UNIVERSIDADE FEDERAL DE PERNAMBUCO

Mary Ângela Aranda de Souza

AVALIAÇÃO DO MECANISMO DE MORTE CELULAR INDUZIDA PELA LECTINA DA PEÇONHA DE *Bothrops leucurus* (BlL): CORRELAÇÃO COM TRANSIÇÃO DE PERMEABILIDADE MITOCONDRIAL

Recife

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CENTRO DE CIÊNCIAS BIOLÓGICAS
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Orientadora: Maria Tereza dos Santos Correia
Co-orientador: Aníbal Eugênio Vercesi

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Recife

2012

"If the doors of perception were cleansed, every thing would appear to man as it is: infinite" (em pt: **Se as portas da percepção fossem abertas, tudo apareceria como realmente é: infinito**).

Aldous Huxley

SUMÁRIO

	Pág.
AGRADECIMENTOS	ix
RESUMO	x
ABSTRACT	xi
LISTA DE ABREVIATURAS	xii
LISTA DE FIGURAS	xiv
LISTA DE TABELAS	xv
1. INTRODUÇÃO	16
1.1 Lectinas	16
1.1.1 Peculiaridades	16
1.1.2 Classificação	18
1.1.2.1 Classificação das lectinas quanto a especificidade ao carboidrato	18
1.1.2.2 Estrutura molecular das lectinas	20
1.1.3 Purificação e caracterização de lectinas	22
1.1.4 Lectinas de animais	23
1.1.4.1 Lectinas Tipo C	24
1.2 Bothrops leucurus	24
1.3 Mitocôndrias	26
1.3.1 Bioenergética mitocondrial	26
1.3.2 Geração Mitocondrial de Espécies Reativas de Oxigênio	28
1.3.3 Transição de Permeabilidade Mitocondrial e Morte Celular	29

1.4 Morte Celular, Mitocôndrias e Lectinas 32

2. OBJETIVOS 34

2.1. Objetivo Geral 34

2.2. Objetivos Específicos 34

3. RESULTADOS 35

3.1. PARTE I 35

3.2. PARTE II 41

4. CONCLUSÃO 55

BIBLIOGRAFIA 56

CAPÍTULO I 65

CAPÍTULO II 73

ANEXO I 78

ANEXO II 80

ANEXO III 82

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Resumo

As lectinas são proteínas ou glicoproteínas com a capacidade de aglutinar eritrócitos e outras células, precipitar polissacarídeos e outras glicoproteínas por possuírem um (ou mais) centro de ligação, específico e reversível, a carboidratos. BlL é uma lectina ligante de galactosídeo, e foi purificada do veneno da serpente *Bothrops leucurus* através da cromatografia de afinidade em gel de guar. A atividade antitumoral de BlL foi avaliada em relação ao seu potencial citotóxico em linhagem tumoral (K562, Hep-2 e NCI-H292) e quanto à sua capacidade hemolítica. BlL apresentou uma significante atividade citotóxica em todas as linhagens tumorais testadas e não exibiu atividade hemolítica na máxima concentração testada (2000 μg/mL). Além disso, foi realizada em células K562, a análise da externalização da fosfatidilserina e potencial de membrana mitocondrial, utilizando microscópio de fluorescência. Tratamento com BlL induziu externalização da fosfatidilserina e despolarização mitocondrial, indicando morte celular por apoptose. A diminuição significativa da viabilidade de células B16-F10 de melanoma após tratamento com BlL também foi observada. A morte celular foi provavelmente decorrente dos aumentos prévios nas concentrações de cálcio intracelular e de espécies reativas de oxigênio (EROs) mitocondrial. A incubação de células (10^6/mL) na presença de BlL (75 μg/mL) e Ca^{2+} (20 μM) induziu diminuição da permeabilização de membrana plasmática induzida por digitonina (20 μM), seguida possivelmente por influxo da lectina que se acumulou preferencialmente na mitocôndria. Quando testada em mitocôndria de fígado de rato (MFR), a velocidade de consumo de oxigênio durante o estado 3, utilizando ADP como substrato respiratório, não apresentou redução significativa na presença de BlL (10 μg/mL). No entanto, BlL apresentou efeito estimulatório na respiração durante o estado 4, sendo a lectina responsável por um estímulo de 57,1%. Este estímulo do consumo de oxigênio no estado respiratório 4 se refletiu na redução do controle respiratório. A razão ADP/O em suspensões mitocondriais na condição controle e na presença de BlL foi de 2,5 ± 0,07 e 2,3 ± 0,04 ($p<0,05$), respectivamente, esta diferença indica uma perda da eficiência da fosforilação oxidativa de 8 %. Os parâmetros de potencial de membrana mitocondrial (ΔΨm) também foram avaliados após incubação das suspensões mitocondriais com BlL. Houve significativa diminuição do ΔΨm, sensível a Ciclosporina A (CsA), e estímulo a abertura do poro de transição de permeabilidade mitocondrial (PTPM). Em conjunto, estes resultados indicam que o efeito citotóxico de BlL provavelmente está relacionado com o efeito de transição de permeabilidade mitocondrial (TPM).

Palavras chave: *Bothrops leucurus*, lectina, morte celular, mitocôndria

Abstract

Lectins are proteins or glycoproteins which agglutinate erythrocytes and other cells and polysaccharides, since they have one or more specific carbohydrate-binding sites. BlL is a galactoside-binding lectin, and was purified from the venom of *Bothrops leucurus* through affinity chromatography on guar gel. Antitumor activity of BlL was assessed in relation to their cytotoxic potential in type of tumor (K562, Hep-2 e NCI-H292) and the its hemolytic capacity. BlL showed a significant cytotoxic activity in all tumor cell lines tested and showed no hemolytic activity at maximum concentration tested (2000 µg/mL). Furthermore, it was held in K562 cells, analysis of externalization of phosphatidylserine and mitochondrial membrane potential using fluorescence microscope. BlL treatment induced phosphatidylserine externalization and mitochondrial depolarization, indicating cell death by apoptosis. The significant decrease in B16-F10 melanoma viability after treatment with BlL was also observed. Cell death was probably due to previous increases in the concentrations of intracellular calcium and mitochondrial reactive oxygen species (ROS).Incubation of cells (10^6/mL) in the presence of BlL (75 mg / mL) and Ca^{2+} (20 mM) induced decrease in plasma membrane permeability induced by digitonin (20 mM), followed possibly by influx of lectin which has accumulated preferably in mitochondria. When tested in rat liver mitochondria (RLM), the rate of oxygen consumption during state 3, using ADP as substrate respiratory, showed no significant reduction in the presence of BlL (10 mg/mL). However, BlL present stimulatory effect on respiration in state 4, the lectin being responsible for the stimulation of 57.1%. This stimulation of oxygen consumption in respiratory state 4 was reflected in a reduction in respiratory control. The ADP/O ratio in the mitochondrial suspensions and the control condition in the presence of BlL was 2.5 ± 0.07 and 2.3 ± 0.04 ($p < 0.05$) respectively, the difference indicates a loss of efficiency in the oxidative phosphorylation of 8%. The parameters of mitochondrial membrane potential (ΔΨm) were evaluated after incubation of mitochondrial suspensions with BlL. A significant decrease in ΔΨm sensitive to cyclosporine A (CsA), and opening stimulation of the mitochondrial permeability transition pore (PTP). Together, these results indicate that the cytotoxic effect of BlL is probably related to the effect of mitochondrial permeability transition (MPT).

Keywords: *Bothrops leucurus*, lectin, cell death, mitochondria

LISTA DE ABREVIATURAS

$[Ca^{2+}]$: concentração de cálcio

AA: antimicina A

AH: Atividade hemaglutinante

AHE: Atividade hemaglutinante específica

ANT: “adenine nucleotide transporter” (translocador de nucleotídeos de adenina)

AT(D)P: adenosina tri (di) – fosfato

BSA: Albumina sérica bovina

BSA: Albumina sérica bovina

CCCP: carbonyl cyanide p-(Trifluoromethoxy) hydrazone

CDR: domínio de reconhecimento de carboidrato

CDR: domínio de reconhecimento de carboidrato

ConA: Concavalina A

CsA: ciclosporina A

CTBS: Tampão Tris em NaCl contendo $CaCl_2$

CTE: cadeia de transporte de elétrons

CyD: ciclofilina-D

Cyt-c: citocromo c

EB: Extrato bruto

EDTA: Ácido etilenodiaminotetracético

EGTA: etileno glico – bis(b-aminoetil éter)-N,N,N´,N´-ácido tetraacético

EROs: espécies reativas de oxigênio

$FADH_2$: flavina adenina dinucleotídeo reduzido

Gal: Galctose

GalNac: N-acetilgalactosamina

GlcNac: N-acetilglicosamina

H_2O_2: peróxido de hidrogênio

HA: Hemagglutinating activity

HEPES: (N-[2-Hydroxyethyl]piperazine-N´-[2-ethanesulfonic acid])

LacNac: N-acetil-D-Lactosamina

ManNac: N-acetil-D-manosamina

MnSOD: Mn–superóxido dismutase

NAD^+ : nicotinamida adenina dinuclotídeo (estado oxidado)

NADH: nicotinamida adenina dinuclotídeo (estado reduzido)

$NADP^+$: nicotinamida adenina dinuclotídeo fosfato (estado oxidado)

NADPH: nicotinamida adenina dinuclotídeo fosfato (estado reduzido)

$O_2\bullet^-$: ânion superóxido

OH-: radical hidroxil

pH: Potencial de Hidrogênio

PHA: aglutinina (fitoaglutinina) de Phaseolus vulgaris

Pi: fosfato inorgânico

PTPM: poro de transição de permeabilidade mitocondrial

Rot: rotenona

SDS: Sulfato sódico de dodecila

SDS-PAGE: Eletroforese em gel de poliacrilamida conteno sulfato sódico de dodecila

SHA: Specific HA

TEMED: N,N,N',N'-tetrametil-1,2-diaminometano

TPM: transição de permeabilidade mitocondrial

UCP: proteína desacopladora

UQ: ubiquinona (forma oxidada da coenzima Q)

$\Delta\Psi$ - potencial elétrico de membrana

$\Delta\Psi m$ – potencial elétrico de membrana mitocondrial

LISTA DE FIGURAS

INTRODUÇÃO **Pág.**

Figura 1. Representação esquemática do sítio de ligação glicídico de um monômero de lectina com o efeito de ligações múltiplas addicionais. 17

Figura 2. Classificação das lectinas vegetais conforme característica estrutural 21

Figura 3. *Bothrops leucurus* 25

Figura 4. Versão simplificada da teoria quimiosmótica aplicada à mitocôndria 27

Figura 5. Modelo proposto para explicar a formação do poro de transição de permeabilidade induzido por Ca2+ e EROs na membrana mitocondrial interna 30

RESULTADOS - PARTE I

Fig. 1. Effect of BlL on cell population determined by epifluorescence microscopy. 38

RESULTADOS - PARTE II

Fig 1. BlL reduce B16-F10 melanoma cell viability. 56

Figure 2. GN-13 fibroblast viability after BlL treatment. 60

Figure 3. B16-F10 treatment with BlL (75 µg/mL) promotes oxidative stress and increases [Ca^{2+}]cyt in B16-F10 melanoma cells. 61

Fig. 4 Effect of BlL on Ca^{2+} movements by permeabilized B16-F10 melanoma cells. 63

Figure 5. BlL effect in the mitochondrial respiration. 64

Figure 6. Ca^{2+}-induced dysruption of mitochondrial transmembrane potential ($\Delta\Psi$): Effect of the BlL (10 µg/mL). 68

Figure 7. Mitochondrial swelling induced BlL in vitro. 69

LISTA DE TABELAS

Pág.

INTRODUÇÃO

Tabela 1. Especificidade de ligação de lectinas vegetais a carboidratos 19

Tabela 2. Classificação de lectinas do grupo II em famílias 20

Tabela 3. Categorias de classificação de lectinas animais 22

CAPÍTULO II

Table 1. Mitochondrial O_2 consumption and phosphorylation efficiency. 49

1. INTRODUÇÃO

1.1 Lectinas

Proteínas são as moléculas mais abundantes nos sistemas vivos, um organismo vivo possui cerca de 15% do seu peso seco constituído por elas. Neste contexto, as lectinas apresentam-se como um grupo de macromoléculas da classe das proteínas com as mais variadas funções como: hormonal, enzimática, defesa, nutricional, receptor de sinais celulares, proteínas de transporte bem como funções estruturais (SHARON e LIS 2004).

O termo "lectina" (do latim *lectus*, significa selecionado, escolhido) foi proposto por Boyd e Shapleigh em 1954 para designar um grupo de proteínas que apresentava a característica comum de seletividade na interação com carboidratos. O termo aglutinina é usado como sinônimo para lectina, em referência à habilidade de aglutinar eritrócitos ou outras células (PEUMANS e VAN DAMME 1995). Porém, a definição de lectina mais completa foi formulada por Kocourek & Horejsi citados por Etzler (1985), de acordo com estes autores lectinas são proteínas não pertencentes ao sistema imunológico, porém capazes de reconhecer sítios específicos em moléculas e ligar-se reversivelmente a carboidratos, sem alterar a estrutura covalente das ligações glicosídicas dos sítios (PEUMANS e VAN DAMME 1995, PEUMANS et al. 2001).

A primeira purificação de aglutinina vegetal foi realizada por Summer, em 1919, quando obteve a concanavalina A, Con A (lectina de semente de *Canavalia ensiformis*) através de precipitação salina e cristalização (SHARON e LIS 1987). Agraw e Goldstein, em 1965, descreveram um método pioneiro de purificação de lectinas baseando-se na especificidade de ligação aos carboidratos; Con A, lectina com especificidade de ligação a D-glicose, foi purificada através da adsorção específica em coluna de Sephadex-Dextran, seguida de eluição com solução de D-glicose.

1.1.1 Particularidades de lectinas

As lectinas diferem entre si quanto a sequência de aminoácidos, requerimentos de metais, peso molecular e estrutura tridimensional. Algumas lectinas são específicas, em suas reações com grupos sanguíneos, humanos ABO e MN e subgrupo A1 (SHARON e LIS 1972). Todos estes efeitos são produzidos pela habilidade das lectinas de se ligarem a tipos específicos de açúcares na superfície celular (DESHPANDE e DAMODARAN 1990). Além dessas propriedades, as lectinas podem promover estimulação mitogênica de linfócitos e aglutinação de células cancerosas (LIS e SHARON 1973).

As lectinas, por possuírem sítios específicos de ligação a carboidratos, são capazes de interagir com diversas moléculas dos fluidos biológicos e receptores de superfície celular, agindo como decodificadores das informações trocadas entre moléculas, células e organismos (MISQUITH et al. 1994). Desta forma, estas proteínas vêm sendo utilizadas na investigação científica como uma ferramenta útil na avaliação e no entendimento de diversos sistemas biológicos. Dentre as diversas atividades biológicas atribuídas às lectinas, destacam-se a identificação de grupos sanguíneos, a caracterização de microorganismos, a estimulação mitogênica de células imunes, a detecção e o isolamento de carboidratos em solução nas macromoléculas ou em superfície celular (SHARON e LIS 2001, BEUTH et al. 1995).

