003; Felicori et al., 2006, 2009; Dias-Lopes et al., 2010). The cDNA encoding this protein was shown to display a similarity with the genes of the known *Loxosceles* phospholipase D toxins (Kalapothakis et al., 2007). The recombinant protein rLiD1 was strongly recognized by anti-*L. intermedia* crude venom and was also able to generate reactive antibodies against the native dermonecrotic proteins and whole *L. intermedia* venom. Using these antibodies and overlapping synthetic peptides covering the whole (LiD1) sequence, regions with an epitope function were revealed. The N-terminal (residues 13–27), central (residues 31–45, 58–72, 100–114, and 160–174) and C-terminal (residues 247–261) parts of the protein have been shown to contain continuous epitopes with neutralizing potential. The antibodies elicited by these epitopes were found to protect against the dermonecrotic-, hemorrhagic- and edema-forming activities induced by LiD1 and whole venom. To visualize the three-dimensional position of the experimentally determined epitopes, the LiD1 protein was modeled by homology using the solved structure of phospholipase D from *L. laeta as a template*. The localization of the epitopes in the context of the three-dimensional structure of the dermonecrotic protein is shown in Fig. 8. As shown, most of epitope regions determined were localized in the α-helix-loop regions. However, 3 of the 8 α-helix regions were not antigenic (Felicori et al., 2009).

Studies using monoclonal antibodies raised against the toxins of the *L. intermedia* whole venom revealed that one antibody (LimAb7) recognized several venom proteins, including LiD1 (Alvarenga et al., 2003). Because LimAb7 reacts with rLiD1, the LiD1 epitope recognized by LimAb7 was mapped. None of the overlapping peptides that

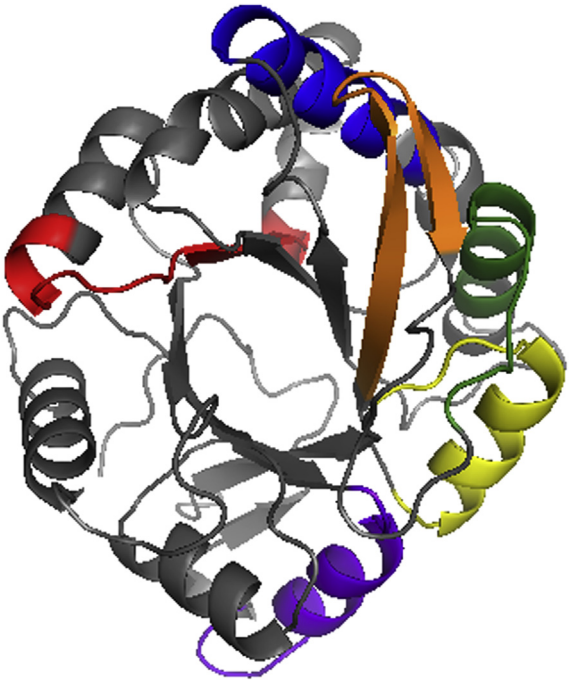

Fig. 8. LiD1 structural model indicating the position of the selected epitopes that reacted with the horse anti-*L. intermedia* venom serum. In yellow, the N-terminal epitope (residues 13–27); in orange, the central epitope (residues 31–45); in green, the central epitope (residues 58–72); in blue, the central epitope (residues 100–114); in red, the central epitope (residues 160–174); and in purple, the C-terminal epitope (residues 247–261). Figure color codes refer to the on-line images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

covered the sequence of LiD1 were recognized, indicating that the epitope is not continuous but rather, discontinuous (de Moura et al., 2011). Consequently, the phage-display technique was used, and this method allowed for the identification of mimotopes without homology between the amino acid sequences of the phage-selected peptides and the sequence of LiD1 (de Moura et al., 2011). The potential epitope regions in LiD1 based on the amino acid sequences of the selected mimotopes and on the 3D LiD1 protein model were predicted, and the MIMOP residues C^{197}, Y^{224}, W^{225}, T^{226}, D^{228}, K^{229}, R^{230}, T^{232} and Y^{248} were identified as being the putative epitope bound by LimAb7 (de Moura et al., 2011).