As lectinas apresentam grande diversidade estrutural, e o aspecto comum entre elas é a presença de, ao menos, um sítio específico de ligação a carboidrato, denominado "o domínio de reconhecimento de carboidrato" (Figura 1). Em sua plenitude, se ligam a carboidratos ou glicoconjugados em solução ou que estejam conectados ao envoltório celular (PEUMANS et al. 2001, WILLIAM e DRICKAMER 1996). Algumas proteínas possuem um único sítio de ligação a carboidratos; este sítio único não lhes confere a característica funcional de lectinas, como a hemaglutinação; entretanto, não as exclui de pertencer a este grupo. Estas interpretações estendem o termo "Lectina" para moléculas que não foram, inicialmente, caracterizadas como tais (HARISSON 1991).

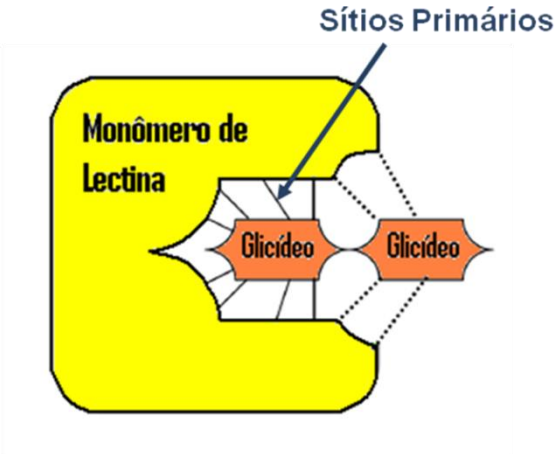

Figura 1. Representação esquemática do sítio de ligação glicídica de um monômero de lectina com o efeito de ligações múltiplas adicionais. As linhas cheias representam o sítio de especificidade primária, ou seja, o sítio determinante do monossacarídeo específico. As linhas tracejadas representam as interações adicionais entre os subsítios da lectina e os demais glicídios que formam o oligossacarídeo. **Adaptado de Kennedy et al. (1995).**

Embora sejam ligantes específicas de carboidratos, as lectinas possuem uma afinidade relativamente baixa por monossacarídeos (na ordem de mM). A seletividade das lectinas por seus alvos naturais dá-se em funcão de múltiplos sítios de ligação adicionais aqueles que determinam a especificiade. Os sítios determinantes da especificidade são denominados sítios primários. A multiplicidade de ligações é proporcionada por subsítios de ligação (ou sítios extendidos) ou por subunidades multivalentes. Em ligações que envolvem subsítios, um monosacáride (carboidrato específico), usualmente terminal, está intimamente ligado no sítio primário da lectina juntamente com os monossacárides posteriores da cadeia de oligossacarídeo (ELGAVISH e SHAANAN 1997).

Em relação às cadeias polipeptídicas, as lectinas são caracteristicamente ricas e aminoácidos ácidos e hidroxilados, associados por interações hidrofóbicas, pontes de hidrogênio e, em alguns casos, pontes dissulfetos (KENNEDY et al. 1995). As especificidades e afinidades dos sítios são alcançadas principalmente por pontes de hidrogênio, com o auxílio de forças de van der Waals e interações hidrofóbicas com resíduos de aminoácidos aromáticos que estão próximos as porções hidrofóbicas de monossacarídeos (SHARON e LIS 2002), contribuindo para estabilidade dos complexos formados.

Os cátions divalentes, tais como o Cálcio (II) e o Manganês (II), estão envolvidos indiretamente no mecanismo molecular de reconhecimento glicídico. As cadeias laterais dos aminoácidos, ácido aspártico e asparagina coordenam esses cátions, a asparagina também faz a ligação de hidrogênio com o glicídio ligante. Portanto, além de servirem para manutenção da integridade das subunidades dessas lectinas, os íons metálicos ajustam os resíduos de aminoácidos envolvidos no sitio ligante glicídico, auxiliando-os na interação com o carboidrato; no entanto não interagem diretamente com o mesmo. Nas lectinas animais Tipo C, o carboidrato ligante coordena diretamente com íon Cálcio (II) por meio de duas hidroxilas e as demais são estabelecidas com as cadeias laterais dos aminoácidos asparagina e ácido glutâmico (conservados nas lectinas tipo C), próximos ao sítio de ligação do carboidrato. As demais ligações e coordenação do íon Cálcio (II) são estabelecidas com quatro grupamentos carbonila provenientes desses mesmos aminoácidos citados (SHARON 1993).

1.1.2 Classificação

1.1.2.1 Classificação das lectinas quanto a especificidade ao carboidrato

Conforme Sharon e Lis (2004) as lectinas podem ser caracterizadas de acordo com sua estrutura, composição da sua estrutura primária, bem como seu nível de glicosilação. Além

disso, podem ser divididas tendo como referência a sua especificidade a carboidratos e estrutura molecular.

Makela, no ano de 1957, apresentou uma classificação segundo a afinidade por açúcar simples. Goldstein e Poretz, no ano de 1986, ampliaram esta classificação e, Van Damme et al. (1998) propuseram uma classificação mais abrangente com seis grupos de lectinas vegetais, conforme tabela 1.

Tabela 1. Especificidade de ligação de lectinas vegetais a carboidratos

Grupo	Especificidade ao Carboidrato
Fucose	L-fucose
Galactose/ N-acetilgalactosamina	Galactose > Neu5Acα(2,6)Gal/GalNAc
Exemplo: SBA	Galactose = Neu5Acα(2,6)Gal/GalNAc
	Galactose < Neu5Acα(2,6)Gal/GalNAc
N-acetil-D-glicosamina	N-acetil-D-galactosamina
Exemplo: WGA	$(GlcNAc)_n$
Manose	Manose
Exemplo> ConA	Manose/ glicose
	Manose/ maltose
Ácido siálico	Ácido siálico
Exemplo: Lectina de *Limax flavus*	Neu5Acα(2,6)Gal/GalNAc
	Neu5Acα(2,3)Gal/GalNAc
Grupo de glicanos complexos	Complexos conhecidos
Exemplo: PHA	Complexos desconhecidos

Van Damme et al. (1998).

Com a finalidade de identificar e agrupar lectinas pela compreensão dos mecanismos de reconhecimento lectina-carboidrato, torna-se conveniente classificar essas proteínas de acordo com as características topológicas do sítio ligante glicídico. Essa ligação é extremamente importante, uma vez que a topologia define a categoria da atividade da lectina, pois a acessibilidade do ligante ao sítio de reconhecimento determina mecanismos de reconhecimentos

distintos. Sendo assim, tais proteínas podem ser divididas em dois grupos principais (Elgavish & Shaanan, 1997).

- **Grupo I**: lectinas de transporte, tais como as proteínas transportadoras periplasmáticas bacterianas e as enzimas. Os sítios ligantes glicídicos dessas proteínas se encontram topologicamente mais internos ("enterrados" engolfando o ligante completamente) e apresentam padrões de reconhecimento que lhes são próprios.

- **Grupo II**: lectinas que apresentam um sítio glicídico mais externo e topologicamente mais raso; formando, na maioria das vezes uma depressão superficial molecular que acomoda o carboidrato ligante. Nesse grupo, encontram-se grande parte das lectinas conhecidas, divididas em famílias clássicas de acordo com sua procedência ou mesmo especificidades glicídicas.

Tabela 2. Classificação de lectinas do grupo II em famílias

Tipo de lectina	Especificidade	Massa Molecular dos Monômeros (kD)	Número de Monômeros	Sítios ligantes glicídicos por monômeros	Ligações Dissulfeto	Íons Metálicos coordenados
Lectinas de plantas						
Legumes	Diversa	25-30	2 ou 4	1	-	Ca^{+2}, Mn^{+2}
Cereais	NacGlc	~18	~2	2	++	-
Lectinas de animais						
Lectinas tipo- C	Diversa	>>15	Variável	1-8	+	Ca^{+2}
Lectinas tipo- S	Galactose	14-35	?	1	-	-

Shaanan, 1993.

1.1.2.2 Estrutura molecular das lectinas

As lectinas apresentam uma grande diversidade estrutural, e o aspecto comum entre estas moléculas é a presença de pelo menos um sítio específico de ligação a carboidrato o "domínio de reconhecimento de carboidrato" em cada cadeia polipeptídica (ZANETTI 2007). Conforme o domínio de reconhecimento de carboidrato, Van Damme et al. (1998), propuseram que as lectinas poderiam ser classificadas em quatro grupos, conforme a figura 2.

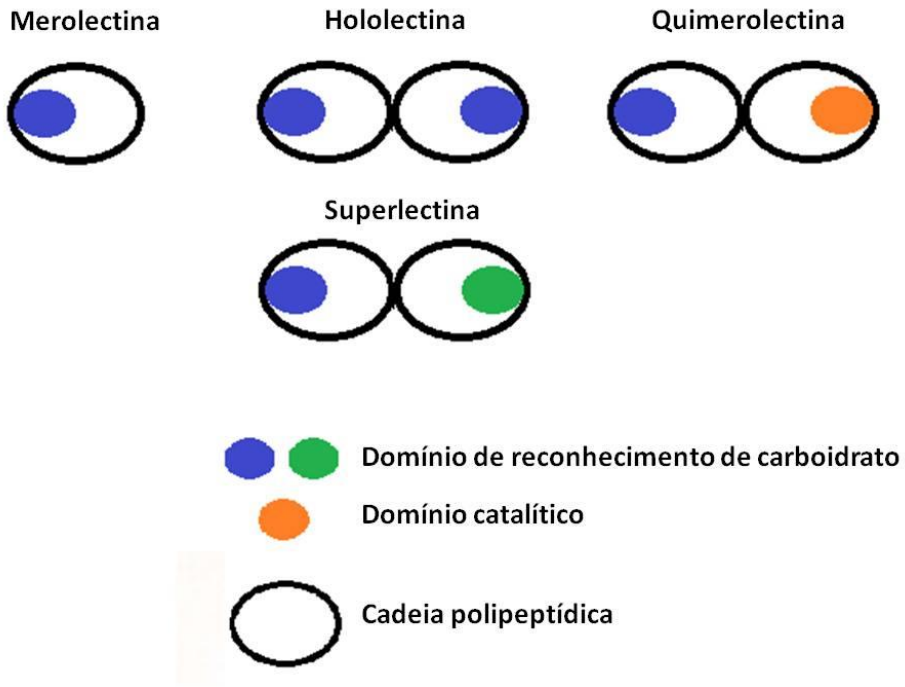

Figura 2. Classificação das lectinas vegetais conforme característica estrutural. Adaptado de Van Damme et al. (1998).

1. **Merolectinas:** Moléculas proteicas com apenas um domínio de reconhecimento de carboidratos. Estas lectinas não apresentam atividade hemaglutinante. Um exemplo de merolectina é a heveína, isolada do látex de *Hevea brasiliensis* (Willd.) Muell. Arg. (VAN DAMME et al. 1998, WITTSUWANNAKUL et al. 1998).
2. **Hololectinas:** Apresentam ao menos dois domínios de reconhecimento de carboidratos idênticos, ou com alto grau de carboidratos idênticos, ou com alto grau de homologia. Estas lectinas são capazes de aglutinar células ou precipitar glicoconjugados. Conforme Van Damme et al. (1998), a Con A é um exemplo de hololectina.
3. **Quimerolectinas:** Sob esta denominação encontram-se as lectinas com um ou mais domínio(s) de ligação a carboidratos associado(s) a outro domínio com atividade biológica como, por exemplo, atividade enzimática, independentemente da ligação a carboidratos. A ricina, obtida de *Ricinus communis* L. e a lectina de *Viscum album* L., exemplificam este grupo de lectinas (OLSNES e KOZLOV 2001, VAN DAMME et al. 1998).
4. **Superlectinas:** Este grupo de lectinas apresentam exclusivamente ao menos dois domínios de reconhecimento de carboidratos, como as hololectinas, porém reconhecem carboidratos com estruturas diferenciadas. Podem ser consideradas como um grupo especial de quimerolectinas, compostas de dois domínios de ligações para carboidratos

independentes estrutural e funcionalmente, como a lectina TGL da *Tulipa gesneriana* L., com um domínio e ligação à manose e outro para N-acetil-D-galactosamina (VAN DAMME et al. 1998).

1.1.3 Purificação e caracterização de lectinas

O desenvolvimento de métodos para a separação e purificação de proteínas vem sendo um importante pré-requisito para muitos avanços na indústria biotecnológica. As lectinas têm sido purificadas devido às suas diversas aplicações. A estratégia para o isolamento de lectinas depende da natureza do material biológico (sementes, líquidos corporais, homogenatos de tecidos, etc.), assim como, das propriedades exibidas pelas mesmas, como carga elétrica, tamanho, solubilidade e afinidade específica de ligação, as quais variam de uma proteína para outra. São diversas as técnicas utilizadas para eliminar moléculas que não sejam de interesse (contaminantes).

A diálise separa as lectinas de moléculas pequenas através da utilização de uma membrana (celulose) semipermeável (KABIR et al. 1998). As moléculas com dimensões maiores são retidas dentro do saco de diálise e as menores e os iontes atravessam os poros e permanecem na solução.

De acordo com Lehninger (2006), três métodos cromatográficos são utilizados na purificação de proteínas:

(A) Cromatografia de Troca Iônica, separa as proteínas pelas diferenças na magnitude das taxas de rede elétrica de proteínas em um dado pH. A matriz da coluna é um polímero sintético ligado a grupos carregados, aqueles vinculados a grupos catiônicos são chamados de ânions trocadores. A afinidade de cada proteína para os grupos presentes na coluna é afetada pelo pH (que determina o estado de ionização da molécula) e a concentração de cátions sem sal em solução circundante. A separação pode ser otimizada de forma gradual com a alteração do pH e/ou da concentração de sal da fase móvel de modo que crie um gradiente de pH ou sal.

(B) Cromatografia por exclusão de tamanho, também chamado de filtração em gel, que separa proteínas de acordo com o tamanho. A matriz da coluna é um polímero reticulado com poros de tamanho selecionado. As proteínas maiores migram mais rapidamente do que as menores, porque eles são muito grandes, impedidas de entrar nos poros e, portanto, tomam uma forma mais direta no percurso através da coluna. As proteínas menores são retardadas entre os poros pelo caminho mais labiríntico da coluna.

(C) Cromatografia de afinidade separa as proteínas por suas especificidades de ligação. As proteínas retidas na coluna são aqueles que ligam especificamente a um ligante. (Em bioquímica, o termo "Ligante" é usado para se referir a um grupo ou uma molécula que se liga a um macromolécula como uma proteína). Depois que proteínas que não se ligam ao ligante são lavadas da coluna, as proteínas de interesse são eluídas (lavados da coluna) por uma solução contendo o ligante livre.

Métodos eletroforéticos são utilizados para a caracterização estrutural de lectinas e se baseiam no princípio que uma molécula com carga elétrica líquida mover-se-á em um campo elétrico. A velocidade de migração de uma proteína depende da intensidade do campo, da carga líquida e do coeficiente de atrito. Os géis de poliacrilamida são os meios de suporte escolhidos para eletroforese porque são quimicamente inertes e prontamente formados pela polimerização da acrilamida (STRYER 2004). Quando submetidos a condições desnaturantes (presença de sulfato sódico de dodecila) e redutoras (presença de β-mercaptoetanol) revelam o grau de pureza da proteína, a composição de subunidades e através de coloração específica, a natureza glicoproteica (COELHO e SILVA 2000). A eletroforese em gel de poliacrilaminda para proteínas em condições não desnaturantes é utilizada para analisar a pureza de estruturas moleculares nativas. A eletroforese em condições não desnaturantes caracteriza a proteína em relação à sua composição aminoacídica e a eletrofocalização define o ponto isoelétrico da proteína (PAIVA e COELHO 1992). Para caracterizar a atividade biológica da lectina avalia-se o efeito do pH, da temperatura, de íons e de inibidores na atividade hemaglutinante (REGO et al. 2002).

1.1.4 Lectinas de animais

As proteínas são conhecidas há mais de um século como constituintes de plantas (CUMMINGS 1997). Esta concepção está fortemente associada ao conceito de que as lectinas seriam encontradas excusivamente em plantas. Porém, as lectinas podem ser encontradas em diferentes fontes, incluindo animais, plantas, fungos, bactéria, vírus (RINI 1995). Desta maneira, nas últimas décadas têm-se testemunhado a descoberta de diversas lectinas animais (GABIUS 1994a) que foram agrupadas conforme descrito na tabela 3.

O isolamento, em 1974, da primeira lectina de mamífero, a asialoglycoprotein hepática receptor galactose-específicas, foi um dos resultados da pequisa de Gilbert Ashwell no NIH, juntamente com Anatol G. Morell na Escola de Medicina Albert Einstein (Nova York). Ao mesmo tempo, Vivian Teichberg informou (TEICHBERG et al. 1975) o isolamento do primeiro membro da família das lectinas b-galactose-específicas, designadas galectinas (BARONDES et al. 1994), dos quais mais de uma dúzia de membros têm caracterizado até agora. Desde o início

do 1980, o número de lectinas animais purificada também começou a crescer rapidamente, em grande parte graças ao advento da recombinação técnicas (SHARON e LIS 2004).

Tabela 3. Categorias de classificação de lectinas animais

Família	Estrutura	Carboidrato ligante
Tipo C	CDR conservado	Variável (manose, galactose, fucose)
Tipo I	CDR ligado a imunoglobulina	Variável ($Man_6GlcNAc_2$, epítopo HNK-1, ácido hialurônico)
Galectinas (Tipo S)	CDR conservado	B-galactosídios
Tipo P	Similar, porém CDR ainda não está definido	Glicoproteínas contendo Manose-6-fosfato

Adaptado de Gabius (1996).

1.1.4.1 Lectinas Tipo C

A dependência da ligação ao açúcar na presença de íons Cálcio e a preservação na sequência comum de 14 aminoácidos na região invariável e 18 na constante foram os dois pré-requisitos para a definição de uma lectina tipo C (DRICKAMER 1993, DRICKAMER e TAYLOR 1993). As lectinas tipo C do veneno de serpentes são Cálcio dependentes e são exclusivamente homodímeros ou homooligômeros (YAMAZAKI 2007). Têm como carboidratos ligantes a manose, galactose, fucose, N-acetilglucosamina, N-acetilgalactosamina e seus derivados e localizam-se principalmente na membrana celular e região extracelular. Possuem funções diversas como, por exemplo, participação na interção célula-célula, na imunidade inata e adquirida, montagem de rede da matriz extracelular, eliminação de glicoproteínas (enzimas lisossomais, hormônios, colágeno e de células com glicosilação anormal).

1.2 *Bothrops leucurus*

A família *Viperidae* é o mais importante grupo de serpentes para a saúde pública no Brasil, pois são responsáveis pela maioria e mais graves acidentes ofídicos registrados. Dentre os acidentes por serpentes, o acidente botrópico destaca-se pela sua elevada incidência (MISE 2007). Sintomas neurológicos (principalmente cefaléia) e respiratórios (bradipneia) ocorreram e podem estar relacionados à ação neurotóxica pré-sináptica do veneno de *Bothrops leucurus* (Figura 3) observada *in vitro* sobre a preparação nervo-frênico diafragma de camundongo

(LIRA-DA-SILVA 2001). Essa peculiaridade do veneno da *B. leucurus* sugere que os agentes de saúde devem estar aptos a executar a terapêutica adequada aos sintomas sistêmicos. A letalidade de 1% é alta, se comparada com a média nacional (0,45%) e nordestina (0,81%) (MISE 2007).

Figura 3. *Bothrops leucurus*.

As peçonhas das serpentes são muito complexas, pois contêm vinte ou mais componentes diferentes, sendo mais de 90% de seu peso seco constituído por enzimas, toxinas não enzimáticas, proteínas e proteínas não tóxicas. As frações não proteicas são representadas por carboidratos, lipídios, aminas biogênicas, nucleotídeos e aminoácidos livres (FRANÇA 2003). Vários componentes têm sido isolados de venenos de Bothrops incluindo proteases, como serino e metaloproteases, Fosfolipase A2, oxidase L-aminoácido, 50-nucleotidase, hialuronidase e lectinas tipo-C (DOLEY e KINI 2009).

B. leucurus (Jararaca-de-rabo-branco) é uma importante serpente venenosa que habita o Nordeste do Brasil. Um estudo epidemiológico realizado na Bahia em 2001 revelou que todos os casos de envenenamento botrópico confirmado com a identificação de serpentes foram causados por *B. leucurus* (MISE et al. 2007). Uma melhor caracterização das atividades biológicas dos venenos de serpentes do gênero Bothrops é muito importante, não só para elucidar os mecanismos moleculares da ação do veneno, mas também para buscar novas abordagens para o tratamento do paciente. Serpentes deste gênero são responsáveis pela grande maioria dos acidentes ofídicos na América Central e do Sul (QUEIROZ 2008).

Em estudos das atividades biológicas de venenos de serpentes da América do Sul, Sanches *et al*. (1992) relataram que a letalidade (intravenosa e intraperitoneal), como também a atividade coagulante, edematogênica, hemorrágica e necrótica de *B. leucurus* é similar a outras espécies botrópicas, incluindo *B. jararaca*. Calmey *et al*. (2002), utilizando o ensaio ELISA, registraram baixos títulos de anticorpos, na reação com soro comercial antibotrópico com a

peçonha de *B. leucurus*, comparado com outras espécies botrópicas (*B. jararaca*, *B. alternatus*, *B. moojeni*, *B. neuwiedi* e *B. jararacussu*) incluídas no "pool" antigênico. Prianti Jr. *et al*. (2003), estudando o efeito do veneno de *B. leucurus* sobre preparações de nervos-músculos de aves (pintos), observaram que o mesmo inibe a transmissão neuromuscular de maneira tempo e dose-dependente, além de produzir alterações morfológicas nas células musculares esqueléticas, indicando a presença de ação miotóxica nessa peçonha.

A peçonha de *B. leucurus* foi avaliada quanto à presença de lectinas, obtendo uma lectina tipo-C (BlL) a partir do veneno total. BlL, foi purificada através de um protocolo eficiente de cromatografias de gel de Guar. Esta lectina é dependente de cálcio e inibida por açúcares contendo galactosídeos. Estruturalmente é um dímero composto de duas subunidades de 15 kDa unidas por ligações dissulfeto. O Dicroísmo Circular revelou que BlL pode ser classificada como uma proteína toda-β (NUNES et al. 2011).

Em relação a sua aplicação biológica, BlL apresentou atividade antibacteriana contra bactérias Gram-positivas (*Staphylococcus aureus*, *Enterococcus faecalis* e *Bacillus subtilis*) (NUNES et al. 2011). Quando BlL foi submetida a irradiação, a formação da fibra amiloide através do fluoróforo Tioflavina T não foi verificada. Consequentemente, a irradiação (1 e 2 kGy) também aboliu a citotoxicidade induzida por BlL não irradiada em todas as linhagens tumorais testadas. Tais resultados confirmaram que a radiação gama de ^{60}Co foi capaz de causar alterações funcionais e estruturais em BlL, abolindo, desta forma, sua atividade citotóxica em linhagens tumorais (NUNES et al. 2012).

1.3 Mitocôndrias

As mitocôndrias são organelas intracelulares com dupla membrana que têm como função predominante a geração de ATP pela fosforilação oxidativa, sendo a fonte primária de compostos altamente energéticos na célula. Além disso, elas desempenham outras funções importantes como a biosíntese de aminoácidos e esteróides, a beta-oxidação de ácidos graxos, produção e detoxificação de espécies reativas de oxigênio (EROs), regulação do cálcio intramitocondrial e citoplasmático e transdução de sinais nas vias de sinalização intracelular em algumas formas de apoptose (BEAL 2005, BRAND e NICHOLLS 2011).

1.3.1 Bioenergética mitocondrial

A metabolização de carboidratos, lipídeos e aminoácidos, realizada em organismos aeróbios, resulta na transferência de elétrons desses nutrientes para o O_2, gerando H_2O e liberando energia que é armazenada na forma de um potencial eletroquímico de prótons (Figura 4). Tal energia é utilizada pela ATP-sintase para promover a conversão de ADP à ATP. E este

processo, denominado fosforilação oxidativa, é controlado pela cadeia transportadora de elétrons mitocondrial (MITCHELL 1966).

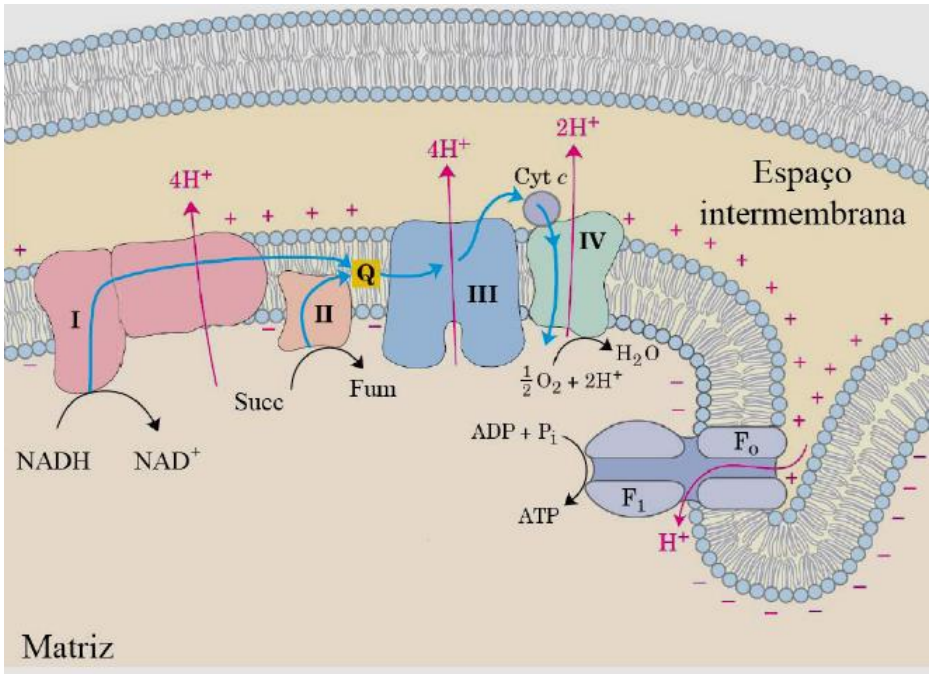

Figura 4. Versão simplificada da teoria quimiosmótica aplicada à mitocôndria
Os elétrons do NADH e de outros substratos oxidáveis passam através de uma cadeia de transportadores arranjados assimetricamente na membrana. O fluxo de elétrons é acompanhado pela transferência de prótons através da membrana mitocondrial, produzindo tanto um gradiente químico (ΔpH) quanto elétrico (ΔΨ). A membrana mitocondrial interna é impermeável aos prótons, os quais podem reentrar na matriz através de canais específicos de prótons (F0). A força próton-motora que impulsiona os prótons de volta para a matriz fornece a energia para a síntese do ATP, catalisada pelo complexo F1 associado com F0. Modificado de **Lehninger (2004).**

Normalmente, elétrons provenientes das coenzimas NADH e $FADH_2$, reduzidas durante a oxidação de carboidratos, aminoácidos e ácidos graxos, são transferidos à NADH desidrogenase. O complexo I transfere seus elétrons à forma oxidada da coenzima Q (UQ), gerando a forma reduzida desta coenzima (UQH_2). Elétrons originados a partir do succinato passam para a UQ através do complexo II, resultando também na redução da coenzima Q. Em alguns tecidos a coenzima Q pode também ser reduzida pelo glicerol-3-fosfato desidrogenase (na presença de glicerol-3-fosfato citosólico) ou pela ubiquinona oxiredutase (como resultado da β-oxidação de ácidos graxos). A UQH_2 é então desprotonada, resultando na formação da espécie aniônica semiquinona ($UQH^{\bullet}$), a forma que doa elétrons ao citocromo c. Este transfere elétrons à citocromo oxidase (complexo IV) que é responsável pela transferência de elétrons para o oxigênio, resultando na geração de água, em um processo envolvendo quatro passos consecutivos de transferência de um elétron (NICHOLLS 2002).

Complexo I é um importante sítio de produção de EROs, e estudos utilizando linhagens de células de pacientes que contêm deficiência do complexo I revelaram uma correlação entre a produção de EROs e a morfologia mitocondrial. Os resultados em casos de comprometimento grave do Complexo I revelaram um aumento na produção de EROs e uma fragmentação mitocondrial, enquanto que em casos de comprometimento leve observou-se apenas uma elevação discreta de EROs. Uma explicação interessante para isso seria que a fragmentação mitocondrial restringe os efeitos locais do EROs, limitando a extensão do dano (DISTELMEIER et al. 2009).

A ativação das proteínas desacopladoras (UCPs – *uncoupling proteins*) e do canal de potássio sensível ao ATP (K_{ATP}) presentes na membrana mitocondrial interna ainda permite o transporte de H^+ de volta para a matriz mitocondrial sem que este "sirva" o complexo da ATP-sintase, resultando em desacoplamento da fosforilação oxidativa. Estes mecanismos de desacoplamento da fosforilação oxidativa estimulam a respiração, reduzem levemente o potencial de membrana mitocondrial e diminuem significativamente a formação de EROs (BOVERIS e CHANCE 1973, KORSHUNOV 1997, KOWALTOWSKI 1998). A redução da formação de EROs pelo estímulo da respiração pode ser explicada, pelo menos em parte, pela diminuição da meia-vida do radical semiquinona na CTE, o que diminuiria a probabilidade da formação de O_2^- pela doação de elétron do radical semiquinona ao O_2 (SKULACHEV 1996).