In a recent study, a chimeric protein (rCpLi) expressing the epitopes of LiD1 previously defined as residues 25–51 and 58–72 and a conformational epitope identified by the phage display technique were generated by cloning the respective synthetic genes in a pET 26b vector. ELISA and immunoblot assays revealed that the mini-protein displayed antigenic activity against the antibodies of the anti-individual epitopes. Anti-*Loxosceles* sp. crude venoms also reacted with rCpLi. Because the protein is non-toxic, it is considered to be an important immunogen target for vaccines against this dangerous regional spider (Mendes et al., 2013).

10. Biotechnological use of brown spider venom components

Spider venoms are mixtures of several hundred biologically active proteins, glycoproteins and peptides that act synergistically as an adaptation to defend against predators and to paralyze and kill insect prey. Because these toxins are active on different cells and tissue structures and effectively modulate distinct physiological responses in insects and vertebrates, they are potential models to study the design of pharmacological tools, drugs and/or biochemical, immunological and cell biology reagents.

The first biotechnological application of brown spider venom constituents consisted of the antiserum-based products used for the therapy of spider bite victims. For example, anti-arachnid serum (obtained using the venom of *L. gaucho*) was produced by the Butantan Institute, São Paulo, Brazil; anti-*Loxosceles* serum using the *L. intermedia*, *L. gaucho and L. laeta* crude venoms was produced by the Production Center of Immunobiologic Products, Parana, Brazil; and anti-*Loxosceles* serum raised against *L. laeta* venom was produced by the National Institute of Health (Peru) (Roodt et al., 2002; da Silva et al., 2004; Pauli et al., 2009). With the recombinant brown spider venom toxins available, a new generation of loxoscelic antiserum could be produced directly by using the antigenic active recombinant toxins from different *Loxosceles* species or by enriching the crude venom with biologically active recombinant molecules to increase antibody production and venom neutralization. In fact, a recombinant phospholipase D was used to produce an anti-*Loxosceles* serum that was able to neutralize the toxic effects induced mainly by the *L. intermedia* and *L. laeta* or slightly weaker the activity of *L. gaucho* venoms (de Almeida et al., 2008). Additional recombinant antigens should be used as antigenic sources for vaccines or antivenom development (See **topic** 10).

Another *Loxosceles* venom-based product is named ARACHnase (Hemostasis Diagnostics International Co., Denver, CO, USA). It consists of plasma containing *L. reclusa* crude venom that mimics the presence of a lupus anticoagulant and should be a useful positive control for lupus anticoagulant testing (McGlasson et al., 1993).

Based on their properties, brown spider recombinant phospholipases D could be used as putative models for the application in the different areas of cell biology, immunology, pharmacology and biochemistry. They can be used, for example, as reagents for biochemical lipid research protocols by generating bioactive lipids, such as ceramide-1-phosphate from the hydrolysis of sphingomyelin and lysophosphatidic acid from the hydrolysis of lysoglycerophospholipids (Lee and Lynch, 2005; Chaim et al., 2011b). In addition, they may be applied in cell biology studies that investigate the biological activities triggered by ceramide-1-phosphate, lysophosphatidic acid and their derived molecules, such as the control of cell proliferation, death, differentiation and migration (Anliker and Chun, 2004; Chalfant and Spiegel, 2005). *Loxosceles* recombinant phospholipases D or mutated isoforms (Kusma et al., 2008; Chaim et al., 2011b; Mendes et al., 2013) could also be used as immunological adjuvant molecules for stimulating