1.3.2 Geração Mitocondrial de Espécies Reativas de Oxigênio

O sistema de transporte de elétrons mitocondrial é uma importante fonte de espécies reativas de oxigênio (EROs), sendo que seus principais sítios de formação são os complexos I e III (BOVERIS e CHANCE 1973, TURRENS 2003). A citocromo oxidase promove a redução completa de uma molécula de O_2 em duas moléculas de água e, para isto, são necessários 4 elétrons. No entanto, como consequência de sua configuração eletrônica, a molécula de O_2 tem forte tendência em receber um elétron de cada vez formando uma série de intermediários tóxicos e reativos (HALLIWELL e GUTTERIDGE 1984), tais como: radical ânion superóxido ($O_2^{\bullet -}$), o peróxido de hidrogênio (H_2O_2), e o radical hidroxil ($OH^\bullet$) (HALLIWELL e GUTTERIDGE 1984).

Em condições fisiológicas, o H_2O_2 é formado na mitocôndria a partir do superóxido (CHANCE et al. 1979), numa reação catalisada pela enzima superóxido dismutase (SOD) (FRIDOVICH 1978). A isoforma da SOD presente na matriz mitocondrial é dependente de manganês (MnSOD), enquanto a isoforma da SOD presente no citosol é dependente de cobre e zinco (CuZnSOD) (FRIDOVICH 1978). O H_2O_2 é uma espécie química permeável por

membranas e mais estável, podendo ser removida por diferentes enzimas com atividade peroxidásica. A EROs mais reativa e citotóxica é o OH•, que pode ser gerado pelo H_2O_2 quando reage com íons cobre (Cu^+) ou ferro (Fe^{2+}), reação conhecida como reação de Fenton (HALLIWELL e GUTTERIDGE 1997).

O Ca^{2+} parece ser o principal agente estimulador da geração mitocondrial de EROs. Íons Ca^{2+} provocam alterações estruturais da membrana mitocondrial interna, incluindo aumento do empacotamento lipídico e separação lateral de fase (GRIJALBA 1999), aumentando a geração mitocondrial de EROs, provavelmente devido à desorganização da cadeia respiratória, cujos componentes estão, em grande parte, imersos na membrana mitocondrial interna (NICHOLLS 1982). O Ca^{2+} liga-se à cardiolipina, um lipídeo que possui cabeça polar eletronegativa, presente em altas concentrações (14-23%) na membrana mitocondrial interna em uma grande variedade de tecidos (DAUM 1985). A geração mitocondrial de EROs induzida pelo Ca^{2+} pode ser estimulada pela presença concomitante de fosfato inorgânico (P_i) (KOWALTOWSKI 1996a, KOWALTOWSKI 1996b).

Nas situações onde a geração mitocondrial de $O_2•^-$ é aumentada, ou quando os sistemas antioxidantes estão depletados, as EROs podem acumular e gerar uma condição de estresse oxidativo na mitocôndria (HALLIWELL e GUTTERIDGE 1989). Nestas situações, os danos oxidativos à proteína, lipídios e DNA podem levar à disfunção mitocondrial e até mesmo à morte celular (KOWALTOWSKI e VERCESI, 1999).

1.3.3 Transição de Permeabilidade Mitocondrial e Morte Celular

Durante a segunda metade do século XX, a mitocôndria era considerada uma organela cuja função se restringia à transformação de energia através da fosforilação oxidativa. Contudo, cerca de uma década atrás, ficou claro que a mitocôndria tinha uma segunda função crucial: o controle da morte celular (Kroemer et al., 2007). A célula, a mitocôndria em particular, e a homeostase do cálcio regulam a geração de EROs mitocondrial e de proteína de membrana *tiol cross-linking*, causando transição de permeabilidade da membrana mitocondrial (MPT) (CASTILHO 1995, KOWALTOWSKI 1995, GRIJALBA 1999, MACIEL 2001), uma condição caracterizada pela abertura de uma condutância elevada, poro proteico não específica, o poro de transição de permeabilidade (HUNTER 1979) que leva a disfunção mitocondrial (KOWALTOWSKI 1999; KOWALTOWSKI 2001) e morte celular seja por apoptose ou necrose (KIM 2002, BERNARDI 2006, LEMASTERS 2009, MARTIN 2010). A ativação do MPT é considerada uma das principais causas de morte celular sob uma variedade de condições fisiopatológicas, incluindo isquemia/reperfusão, doenças neurodegenerativas, lesões cerebrais traumáticas, distrofia muscular e toxicidade de drogas (STARKOV 2004, BERNARDI 2006,

KROEMER 2007, MBYE 2009, LIU e MURPHY 2009, NICHOLLS 2009, GLEICHMANN 2011).

A sinalização de Ca^{2+} para geração de EROs mitocondrial ocorre no interior da organela, por ação de mecanismos de transporte de Ca^{2+} conduzidos pela mitocôndria. Embora este mecanismo tenha sido descoberto na década de 60 (DELUCA 1961, VASINGTON 1962), a natureza molecular do canal foi apenas recentemente identificada como um resultado do conhecimento da distribuição dos canais *uniporter* em diferentes eucariotos e do progresso no sequenciamento do genoma. (BAUGHMAN 2011). Além do *uniporter*, mitocôndrias possuem dois outros sistemas de mediadores do influxo de Ca^{2+}: um modo de absorção chamado de modo rápido ou RAM (SPARAGNA 1994) e um mecanismo de absorção de Ca^{2+} mediada por um receptor de Rianodina (mRyR), identificado em células excitáveis (BEUTNER 2001).

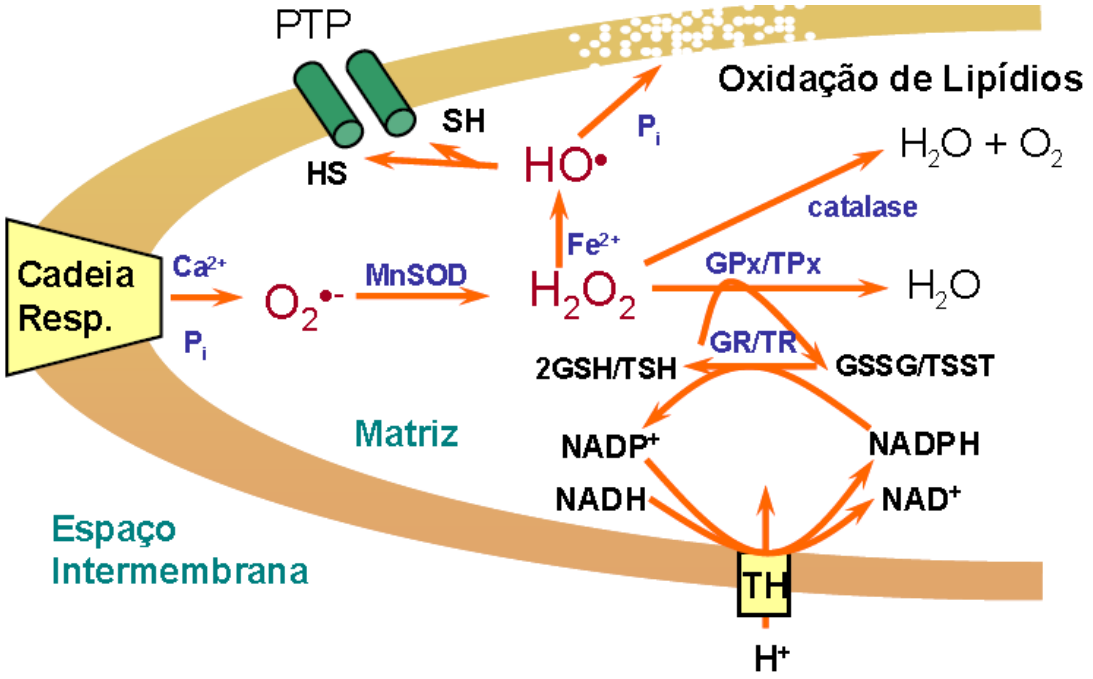

Figura 5. Modelo proposto para explicar a formação do poro de transição de permeabilidade induzido por Ca^{2+} e EROs na membrana mitocondrial interna. A cadeia respiratória, inserida na membrana mitocondrial interna, constantemente gera pequenas quantidades de radicais O_2 •-. Estes radicais são normalmente removidos pela Mn-superóxido dismutase (MnSOD), que promove a geração de H_2O_2. O H_2O_2 é então reduzido à H_2O pela glutationa peroxidase (GP), tioredoxina peroxidase (TP) ou catalase (em mitocôndria de coração). GSH, oxidado pela GP, e TSH, oxidado pela TP, são recuperados pelo sistema enzimático glutationa e tioredoxina redutases (GR e TR), que usam NADPH como doador de elétrons. NADH, que está presente em quantidades reguladas pela respiração, reduz então $NADP_+$ usando a NAD(P) transidrogenase (TH). Quando a geração de O_2 •- aumenta na presença de Ca^{2+} e Pi, e/ou os mecanismos de remoção de H_2O_2 estão inativados, H_2O_2 acumula-se e na presença de Fe^{2+}, gera o radical OH• altamente reativo. OH• oxida grupos tiólicos (-SH) do complexo do poro de TPM, levando à formação e abertura do poro. Alternativamente, OH• pode promover permeabilização da membrana através da peroxidação lipídica, um processo fortemente estimulado por Pi. **Kowaltowski et al. (2001).**

Em condições fisiológicas, a captação de Ca^{2+} por mitocôndrias transfere, para a matriz, o sinal trazido por Ca^{2+} citosólico transiente (GLANCY 2012). As alterações oxidativas das proteínas da membrana mitocondrial interna que ocorrem na presença de Ca^{2+} levam a uma permeabilização não específica da membrana mitocondrial interna, conhecida como transição de permeabilidade mitocondrial (KOWALTOWSKI 2001, VERCESI 1997, ZORATTI 1995) A TPM é caracterizada por uma permeabilização progressiva da membrana mitocondrial interna, que gradativamente se torna permeável a prótons, íons, suporte osmótico e até mesmo pequenas proteínas (VERCESI, 1997). Esta permeabilização é dependente da presença de Ca^{2+} no espaço intramitocondrial, e é inibida por concentrações submicromolares de ciclosporina A, um imunossupressor (CROMPTON 1988, BROEKEMEIER 1989), provavelmente devido à ligação da ciclosporina a ciclofilinas da membrana mitocondrial interna, que seriam necessárias para a abertura do poro da TPM (CONNERN 1994, NICOLLI 1996). Assim sendo, o nome "transição de permeabilidade" é utilizado devido à observação de que a permeabilização mitocondrial nesta situação pode ser parcialmente revertida pela adição de quelantes de Ca^{2+} ou redutores ditiólicos logo após a permeabilização (VALLE 1993, CASTILHO 1996, HUNTER 1979).

Relacionando a geração de espécies reativas de oxigênio (EROs) à transição de permeabilidade é sabido que H_2O_2 produzidos pela SOD e de origem mitocondrial podem desempenhar um papel na sinalização redox da mitocôndria para outros compartimentos celulares. Este processo se dá pela modulação da atividade de proteínas alvo através de oxidação reversível de grupos tiol de quinases, fosfatases e fatores de transcrição (HURD 2007). Embora a oxidação de grupamentos tiólicos na TPM seja um processo extenso (CASTILHO 1995, KOWALTOWSKI 2001), é provável que a permeabilização da membrana mitocondrial seja promovida pela oxidação de um grupo específico de proteínas, o que explicaria o caráter parcialmente reversível e regulado da TPM (CASTILHO 1996, LEHNINGHER 1978).

O translocador de ADP/ATP é a proteína mais abundante da membrana mitocondrial interna, possui quatro resíduos de cisteína (KLINGENBERG 1989) e certamente está envolvido no processo de TPM. Ligantes do translocador de ADP/ATP que o mantém na sua configuração c (o sítio de ligação para o nucleotídeo está voltado para o lado citoplasmático), como ADP e bongkrekato, inibem e até revertem a TPM, enquanto ligantes que mantém o translocador em sua conformação m (o sítio de ligação para o nucleotídeo está voltado para o lado da matriz mitocondrial), como o carboxiatractilosídeo, promovem TPM (CASTILHO 1996, VERCESI 1984). O ADP também é capaz de inibir parcialmente, de modo sensível a carboxiatractilosídeo, a oxidação de grupamentos tiólicos de proteínas de membrana mitocondrial promovida por TPM (CASTILHO 1996). Por outro lado, Novgorodov e colaboradores demonstraram que o translocador de ADP/ATP participa indiretamente da formação do poro de TPM. Estes

resultados sugerem que alterações na configuração do translocador de ADP/ATP podem mudar o posicionamento de grupamentos tiólicos de proteínas de membrana, promovendo ou inibindo sua oxidação, e, conseqüentemente, a ocorrência de TPM (VERCESI 1997).

Ampliando para um contexto de morte celular necrótica, o aumento do Ca^{2+} citosólico ocorre por falência dos mecanismos homeostáticos do cátion, o que pode levar a TPM generalizada, depleção de ATP e morte celular. No caso da morte por apoptose, relacionada ao aumento de Ca^{2+}, a TPM seria um evento localizado a sítios de aumento regulado deste íon e a produção do ATP, necessário para este tipo de morte, seria preservada às custas de mitocôndrias não atingidas. A abertura do poro de transição de permeabilidade facilitaria a liberação de fatores pró-apoptóticos mitocondriais tais como Pró-caspase 9, Smac/Diablo, fator indutor de apoptose (AIF) e citocromo c. A presença destes fatores no citosol leva a ativação de proteases denominadas caspases, que iniciam a fase efetora da apoptose e que culminam com o encolhimento e fragmentação celular, formando os corpos apoptóticos. No núcleo ocorre condensação da cromatina e fragmentação do DNA. Finalmente os restos celulares são fagocitados por células vizinhas. Este processo é fisiológico e necessário para a vida de seres multicelulares, pois elimina células desnecessárias ou disfuncionais, sem desencadear um processo inflamatório.

1.4 Morte Celular, Mitocôndrias e Lectinas

A morte celular por apoptose difere da necrose com base em diversos aspectos bioquímicos e morfológicos. A apoptose está relacionada com insultos celulares mais amenos, que não resultam em inflamação e sua ativação depende da produção energia, ATP, ativação de caspases e outros fatores pró apoptóticos. A morfologia da apoptose é caracterizada pela integridade das organelas celulares, incluindo a da mitocôndria, condensação da cromatina, fragmentação do DNA nuclear e formação de corpos apoptóticos. Por outro lado, a necrose está relacionada a intesas agressões nas células associadas com a inflamação, processo que resulta na queda da produção de ATP e ou lesão da membrana celular, morfologicamente caracterizada por: tumefação, rompimento celular e das organelas, particularmente das mitocôndrias, aparecimento de vacúolos, acidofilia citoplasmática e em suas etapas finais a necrose é responsável pela degradação total das células (MCCONKEY 1998, ELMORE 2007, KROEMER et. al. 2009).

Diversos mediadores, organelas e processos celulares têm sido implicados na morte celular necrótica, mas ainda não é claro como esses eventos interagem uns com os outros (KROEMER et al. 2009). Entretanto, sabe-se que esse fenômeno inclui alterações mitocondriais (desacoplamento, produção de espécies reativas de oxigênio, estresse nitroxidativo por óxido

nítrico ou compostos similares e permeabilização de membrana mitocondrial, freqüentemente controlada pela ciclofilina D), alterações lisossomais (produção de espécies reativas de oxigênio por reação de Fenton e permeabilização de membrana lisossomal), mudanças nucleares (hiperativação de PARP-1 e concomitante hidrólise de NAD+), degradação lipídica (ativação de fosfolipases, lipoxigenases e esfingomielinases), aumento na concentração de Ca^{2+} citosólico que resulta em sobrecarga mitocondrial e ativação de proteases não caspases (calpainas e catepsinas). Ainda hoje, apesar do conhecimento de todas essas alterações, a caracterização de uma morte celular por necrose é avaliada principalmente por permeabilização de membrana plasmática precoce e ausência de marcadores apoptóticos ou autofágicos (KROEMER et al. 2009).

Na diferenciação e transformação maligna, a biossíntese das cadeias de oligossacarídeos presentes nas glicoproteínas encontra-se frequentemente alterada. Algumas dessas mudanças podem ser reconhecidas por proteínas ligantes de carboidratos, as lectinas (GAJ et al. 2009). Nos últimos anos lectinas têm recebido atenção especial devido as suas importantes atividades biológicas exploráveis, como citoaglutinação, sonda histoquímica, atividade mitogênica, citotoxicidade, ação antiproliferativa e indutora da apoptose (SOBRAL 2010, LAM e NG 2010, YAN et al. 2010, ZHANG et al. 2010).

Diversas lectinas apresentam efeito citotóxico e desencadeiam morte celular, seja por apoptose, necrose ou autofagia, e em alguns há alteração na bioenergética mitocondrial. Em 1996, Agrawal et al. mostraram que a apoptose causada por uma lectina aconteceria após a ligação com receptores presentes na membrana plasmática de neurônios. Recentemente, Liu et al. (2009 a,b) esquematizaram um modelo para a morte celular induzida por PCL (*Polygonatum cyrtonema* lectin). Neste modelo, PCL primeiramente se liga a um receptor contendo carboidrato presente na membrana plasmática. Depois disso, a lectina seria internalizada e se localizaria preferencialmente na membrana da mitocôndria. Na mitocôndria, a lectina seria responsável por promover perda do citocromo c, ativação de caspase-9 e caspase-3. Em adição, há promoção de produção de ROS e ativação de p38 e p53, aumento dos níveis de Bcl-2 e Bax, induzindo a morte celular por apoptose via mitocondrial. Outros estudos realizados com a Con A corroboram este modelo, pois indicam que o potencial antitumoral se dá após a ligação da lectina ao receptor de membrana celular contendo manose (LI 2011, LIU 2009, LEI e CHANG 2007).

2. OBJETIVOS

2.1. Objetivo Geral

Estudar o mecanismo de atuação de BlL (*Bothrops leucurus* lectin) sobre a morte celular e sobre a função mitocondrial.

2.2. Objetivos Específicos

- Determinar a citotoxicidade da lectina em linhagens tumorais humanas (Hep-2, K562 e NCI-H292) *in vitro*;
- Examinar a atividade hemolítica da lectina em eritrócitos de camundongos;
- Avaliar o mecanismo de ação envolvida na atividade citotóxica da lectina em células K562 *in vitro*;
- Avaliar o efeito da lectina na viabilidade de células B16-F10 e GN-13, bem como determinação do tipo de morte celular;
- Avaliar a homeostase intracelular do Ca^{2+} e a produção de EROs mitocondrial em células tratadas com BlL;
- Analisar os efeitos de BlL diretamente na função mitocondrial de células B16-F10 através do monitoramento do influxo de Cálcio para o citoplasma através da membrana plasmática.
- Analisar o mecanismo de ação de BlL em mitocôndrias isoladas de fígado de ratos Wistar em relação ao Potencial elétrico de membrana mitocondrial e consumo de oxigênio;
- Avaliar o efeito de BlL sobre a abertura do poro de transição de permeabilidade mitocondrial (TPM);

3. RESULTADOS

3.1 PARTE I

Cytotoxic effect and apoptosis induction by *Bothrops leucurus* venom lectin on tumor cell lines

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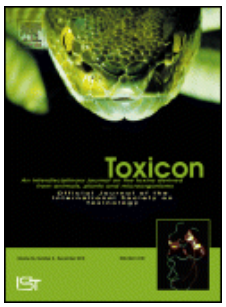

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Short communication

Cytotoxic effect and apoptosis induction by *Bothrops leucurus* venom lectin on tumor cell lines

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ABSTRACT

Neoplastic transformation is the abnormal proliferation of cells. These transformations are often related to changes in cell surface glycoconjugates which can be detected by lectins. We evaluated the anti-tumor potential of BlL, a galactoside-binding lectin isolated from *Bothrops leucurus* venom as well as its cytotoxicity and hemolysis activity. The phosphatidylserine externalization and mitochondrial membrane potential were also determined. BlL exhibited cytotoxic activity against all tumor cell lines tested by induced phosphatidylserine externalization and mitochondrial depolarization, indicating cell death by apoptosis.

Cancer is the second cause of mortality worldwide (Hemalswarya and Doble, 2006) and in Brazil the estimate for the year 2010 (also valid for 2011) is the occurrence of 489,270 new cases of cancer (Instituto Nacional de Câncer, 2009). Etiologic factors associated with cancer include improper diet, genetic predisposition and environment conditions; the majority of human cancers result from exposure to environmental carcinogens (Reddy et al., 2003). Glycosylation is the most frequent form of post-translational modifications of proteins (Chen et al., 2007; Rek et al., 2009) and alterations in the pattern of cell surface glycoconjugates are remarkably characteristic of malignant cells associated with induction of tissue invasion and metastasis (Hakomori, 2002; Kobata and Amano, 2005; Reis et al., 2010). Due to their peripheral location, oligosaccharide epitopes of glycoproteins and glycolipids are recognized by membrane-anchored carbohydrate-recognition domains of different molecules, including lectins (Jiménez-Castells et al., 2008).

Lectins comprise proteins or glycoproteins which bind specifically to mono- or oligosaccharides and glycoconjugates (Wu et al., 2009). Carbohydrate-specificity of lectins has been shown to be a versatile and useful molecular tool for study of glycoconjugates on the cell surface, in particular the changes that cells suffer in malignancy (Sharon and Lis, 2004). Thus, lectins are excellent candidates to be explored in cancer research as therapeutic agents. Lectins from snake venoms exhibit several biological activities like the ability to inhibit integrin-dependent proliferation, migration and invasion

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of tumor cells (Sarray et al., 2004, 2007) as well as the ability to reduce the growth of tumor and endothelial cells (Carvalho et al., 2001). The induction of tumor cell apoptosis by snake venom lectins has been observed (Nolte et al., 2012). However, different mechanisms of action induction of apoptosis can be involved and therefore need to be investigated. The BlL is a galactoside-binding lectin isolated from the venom of *Bothrops leucurus* (white-tailed-jararaca). BlL is a Ca^{2+}-dependent protein of 30 kDa composed of dissulfide-linked dimers of 15 kDa and exhibits antibacterial activity against human pathogenic Gram-positive bacteria (Nunes et al., 2011).

Apoptosis (programmed cell death) is an essential cellular homeostasis mechanism that ensures the correct development and function of multi-cellular organisms. However, cancer cells show a reduced sensitivity towards apoptosis and tumors are dependent on the mechanisms of this resistance to persist and continue development. Therefore, the discovery of drugs that selectively affect the balance of tumor cellular functions towards apoptosis is of enormous therapeutic interest. According Taraphdar et al. (2001), induction of apoptosis is an important strategy for cancer therapy and prevention. The aims of this study were to evaluate the *in vitro* cytotoxicity of BlL on different human tumor cell lines (K562, NCI-292 and Hep-2) and its ability to induce apoptosis in human tumor cells.

An Annexin V FITC Apoptosis Kit was purchased from Calbiochem. All the solvents and other chemicals used were of analytical grade from Gibco™, Invitrogen™, Sigma–Aldrich and Merck. All solutions were prepared with water purified by the Milli-Q® system (Millipore). BlL was purified according to the protocol previously described by Nunes et al. (2011). The cell lines used in the cytotoxicity assays were K562 (chronic myelocytic leukemia), NCI-H292 (human lung mucoepidermoid carcinoma cells) and Hep-2 (human larynx epidermoid carcinoma cells) obtained from the Instituto Adolfo Lutz (São Paulo, Brazil). The non-tumorigenic cell line (HaCaT), derived from human keratinocytes was purchased from Cell Line Service (CLS, Heidelberg, Germany). The cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin and maintained at 37 °C with 5% CO_2.

Cytotoxicity of BlL was tested in tumor cell lines (K562, NCI-H292 and Hep-2) and in non-tumorigenic cell line (HaCaT). The cells (10^5 cells/mL for adherent cells or 0.3×10^6 cells/mL for suspended cells) were plated in 96-well microtiter plates and after 24 h, BlL (0.07–50 μg/mL) dissolved in DMSO was added to each well and incubated for 72 h at 37 °C. Then, MTT (5.0 mg/mL) was added to the plate and growth of tumor cells was estimated by the ability of living cells to reduce the yellow tetrazolium to a blue formazan product (Mosmann, 1983; Alley et al., 1988). Negative control groups received only DMSO; etoposide (1.25–20 μg/mL) was used as a positive control. After 3 h (for suspend cells) or 2 h (for adherent cells), the formazan product was dissolved in DMSO and absorbance was measured using a multi-plate reader (Multiplate Reader Thermoplate). The BlL effect was quantified as the percentage of control absorbance of reduced dye at 450 nm.

The K562 suspension (0.3×10^6 cells/mL) was seeded in 96-well microtiter plates and incubated at 37 °C at 5% CO_2 for 24 h; after this period, BlL at IC_{50} was added. After 48 h the cells were stained with annexin V and propidium iodide using Annexin V–FITC Kit (Calbiochem®) following the protocol provided by the manufacturer and analyzed by an epifluorescence microscope (Carl Zeiss, Gottingen, Germany) at 1000× magnification under oil immersion with filters for LP 515 nm emission and BP 450–490 nm for excitement. A minimum of 200 cells was counted in every sample.

Mitochondrial depolarization was evaluated by incorporation of JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide), a fluorescent lipophilic cationic probe (Kang et al., 2002; Guthrie and Welch, 2006). The probe JC-1 is freely permeable to cells and undergoes reversible transformation from a monomer to an aggregate form (J_{agg}). K562 suspension (0.3×10^6 cells/mL) was seeded into 96-well microtiter plates and incubated at 37 °C and 5% CO_2; after 24 h, BlL at IC_{50} was added and plates incubated for 48 h. Then, 50 μL of treated cell suspension were collected and incubated with JC-1 (10 μL/mL) for 30 min in the dark followed by washing two times with PBS. The cells were fixed with 4% paraformaldehyde (10 μL), mounted on glass slides, and fluorescence was observed using an epifluorescence microscope (Carl Zeiss, Gottingen, Germany), at 1000× magnification under oil immersion with filters for LP 515 nm emission and BP 450–490 nm excitement. A minimum of 200 cells was counted in every sample. Cells with high potential of mitochondrial membrane were stained in red, while cells with low membrane potential were stained in green.

All data are presented as mean ± S.D. The IC_{50} values were obtained by nonlinear regression with 95% confidence interval using the SigmaPlot software (Systal Software Inc., San Jose, USA). The differences between experimental groups were determined using one-way analysis of variance (ANOVA) followed by the Newman–Keuls test at significance level of 1%.

Cytotoxicity of BlL on cell lines was evaluated after 72 h using MTT assay. BlL exhibited cytotoxic activity against all tumor cell lines with IC_{50} values of 11.75 ± 0.035, 6.63 ± 0.052 and 15.42 ± 0.060 μg/mL for Hep-2, NCI-H292 and K562, respectively. Etoposide was used as a positive control and showed IC_{50} values of 6.10 ± 0.19, 2.75 ± 0.10 and 4.48 ± 0.23 μg/mL for Hep-2, NCI-H292 and K562, respectively. Cytotoxic activity against non-tumorigenic cell line was not observed. The involvement of apoptosis induction on K562 (chronic myelocytic leukemia) death was verified by evaluation of phosphatidylserine externalization using the Annexin V-FITC kit and epifluorescence microscope. We observed that after treatment with BlL (15.42 μg/mL), the number of cells in early apoptosis (Ann V^{pos}/PI^{neg}) corresponded to 70.5% (Fig. 1a). Treatment with BlL exhibited values less than 1% of late apoptotic cells ($AnnV^{pos}/PI^{pos}$) and values less than 2% of cell necrosis ($AnnV^{neg}/PI^{pos}$). Fig. 1b also shows that the treatment of K562 cells with BlL caused mitochondrial membrane potential loss, as the epifluorescence microscopy analysis determined that BlL treatment induced a significant increase in cells with

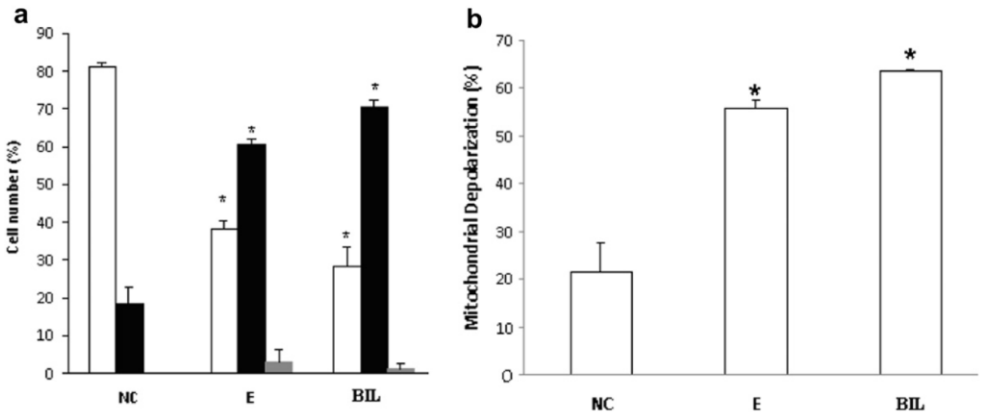

Fig. 1. Effect of BIL on cell population determined by epifluorescence microscopy. (a) Cell number (%) in early apoptosis by evaluation of phosphatidylserine externalization using the Annexin V-FITC kit, after 48 h incubation. (□) viable, (■) apoptotic and (▩) necrotic cells. (b) Mitochondrial depolarization (%) measured by JC-1 incorporation, after 48 h incubation. Negative control (NC), Etoposide (E) and BIL (15.42 μg/mL). NC was the vehicle used (DMSO). Etoposide was used as a positive control. The cell line used in assays was K562 (chronic myelocytic leukaemia). The K562 suspension used was 0.3×10^6 cells/mL. $^*p < 0.01$ in comparison to control by ANOVA followed by the Newman–Keuls test. Data are presented as mean ± S.D. from three independent experiments.

depolarized mitochondria (63.8%) as compared to control cells, as measured by JC-1 incorporation.