immunogenicity because they can modulate inflammation and stimulate the production of cytokines in different cell models. Recombinant phospholipases D could be used as standard laboratory reagents to investigate platelet aggregation, platelet receptor(s), and related molecular pathways. In addition, these recombinant proteins could be used as reagents to induce hemolysis, possibly establishing a new model of hemolysis dependent on phospholipase D and bioactive lipids. Furthermore, recombinant phospholipases D could be used as reagents applied in the diagnosis of loxoscelism because a clinical laboratory diagnosis is currently unavailable. Because brown spider venom phospholipases D are strong antigenic molecules and are highly expressed molecules in crude venom (Ribeiro et al., 2007; Gremski et al., 2010; Wille et al., 2013), recombinant phospholipases D could be used as antigens for generating polyclonal or monoclonal antibodies for the diagnosis of Loxoscelism (de Moura et al., 2011).

ICK peptides have been studied as potential insecticidal bioactive toxin molecules, and recombinant brown spider ICK toxins (Matsubara et al., 2013) could be used as substitutes for chemical defense products as well as in transgenic agricultural models, **if further studies show that they specifically act upon insect channels**. In addition, brown spider ICK peptides could also be useful reagents to probe ion channel structures and functions, as previously described for other similar molecules (Dutertre and Lewis, 2010; Klint et al., 2012).

Recombinant *Loxosceles* hyaluronidase (Ferrer et al., 2013) could be used as a reagent in the biochemical studies of glycosaminoglycan hydrolases and as a tool to design **specific** inhibitors to reduce the spread of venom and toxins **retaining the activity of native hyaluronidases**. Moreover, because hyaluronidases are involved in bacterial pathogenesis, fertilization, and cancer progression, recombinant brown spider hyaluronidase could be utilized to generate hyaluronidase inhibitors that regulate several pathological events involving the balance between the anabolism and catabolism of HA. Finally, it could be used as an adjuvant molecule to increase drug absorption through increased tissue permeabilization (da Silveira et al., 2007c; Ferrer et al., 2013).

Recombinant brown spider venom astacins (da Silveira et al., 2007a; Trevisan-Silva et al., 2010) could be used as tools in the study of extracellular matrix remodeling, for the generation of proteolytic inhibitors and as direct thrombolytic agents for the treatment of vascular diseases.

Finally, other brown spider venom constituents also have putative biological applications. These include serine protease inhibitors, which could be useful agents for the investigation of general proteolysis, and recombinant TCTP (Sade et al., 2012), which could be used to study tumor cell behavior in experimental oncology, to study cell proliferation mechanisms, in the screening of anticancer drugs and as a model for allergy screening. Recently, the N-terminal fragment of TCTP (MIIYRDLISH) was shown to function as a **P**rotein **T**ransduction **D**omain (PTD), which is cell-penetrating peptide. This new feature of TCTP is being studied in drug delivery systems development (Maeng et al., 2013). Detailed data on the putative biotechnological use of brown spider venom toxins have been provided in Senff-Ribeiro et al. (2008) and Chaim et al. (2011a).

11. Future directions

Although many scientific studies have been published in recent years examining the brown spider venom and loxoscelism that have brought insights and improved the knowledge base regarding these topics, there are several opened questions still to be answered, and the challenges and opportunities for researchers are enormous. The use of combined data from molecular biology techniques, bioinformatics, proteomic studies, transcriptome analysis, and the expression of recombinant toxins will open great possibilities in this field.

The challenges concerning brown spiders and loxoscelism can be divided into clinical and basic research. The first clinical challenge is the production of a new generation of antisera using purified recombinant brown spider bioactive and antigenic competent toxins individually or by enriching crude venoms with recombinant toxins. Such antisera would be monospecific and may be used at lower doses, thereby decreasing the deleterious side effects of serum therapy, but with the same or higher efficiency in neutralizing the noxious venom activities.