Uncontrolled proliferation and decreased apoptotic signals are attributes of oncogenic transformation (Hill et al., 2003), and activation of apoptosis constitutes a fundamental mechanism by which drugs may kill tumor cells (Debatin, 2004). Therefore, compounds with the ability to induce apoptosis in tumor cells have potential as anticancer agents (Reed, 2003). MTT assay demonstrated that BIL showed a significant cytotoxic effect indicating that the activity of this lectin was not specific to a particular tumor cell type. Glycoconjugates or saccharides present on the surface of tumor cells are binding sites for lectins (Luo et al., 2007) and differences in sugar patterns between different tumor cells may be a reason for the differential effect of BIL. Differences in the effects of snake venom lectins towards human tumor cell lines have been reported (Pereira-Bittencourt et al., 1999; Carvalho et al., 2001). In addition, cells that do not express specific carbohydrates may be insensitive to cytotoxic lectins (Gorelik et al., 2001).

The morphological and biochemical characteristics of apoptosis are nuclear chromatin condensation, DNA fragmentation, membrane blebbing (Okada and Mak, 2004; Vermeulen et al., 2005), externalization of phosphatidylserine (Hengartner, 2000) and depolarization of the membrane potential (Ly et al., 2003). In this study, apoptosis induction in BIL-treated K562- cells was assessed by epifluorescence microscopy analysis of phosphatidylserine externalization on the cell surface and mitochondrial membrane potential.

The loss of plasma membrane asymmetry represents an early event of apoptosis resulting in translocation of phosphatidylserine from the inner to the outer surface while membrane integrity remains unchanged (Van Engeland et al., 1998; Fadok et al., 2000; Kagan et al., 2000); this externalization provides the recognition and removal of apoptotic cells by phagocytes (Zimmermann et al., 2001; Taylor et al., 2008). The phospholipid-binding protein annexin V has a high affinity for phosphatidylserine and binds to cells fluorescently labeled with FITC (Reyes-Zurita et al., 2009). However, translocation of phosphatidylserine also occurs during necrosis, so propidium iodide is often used to bind to nucleic acids (Gong et al., 2007). We observed by staining with annexin V-FITC simultaneously with propidium iodide dye that BIL was able to increase significantly the number of apoptotic cells. The results suggest that the cytotoxic effect is due to induction of apoptosis.

The mitochondrial apoptotic pathway is one of the major routes to initiate apoptosis (Kuo et al., 2010). Different stimuli cause changes in the inner mitochondrial membrane leading to the opening of the mitochondrial permeability transition pore, loss of the mitochondrial membrane potential (Ly et al., 2003; Saelens et al., 2004) and pro-apoptotic protein release from the intermembrane space into the cytosol (Mayer and Oberbauer, 2003; Borutaite, 2010). Our studies demonstrated that treatment with BIL increased mitochondrial membrane potential loss, which may indicate cell death by apoptosis in K562 cells. Some lectins such as Con A, POL, PCL and MLL may cause disruption of the mitochondrial membrane potential as an event associated with apoptosis (Liu et al., 2009a, 2009b, 2009c; Zhao et al., 2010).

Based on these considerations, the galactoside-binding lectin from *B. leucurus* snake venom (BIL) exhibited cytotoxic activity on tumor cells and induced apoptosis in K562 cells, as verified by phosphatidylserine externalization analysis and mitochondrial membrane potential determination. Moreover, data suggest that BIL fails to induce apoptosis in cultured human nontransformed cells. These results suggest that BIL has a promising potential for application in the therapy and/or diagnosis of cancer.

Future studies are needed to elucidate the details of BlL induced-apoptosis mechanism in several tumor cell lines.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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3.2 PARTE II

Selective cell killing properties of *Bothrops leucurus* venom lectin on B16-F10 melanoma cells and its effects on mitochondrial function

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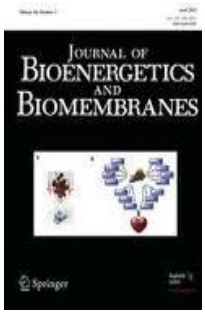

Selective cell killing properties of *Bothrops leucurus* venom lectin on B16-F10 melanoma cells and its effects on mitochondrial function.

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Abstract

Bothrops leucurus lectin (BlL), a Ca^{2+}-dependent and galactose-binding lectin, was reported for its cytotoxicity activity on tumor cell lines. However, the molecular mechanism by which BlL induces the cell killing remains understood. In this paper, we demonstrate that the treatment of B16-F10 melanoma cells with BlL causes a selective cell death by necrosis. Such activity was confirmed by the significant increasing in the production of mitochondrial reactive oxygen species related to elevation in $[Ca^{2+}]$cyt levels, which caused collapse of the mitochondrial membrane potential. Further, our data demonstrated that the death induced by BlL could be correlated with a mitochondria-mediated pathway. In conclusion, these findings demonstrate the involvement of the PTPM in the cell death cause by BlL.

Keywords: *Bothrops leucurus* lectin, melanoma, cytotoxicity, mitochondrial permeability transition

Abbreviations: AA, antimycin A; ADP, adenosine 5'-diphosphate; BlL, *Bothrops leucurus* lectin; EGTA, Ethylene glycol-bis(2-aminoethylether)-*N,N,N ,N* -tetraacetic acid; PI, propidium iodide; ROS, reactive oxygen species; ΔΨm, electrical mitochondrial membrane potential.

Introduction

Melanoma is a very aggressive form of skin cancer that displays a poor prognosis. It derives from malignant transformation of melanocytes, the cells that produce melanin pigment (Chodurek et al. 2012, Riley 2003). Tumor cells may express biochemically altered surface proteins.(Ruoslahti 2002, St Croix et al. 2000). Abnormal glycosylation of membrane proteins is a remarkable characteristic of malignant cells and this feature is associates with the induction of tissue invasion and metastasis (Reis et al. 2010, Kobata and Amano 2005, Hakomori 2002). Thus, the carbohydrate-sensitive surface proteins may be a target for the development of selective tumor-killing compounds (Wang 2000).

C-type lectins are sugar-binding proteins composed by homodimers or homooligomers that are Ca^{2+}-dependent and usually exhibit galactose-binding properties. They are involved in several immune-related and other physiological functions (Koh et al. 2011, Clemetson 2010). The interest in lectins increased after the understanding that they are proteins useful as an experimental tool in sugar cell surface studies (Sharon 2007). Several studies employing lectins have evidenced the presence of sugars on the cell surface (Gorelik et al. 2001). In addition, lectins also have been reported as an inducer of cell death via mitochondrial pathways in diverse cell types (Li et al. 2011).

Snake venom has a variety of biologically active compounds and many of which are of pharmacological value. (Koh et al., 2006; Fox et al., 2007) The venom of *Bothrops leucurus* contain the lectin BlL that is a Ca^{2+}-dependent protein of 30 kDa exhibiting antibacterial activity against human pathogenic Gram-positive bacteria (Nunes et al. 2011). Biological tests have revealed that BlL possesses cytotoxic effect against human tumor cell lines, as it promoted phosphatidylserine externalization and mitochondrial membrane potential dysruption (Nunes et al. 2012).

In this context, we aimed at evaluating the potential cell killing effects of BlL on melanoma B16-F10 cell line. Further studies on BIL toxicity were also conducted with health fibroblast cells and isolated mitochondria.

Materials and Methods

Lectin preparation

BlL was purified according to the protocol previously described by Nunes et al. (2011). Lyophilized crude venom of *B. leucurus* (30 mg) was dissolved in 1 mL of CTBS buffer (20 mM Tris-HCl, 150 mM NaCl and 5 mM $CaCl_2$, pH 7.5) and centrifuged (2000 g, 5 min, 25 °C) to remove insoluble material. The resulting supernatant was applied to a column (10 x 1.0 cm)

of guar gel previously equilibrated with CTBS at a flow rate of 10 mL/h. BlL was eluted from the column with 200 mM galactose in CTBS.

Cell culture

The cells B16-F10 were obtained from American Type Culture Collection-USA and grown in RPMI-1640 (Vitrocell, Brazil) supplemented with 10% fetal bovine serum (Vitrocell), 100 µg/mL gentamycin (Vitrocell), 100 IU/mL penicillin (Vitrocell) and 100 µg/mL streptomycin (Vitrocell). GN-13, a spontaneously transformed human fibroblast cell line, was maintained in DMEM high glucose (Vitrocell) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were maintained at 37 °C in a humidified atmosphere with 5 % CO_2.

Determination of BlL effect on the B16F10 and GN-13 cells viability and proliferation rates

Cell viability and proliferation were evaluated in different lectin concentrations. Thus, the cells were treated with 0,05% tripsin for cells detachment. The fluid was centrifuged (4,000 rpm for 4 min) and the cell pellet resuspended in 3 ml of medium with 10% fetal bovine serum. Trypan blue dye (0.1%) was added to aliquots of cellular suspensions and the percentage of stained cells was determined microscopically.

Flow Cytometry Analysis of cell death on the B16F10 cells after BlL treatment

The percentage of apoptotic and necrotic cells was determined in a FACScalibur flow cytometer (BD Biosciences, USA) equipped with an argon laser and Cell-Quest software (version 4.1). For the analysis, 10^6 cells were incubated in binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM CaCl2) containing annexin V-FITC (1:500, Invitrogen, USA) and PI (20 µg/mL, Molecular Probes, USA) at room temperature for 20 min in the dark. Apoptosis was quantified by FACS analysis as the number of annexin V-FITC-positive and PI-negative cells divided by the total cell number, while necrosis was quantified as the number of PI-positive cells and annexin V-FITC negative cells divided by the total cell number.

Microscopy

B16-F10 melanoma cells (10^6 cells/mL) were treated with BlL in different concentrations for 24 h. Then, the cells were photographed under a Leica DFC360 FX microscope, using the LAS AF software (Leica Microsystems, Wetzlar, Germany).

Measurement of ROS

Following the treatment with BlL for 6h, cells (10^6) were incubed with 5 µM MitoSox (Molecular Probes) at 37°C for 10 min to detect mitochondrial superoxide. ROS levels were analyzed by flow cytometry (Zecchin, 2007).

Spectrofluorimetric determination of cytosolic Ca^{2+} levels

B16-F10 cells ($1x10^6$ cells/ml) were washed twice at 6000 *g* for 4 min at 4 ºC in PBS containing 11 mM Glucose and 0.8 mM $MgSO_4$, pH 7.2. Cells were resuspended in PBS containing 11 mM Glucose and 0.8 mM $MgSO_4$, pH 7.2., and 5 µM fura 2-AM (Sigma). The suspensions were incubated for 45 min and maintained at 37 °C in a humidified atmosphere with 5 % CO_2. Subsequently, the cells were washed with PBS to remove extracellular dye. Cells were resuspended to a final density of 10^6 cells/mL in PBS. For fluorescence measurements, 2 x 10^6 cells was diluted into 2.0 mL of PBS (10^6 cells/mL) in a cuvette placed into a thermostatically regulated (37 ºC) F-4500 Hitachi spectrofluorimeter. Excitation was at 340 and 380 nm and emission was at 510 nm. The fura 2 fluorescence response to $[Ca^{2+}]$cyt was calibrated from the ratio of 340/380 nm fluorescence values after subtraction of the background fluorescence of the cells at 340 and 380 nm as described by Grynkiewicz et al. (1985). $[Ca^{2+}]$cyt was calculated by titration with different concentrations of Ca-EGTA buffers (Vercesi et al. 1993).

Measurement of Ca^{2+} movements

Variations in free Ca^{2+} concentrations in whole cells suspensions were followed by measuring the changes in the fluorescence of Calcium Green-5N (Molecular Probes, Invitrogen, Carlsbad, CA), recorded on an F-4500 Hitachi spectrofluorimeter at the wavelength pair 506 – 532 nm.

Mitochondrial isolation and oxygen consumption measurements

Mitochondria was isolated by conventional differential centrifugation from the livers of adult Wistar strain rats, as described elsewhere (Kaplan and Pedersen 1983, Kowaltowski 1996, Kowaltowski 2001, Figueira 2011). The protein concentration of final mitochondrial suspensions was determined using a modified Biuret assay. Oxygen consumption was measured using a Clark-type electrode (Yellow Springs Instruments Co.) in a 1.3 ml glass chamber equipped with a magnetic stirrer. The initial O_2 concentration in the reaction medium was 220 nmol/ml and filled with standard reaction medium supplemented with EGTA (200 μM). The experiments were done at 28°C in a standard medium containing 125 mM sucrose, 65 mM KCl, 2 mM inorganic phosphate, 1 mM magnesium chloride, and HEPES buffer, pH 7.2. Stimulated respiration by oxidative phosphorylation (State 3 respiration) was elicited by the addition of ADP to a final concentration of 300 μM. Phosphorylation efficiency (ADP/O) was calculated as the molar ratio between the amount of added ADP and the oxygen consumed during State 3. The final concentration of mitochondrial proteins was 0.5 mg/mL in the respiratory and MPT assays.

Assesment of Ca^{2+}-induced Mitochondrial Permeability Transition (MPT)

In suspensions of isolated mitochondria, cyclosporin A-sensitive Ca^{2+}-induced MPT result in osmotic swelling, disruption of transmembrane electrical potential, and Ca^{2+} release to the medium; all of which can be spectrophotofluorometrically followed over time. Mitochondrial swelling was estimated from the decreasing absorbance at 520 nm measured in a Hitachi U3000 spectrophotometer, Tokyo, Japan. ΔΨm was estimated by following Safranine O fluorescence (Kowaltowski 1995, Figueira 2011), recorded on an F-4500 Hitachi spectrofluorimeter operating at excitation and emission wavelengths of 495 and 586 nm, respectively, and a slit width of 2.5 nm. Relative changes in membrane potential were expressed as fluorescence arbitrary units (a.u.).

Statistical analysis

Statistical significance was determined by one way ANOVA and the differences between the groups were analyzed using Student's t-test. The significance level was set at $p<0.05$. All tests were performed using the software SigmaStat 3.1 (Systat, San Jose, CA, USA).

Results

The BlL effect was evaluated in B16-F10 and GN-13 cells using the trypan blue exclusion test and by flow cytometry after 24h of exposure in culture. The figure 1 (a) shows that B16-F10 viable cells decreased when BlL concentrations increased (50-100 µg/mL). A percentage of 46.71 ± 5.59 ($P<0.0001$) of viable cells were counted for the concentration of 75 µg/mL (Fig. 1b), thus establishing the IC_{50} value. Cell death was also analyzed by flow cytometry for the same concentrations with observed staining of 49.1 % ± 6.7 on IC_{50} value ($P<0.002$) (Fig. 1c). PI-positive cells divided by the total number of cells (necrotic indicative) was 38.6 % ± 2.7 (Fig. 1c). Morphological alterations were verified by optical microscopy (Fig. 1d). Characteristics as loss of plasmatic membrane integrity and cell lysis were observed, but there was no formation of cytoplasmatic vesicles, emphasizing cell death by necrosis. The lectin treatment in fibroblast, GN-13, exhibits no significant result when tested with Trypan Blue (Fig. 2).