A second clinical challenge hinges on the improvement of treatment of the injured victims. Currently, loxoscelism treatment is empirical and based on clinical signs, as described above (**topic** 4). A molecular comprehension of brown spider venoms and the mechanism by which the toxins trigger their effects, together by obtaining recombinant toxins and toxin 3D structural/biological data, will open to the possibility of a rational design of synthetic inhibitors directed at the specific venom toxins involved in the local and systemic effects. In addition, inhibitors for the receptors or cellular molecules involved in the metabolism of the bioactive lipid mediators generated by phospholipases D could also produce novel and powerful tools for the treatment of loxoscelism. Another clinical hurdle is the development of a clinical, *in vitro* diagnostic for loxoscelism with the sensitivity and specificity for different brown spider species. This diagnostic would be based on low-invasive molecular biology techniques and would provide sensitivity and specificity for different brown spider species using blood or even urine taken from exposed victims.

Finally, because loxoscelism is a public health problem around the world, the development of rational biological control methods, which are currently not available, is necessary to decrease the number of spiders and domiciliary infestations in the endemic regions. Similar methods are currently used for agricultural plagues, utilizing bacteria, fungus or other natural predators (Boyer et al., 2012; Lockett et al., 2012) to replace the pesticides currently used as a nonspecific biological control method and which cause environmental and human hazards.

The future of basic research on brown spider venoms and/or loxoscelism represents a remarkable challenge. Although considerable growth in this field has occurred, a great number of molecules in brown spider venoms remain

unidentified or their biological effects and mechanisms have not been described, especially for toxins with low level of expression and/or novel toxin isoforms of previously described. In addition, a genomic project focusing on *Loxosceles* species is a rational future direction that will bring novel insights for brown spider biology and loxoscelism and that will create access for several novel research tools.

Another current challenge for the brown spider venom toxinologist is the access to purified recombinant toxins in models other than bacteria. Currently, all recombinant brown spider venom toxins obtained have been produced in bacteria, an inexpensive expression model system that is simple to manipulate. However, because it does not generate co- and post-translational modifications, such as N-glycosylation and disulfide bonds, bacteria model systems often produce recombinant molecules in their unfolded form, with incorrect conformations, water insolubility, and with no biological function. The synthesis of brown spider venom recombinant toxins using alternative expression models with additional features that optimize and refine this process, such as the yeast *Pichia pastoris*, the insect *Drosophila* Schneider cells and mammalian systems, is an immediate challenge.

Another future direction is to obtain native brown spider venom toxins by developing primary cultures of the venom secretory cells. Similar approaches have been successfully established for other venomous animals. Examples include culturing the secretory cells from the venom glands of snakes *Crotalus durissus terrificus* and *Bothrops jararaca* (Duarte et al., 1999; Yamanouye et al., 2007) and from the *Phoneutria nigriventer* spider (Silva et al., 2008). These cultures could produce and secrete sufficient amounts of native toxins to be useful for biological and biotechnological evaluation. The use of mass spectrometry analysis and other proteomic protocols, such as 2-DE, N-terminal amino acid sequencing and high efficiency chromatography, provide great promise for detailed studies of brown spider venoms and hemolymph proteins and peptides. To date, only two studies have addressed this topic: Machado et al. (2005) described eleven isoforms of the phospholipase D toxin in *L. gaucho* venom, and dos Santos et al. (2009) identified 39 proteins in *L. intermedia* venom. There are no reports of this type of study for hemolymph.

Additionally, in the near future, novel data will provide information related to the tridimensional toxin structures, which will require the experimental co-crystallization of putative ligands or substrates to recombinant toxins. The tridimensional analysis of brown spider venom toxins will be critical to elucidate the location of the catalytic sites and sites that interact with natural substrates or ligands, and especially to show how the toxins interact with cell structures. These analyses will allow for the development of synthetic ligands, analogs, or inhibitors.

Finally, an attractive and practically unknown model for studying *Loxosceles* spiders is the analysis of hemolymph contents and its relationship to venom toxins and loxoscelism. From such analysis, natural inhibitors and/or other important molecules could be discovered, which would add great value to the field of toxinology.

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Conflict of interest statement

None.

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