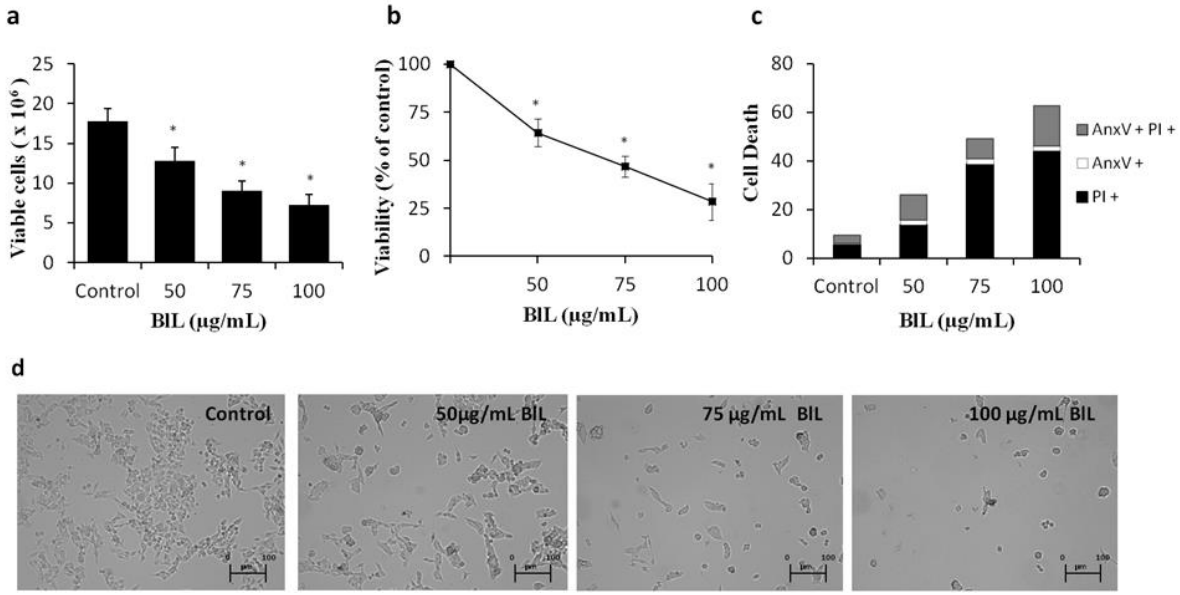

Figure 1. BlL reduce B16-F10 melanoma cell viability. B16-F10 cells were treated with increasing concentrations of BlL for 24h; the number of viable cells were determinate using trypan blue (a and b). The percentages of necrotic (PI+) or apoptotic (AnxV+) cells were determinate by Flow cytometry (c). The cells BlL-treated were photographed under a microscope Leica DFC360 FX, using the LAS AF software (Leica Microsystems) (d). Values are mean ± s.e.m. of at least five independent experiments. * Significantly different from control at $P<0.05$ level, Student's *t*-test.

The production of mitochondrial superoxide after a 6h of treatment with BlL (75 µg/mL) was analyzed in order to understand the mechanisms involved in cell death by necrosis. The data showed that the incubation in the presence of BlL promoted a three-fold increase of mitochondrial ROS levels (Fig. 3a). Interestingly, such increase was related to the increase in the cytosolic levels of calcium ($[Ca^{2+}]$cyt) when the cells were treated with BlL for 4h (Fig. 3b).

These data demonstrate that the increase of mitochondrial ROS production occur after the elevation of $[Ca^{2+}]$cyt in melanoma cells treated with BlL.

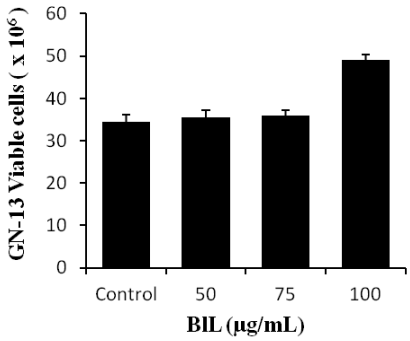

Figure 2. GN-13 fibroblast viability after BlL treatment. After trypsinization, fibroblasts (1 x 10^6 cells/ml) were incubated in DMEM medium with 1% fetal bovine serum in the presence of different BlL concentrations for 24 h. Data are the average ± s.e.m. of four independent experiments. In all the concentrations *p>0.05*.

Previous studies suggested that, the lectins exert a direct effect on mitochondria. This is possible because the lectins can bind to the receptors containing carbohydrate, abundant on tumor cell membranes, which have abnormal glycosilation. The lectin binded to the receptor containing carbohydrate is internalized through clathrin-mediated endocytosis and accumulated preferentially into the mitochondria (Lei and Chang 2007). Thus, we utilized the probe CalciumGreen for the investigation of the effect of BlL on mitochondria of the permeabilized B16-F10 cells in the presence of Ca^{2+}. A representative experiment is depicted in figure 4. The presence of BlL decreases the mitochondrial Ca^{2+} retention capacities and such condition was inhibited for CsA. This data indicates that mitochondrial membrane permeability transition probably has been caused by the direct contact between the lectin and the mitochondria. After this, we investigated the direct effect of BlL on isolated liver mitochondria.

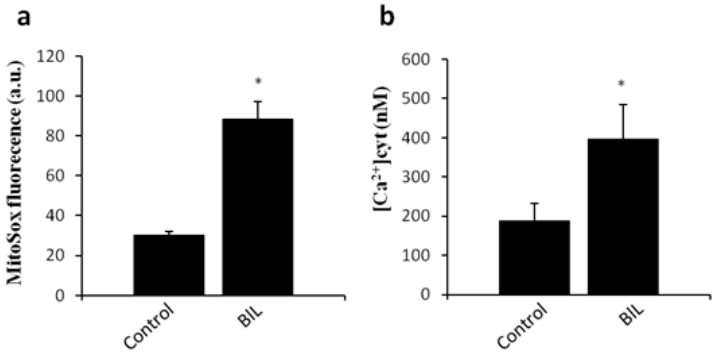

Figure 3. B16-F10 treatment with BlL (75 µg/mL) promotes oxidative stress and increases $[Ca^{2+}]$cyt in B16-F10 melanoma cells. B16-F10 cells were treated with BlL for 6 h, washed and then probed with 5 µM MitoSox (a) or 5 µM Fura 2-AM (b). ROS and $[Ca^{2+}]$cyt production were determined as explained in the Materials and methods section. Values are mean ± s.e.m. of at least 5 independent experiments. * Significantly different from control at P<0.05 level, Student's *t*-test.

Table 1 shows resting (V4) and ADP-stimulated (V3) liver mitochondrial respiration, the respiratory control ratio (RCR), and the phosphorilation efficiency (ADP/O ratio). Figure 5 presents the effect of BlL on ADP-supported state 4. The mitochondrial treatment with BlL (10µg/mL) demonstrate an increase of 57.1% in the state 4 , while no significant alterations were observed in the state 3. However, the ADP/O ratio decreased in 8% when the mitochondria were incubated with BlL.

Table 1. Mitochondrial O_2 consumption and phosphorylation efficiency.

	State 3	**State 4**	**RCR**	**ADP/O**
Control	19.4 ± 2.1	2.8 ± 0.7	6.9 ± 0.5	2.5 ± 0.07
BlL (10 µg/mL)	18.9 ± 2.3	4.4 ± 0.2	4.1 ± 0.9	2.3 ± 0.04

Figure 6 presents the experiments in which the Safranine O fluorescence detected the decrease of the mitochondrial transmembrane electric potential. There was addiction of 20 µM Ca^{2+} and lectin, as well as, the MPT inhibitor cyclosporin A (CsA) 1 µM and 200 µM EGTA. These data suggest a relationship between the treatment of the mitochondrial suspensions with BlL and the mitochondrial membrane permeability transition.

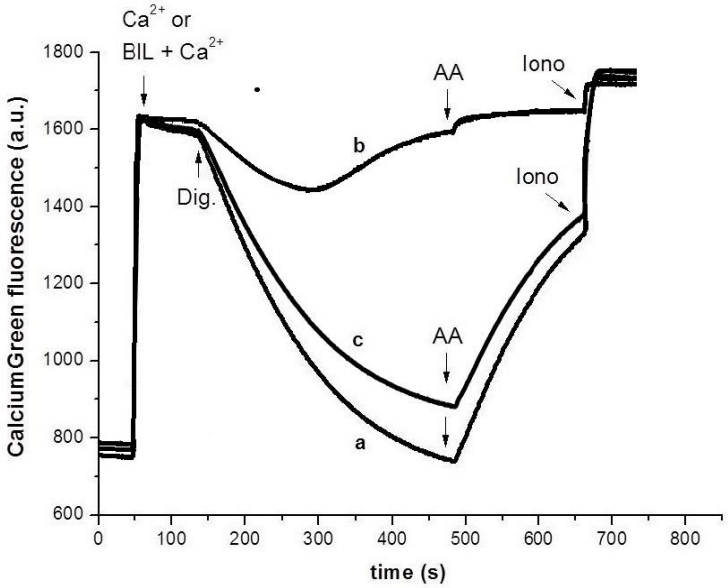

Fig. 4 Effect of BlL on Ca^{2+} movements by permeabilized B16-F10 melanoma cells. 10^6 cells/ml were added to a reaction medium containing 125 mM sucrose, 20 mM HEPES-K^+ and 0.2 µM Calcium Green-5N in a total volume of 1.5 ml. 15µM Digitonin (Dig.), 5µM Antimycin A (AA), 10µM Ionomycin (Iono) and EGTA (0.33 µM) were added at time points indicated by the arrows. The lines represent incubation with: 20 µM Ca^{2+} (*line a), 75* µg/ml BlL (*line b)* and *75* µg/ml BlL plus 1 µM Cyclosporin A *(line c). The* results shown are representative of four independent experiments. a.u., arbitrary units.

In figure 7, the swelling assay showed the relationship between mitochondrial treatment with BlL and MPT. Incubation of liver mitochondria with BlL in a medium containing 20 µM Ca^{2+}, resulted in significant swelling of the organelle. The decreasing turbidity of mitochondrial suspension decreased by two times the absorbance compared to the control. Under these experimental conditions, mitochondrial swelling increased in 22.7 % ± 0.02 when the mitochondria were treated with BlL. To assess whether the treatment with BlL has resulted in the opening of PTPM, the suspensions were incubated in the presence of inhibitors of classical MPT as CsA and EGTA. Under these conditions there was a full protection of mitochondrial swelling. These experiments demonstrated that the mitochondrial permeability Ca^{2+}-dependent was caused by the lectin with consequent opening of PTPM.

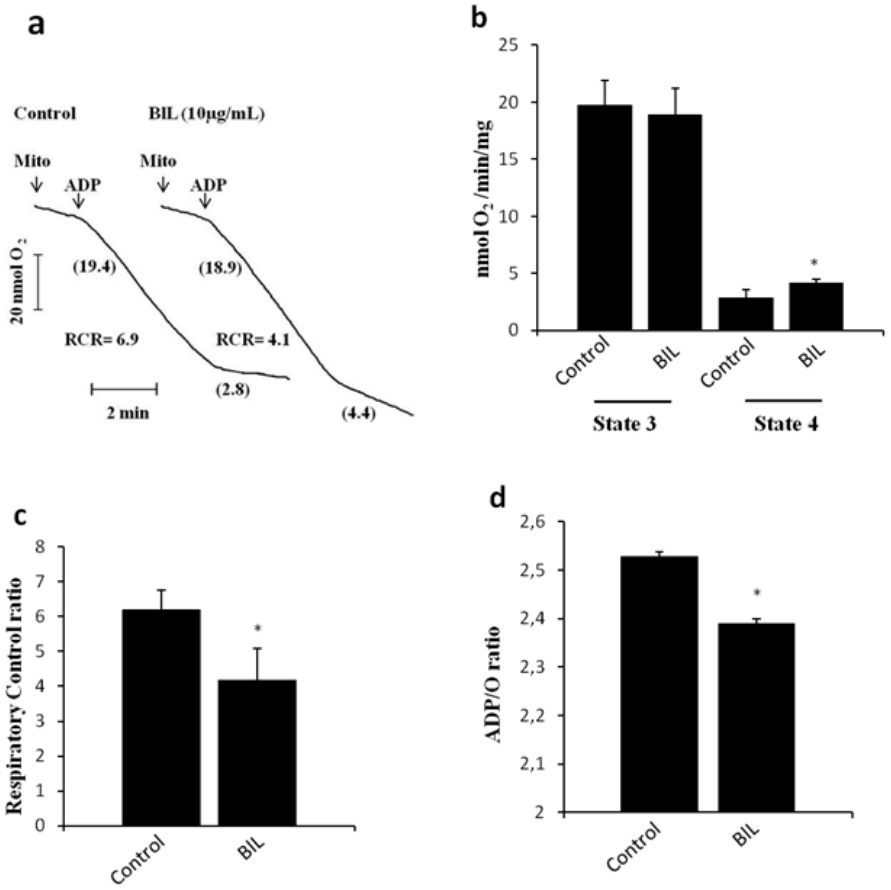

Figure 5. BlL effect in the mitochondrial respiration. Mitochondria isolated from rat liver (0.5 mg / ml) were incubated at 30 ° C in assay medium (pH 7.2) containing 125 mM sucrose, 20 mM HEPES-K $^{+}$ and 200 µM EGTA, in the presence and absence of BlL (10 mg/mL). 300 mM ADP were added after 2 minutes of preincubation to initiate the oxidative phosphorylation. (a and b) BlL effect in the oxygen consumption during state 3 and 4. (c) Effect of BlL on respiratory control ratio (RCR) (d) ADP/O ratio indicating the number of molecules of ADP phosphorylated to ATP when two electrons are transferred from a substrate through the respiratory chain to the reduce of the one oxygen ($^{1}/_{2}$ O_2). Data are the average ± SD of six independent experiments. **p*<0,05.

Discussion

We show here that BLL was selectively cytotoxic to B16-F10 tumorigenic cells, but not to NG-13 health cells. B16-F10 cell death (Fig. 1) was associated with increased [Ca^{2+}]cyt mitochondrial ROS production (Figs. 1 and 3). Previous data have also indicated that lectins allow normal cells to be distinguished from malignant cells (Sabova et al. 2010) because they bind to specific carbohydrate recognition domain (CRD) present in the cell membrane (Nolte et al 2012, Koh et al. 2011, Sharon 2007) that may be differentially expressed in cancer cells.

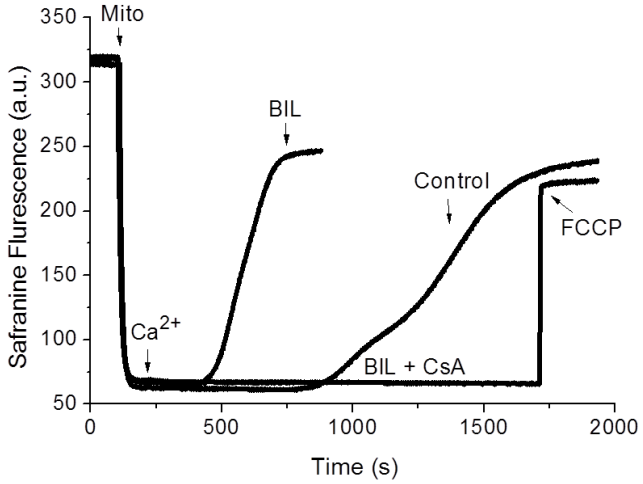

Figure 6. Ca^{2+}-induced dysruption of mitochondrial transmembrane potential (ΔΨ): Effect of the BlL (10 µg/mL). Mitochondrial suspensions (0.5 mg/mL) were added to reaction medium containing BlL and, where indicated, EGTA (200 µM) or cyclosporin A (1 µM) was present as an experimental control. The increase of the safranin fluorescence is related with the decrease in mitochondrial membrane potential. FCCP (2 µM) was added where indicated. Data are of six independent experiments.

Liu et al. (2009) have demonstrated that lectin binds to glycoproteins on the membrane cell and elicits it is internalization and and accumulation into the mitochondria. However little is known about the direct effect of many lectins, including BIL, on mitochondrial function and integrity. Our data (Fig. 4) indicate that the deleterious effects of mitochondrial Ca^{2+} accumulation is potentiated in the presence of BlL.; i.e. BIL stimulated the Ca^{2+}-induced dissipation of mitochondrial transmembrane potential (ΔΨ) in a CsA- sensitive maner.

The mechanism through wich BlL promoted cell death seems to involve mitochondria-mediated necrosis. The elevation of cytosolic Ca^{2+} (Fig. 3b) may have propagated the Ca^{2+} signal to mitochondria where Ca^{2+} overload can trigger MPT opening.and leading to cell killing via dysregulation in ATP and ROS homeostasis (Kowaltowski 2009, Zoratti and Szabo 1995). Submicromolar concentrations of Ca^{2+} stimulate ROS production by mitochondrial α-glycerophosphate dehydrogenase, enzyme present in liver mitochondria (Adam-Vizi and Starkov 2010, Tretter 2007, Wernette 1981). Thus, Ca^{2+} signaling for mitochondrial ROS

generation occurs inside the organelle (Gunter and Sheu 2009) and this dysregulation in Ca^{2+} signaling may compromise cell function and survival via ROS actions (Hidalgo and Donoso 2008). Grijalba et al. (1999) showed that Ca^{2+} bind to cardiolipin and alters the lipid organization of the inner mitochondrial membrane. Such events led to lipid/protein alterations facilitating the propagation of radical reactions (Kowaltowski et al. 2009).

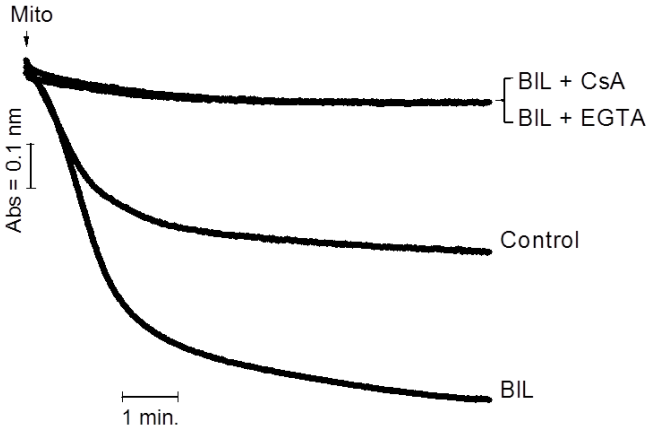

Figure 7. Mitochondrial swelling induced BlL in vitro. Mitochondrial suspensions (0.3 mg/mL) were incubated in the reaction medium containing 20 μM Ca^{2+} in the presence of 10 μg/ml BlL,10 μg/ml BlL plus Cyclosporin A (CsA) 1 μM or 200 μM EGTA. Absorbances at 10 min were: 0.6575 ± 0.015 (Control), 0.5075 ± 0.009 (BlL), 0.8325 ± 0.043 (BlL+CsA) and 0.8153 ± 0.037 (BlL+EGTA). pb0.001 for control vs. all BlL. Data are of six independent experiments.

The increase in mitochondrial state 4 respiration rates is a typical effect of uncouplers, as was observed after incubation of mitochondrial suspensions with BLL. In diverse pathophysiological conditions, the activation of MPT is considered the major cause of cell death (Gleichmann and Mattson 2011, Nicholls 2009, Goldstein and Kroemer 2007). Moreover, a significant decreasing in ΔΨm and the stimulation of the PTP opening were observed when BlL was tested in isolated mitochondria (Figs. 6 and 7). The organization of mitochondrial membrane can also affect the respiratory chain function, including coenzyme Q mobility, leading to monoelectronic oxygen reduction (superoxide radical generation) at intermediate steps of the respiratory chain and leading to mitochondrial oxidative damage followed by cell death (Grijalba et al. 1999). These characteristics were observed when mitochondrial suspensions were submitted to the lectin incubation in presence of Ca^{2+}. In this condition, the mitochondria showed a significant uncoupler-induced mitochondrial permeability transition in a CsA-sensitive mechanism (Fig. 6).

Although there are many reports that relate cell death to the direct action of C-type lectins on the mitochondria, this is the first study that investigates its bioenergetic pathway.

Therefore, our data suggested a relationship between the cell death mediated by BlL and mitochondrial membrane permeability transition.

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4. CONCLUSÃO

De acordo com o trabalho realizado concluímos que o tratamento com BlL das linhagens celulares estudadas:

- BlL apresentou significante atividade citotóxica em células tumorais (K562, Hep-2 e NCI-H292) e induziu morte celular por apoptose em células tumorais K562;
- O tratamento com BlL causou diminuição da viabilidade celular e morte predominantemente por necrose em células B16-F10 de melanoma, mas não houve resultados significativos para células GN-13 de fibroblastos humanos.
- Aumento das concentrações de Ca^{2+} intracelular e consequente produção de espécies reativas de oxigênio mitocondriais.
- Em células permeabilizadas, causa TPM dependente de Ca^{2+}.
- Em mitocôndrias isoladas de fígado de rato, causa abertura de poro de transição de permeabilidade sensível a CsA e EGTA, inibidores clássicos do poro.

Em resumo, a morte de diferentes linhagens de células tumorais após tratamento com BlL está diretamente correlacionada com a ação direta da lectina sobre a função mitocondrial. Desta maneira, este estudo é de grande relevância para o entendimento do mecanismo de atuação desta lectina na morte celular.

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Purification of a lectin with antibacterial activity from *Bothrops leucurus* snake venom

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ABSTRACT

A novel lectin was isolated from *Bothrops leucurus* snake venom using a combination of affinity and gel filtration chromatographies. The lectin (BlL) agglutinated glutaraldehyde-treated rabbit and human erythrocytes with preference for rabbit erythrocytes. Galactose, raffinose, lactose, fetal bovine serum and casein inhibited lectin-induced rabbit erythrocyte agglutination. BlL, with a molecular mass of 30 kDa and composed of two subunits of 15 kDa, showed dependence on calcium. BlL is an acidic protein with highest activity over the pH range of 4.0–7.0 and stable under heating to 70 °C. Fluorescence emission spectra showed tryptophan residues partially buried within the lectin structure. The percentages of secondary structure revealed by circular dichroism were 1% α-helix, 44% β-sheet, 24% β-turn and 31% unordered. BlL showed effective antibacterial activity against Gram-positive bacteria *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus subtilis* with minimal inhibitory concentrations of 31.25, 62.25 and 125 μg/mL, respectively. In conclusion, *B. leucurus* snake venom contains a galactoside-binding lectin with antibacterial activity.

1. Introduction

Lectins are proteins or glycoproteins that bind reversibly to carbohydrates and glycoconjugates (De-Simone et al., 2006). Lectins have been found in a wide range of organisms from microorganisms to plants and animals (Utarabhand et al., 2007). C-type lectins are a large family of Ca^{2+} dependent lectins. Animal C-type lectins can be classified into 17 groups according to structural and functional characteristics (Zelesnky and Gready, 2005). Snake venoms contain C-type lectins included in group VII, which are true sugar-binding lectins composed of homodimers or homooligomers and with Ca^{2+} and usually galactose-binding properties (Clemetson, 2010). Snake venoms also contain C-type lectin-like proteins which are heterodimers or oligomeric complexes of heterodimers called snaclecs (snake venom C-type lectins); this group is more abundant and possesses a loop-swapping or higher-order multimerization (Ogawa et al., 2005; Clemetson et al., 2009; Clemetson, 2010).

Snake venom lectins are able to inhibit or activate specific platelet membrane receptors and blood coagulation factors (Morita, 2004, 2005; Ogawa et al., 2005; Wang, 2008) and can promote a diversity of biological effects, such as lymphocyte proliferation (Mastro et al., 1986), induction of edema (Lomonte et al., 1990; Panunto et al., 2006), induction of Ca^{2+} release from the sarcoplasmic reticulum (Ohkura et al., 1996), inhibition of cancer cell proliferation (Pereira-Bittencourt et al., 1999), erythrocyte agglutination *in vitro* (Kassab et al., 2001), cytotoxicity to tumors and endothelial cell lines (Carvalho et al., 2001), renal effects (Havt et al., 2005) and induction of rolling of leukocytes (Elífio-Esposito et al., 2007).

Glycoconjugates present on bacterial cell surfaces, such as peptidoglycans, lipopolysaccharides and teichoic acids, constitute potential lectin targets (Lee et al., 1998; Santi-Gadelha et al., 2006). Recently, it was reported that snake venom lectins are able to inhibit growth of phytopathogenic bacteria (Rádis-Baptista et al., 2006; Barbosa et al., 2010); however, the interactions between snake venom lectins and human pathogenic bacteria have not been studied.

Bothrops leucurus (white-tailed-jararaca) is an important venomous snake that inhabits northeastern Brazil. *B. leucurus* was responsible for all cases of envenomation from snakebite recorded in the metropolitan region of Salvador (State of Bahia, northeastern Brazil) from January to June 1990 (Lira-da-Silva and Nunes, 1993) and an epidemiological study in Bahia in 2001 revealed that this species was responsible for all confirmed cases of envenomation by *Bothrops*

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species in this state (Mise et al., 2007). Recently, active components from *B. leucurus* venom were isolated, including a fibrinolytic proteinase (Bello et al., 2006), a thrombin-like enzyme (Magalhães et al., 2007), phospholipase A_2 (Higuchi et al., 2007), a P-III metalloproteinase (Sanchez et al., 2007), L-amino acid oxidases (Silva et al., 2007; Torres et al., 2010) as well as the metalloproteinases leucurolysin-a (Gremski et al., 2007; Ferreira et al., 2009) and BleucMP (Gomes et al., 2011).

This paper reports the purification, characterization and antibacterial activity of a novel galactoside-binding lectin isolated from snake venom of *B. leucurus*, a species with great medical importance in northeastern Brazil.

2. Material and methods

2.1. Chemicals

Reference samples of 4.4′-Bis 1-anilinonaphthalene 8-sulfonate (bis-ANS) were purchased from Molecular Probes Inc. (USA). Broad-range protein molecular mass markers, sugars and glycoproteins were purchased from Sigma-Aldrich (USA). All the solvents and other chemicals used were of analytical grade from Sigma-Aldrich (USA) or Merck (Germany). All solutions were prepared with water purified by the Milli-Q® system (Millipore).

2.2. B. leucurus venom

B. leucurus venom was kindly supplied by the Núcleo Regional de Ofiologia e Animais Peçonhentos da Bahia, Universidade Federal da Bahia, Salvador, Bahia, Brazil.

2.3. Protein content and neutral carbohydrate analysis

Protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard. Neutral carbohydrate content was determined by the phenol-sulphuric acid method (Dubois et al., 1956) using mannose as a standard.

2.4. Hemagglutinating activity and carbohydrate specificity

Hemagglutinating activity (HA) was assessed in microtiter plates according to Correia and Coelho (1995) using rabbit and human A, B, AB and O-type erythrocyte suspensions (2.5% v/v; 50 μL) treated with glutaraldehyde (Bing et al., 1967). HA was defined as the lowest lectin concentration able to promote erythrocyte agglutination. Specific hemagglutinating activity (SHA) corresponded to the ratio between HA and protein concentration (mg). Carbohydrate binding specificity was evaluated by determining HA in the presence of sugars (D-galactose, D-glucose, D-fructose, D-lactose, D-mannose, methyl-α-D-glucopyranoside, D-arabinose, L-rhamnose methyl-α-D-mannopyranoside, *N*-acetyl-D-glucosamine, D-xylose and L-raffinose) and glycoproteins (asialofetuin, casein, fetuin and fetal bovine serum).

2.5. Purification of B. leucurus venom lectin

Lyophilized *B. leucurus* venom (30 mg) was dissolved in 1 mL of calcium-Tris-buffered saline buffer (CTBS; 20 mM Tris-HCl, 150 mM NaCl and 5 mM $CaCl_2$, pH 7.5) and centrifuged (2000 *g*, 5 min, 25 °C) to remove insoluble material. The resulting supernatant was applied to a column (10 × 1.0 cm) of guar gel previously equilibrated with CTBS at a flow rate of 10 mL/h. Protein elution was monitored by absorbance at 280 nm. After washing to remove unbound proteins, the adsorbed proteins were eluted from the column with 200 mM galactose in CTBS. Adsorbed fractions with HA were pooled, dialyzed, lyophilized and applied to a Superdex 75 HR 10/300 GL column coupled to an ÄKTA™ purifier system (GE Pharmacia). The column was equilibrated and eluted with 50 mM Tris–HCl buffer (pH 8.0) containing 150 mM NaCl at a flow rate of 0.5 mL/min; fractions of 1 mL were collected and protein elution was monitored by absorbance at 280 nm. Subsequently, the active peak from Superdex 75 chromatography (*B. leucurus* lectin; BlL) was submitted to reverse-phase chromatography in a C-4 column (Vydac-Protein Peptide Ultrasphere) performed in an HPLC system (Shimadzu LC-10 AD-Tokyo, Japan), with elution monitored at 280 nm. The column was equilibrated with 0.1% TFA (solvent A) and eluted using 90% acetonitrile/10% H_2O/0.1% TFA (solvent B) in a non-linear gradient, where B = 0% at t = 5 min, 45% at t = 10 min, 50% at t = 30 min and 100% at t = 35 min.

2.6. Effects of divalent ions, pH and temperature on HA

To evaluate the effect of divalent cations on BlL-induced HA, the lectin was previously dialyzed against 5 mM EDTA (16 h at 4 °C) followed by 150 mM NaCl (6 h at 4 °C) to eliminate EDTA. Subsequently, the HA of dialyzed BlL was evaluated in the presence of 50, 100 and 200 mM Ca^{2+}, Mn^{2+} or Mg^{2+} in 150 mM NaCl. The effects of pH and temperature on HA were evaluated by incubation (45 min at 25 °C) of BlL in selected buffers (10 mM citrate phosphate, pH 4.0–6.0; 10 mM sodium phosphate, pH 7.0; 10 mM Tris–HCl, pH 8.0–9.0) or after heating (30 min) at 30, 40, 50, 60, 70, 80, 90 and 100 °C.

2.7. Polyacrylamide gel electrophoresis (PAGE)

BlL was evaluated by native PAGE for basic [15% (w/v) gel] or acidic [15% (w/v) gel] proteins according to Reisfeld et al. (1962) and Davis (1964), respectively. Electrophoresis in the presence of SDS and β-mercaptoethanol was performed in 15% (w/v) gel according to Laemmli (1970). Polypeptide bands were stained with Coomassie Brilliant Blue in 10% acetic acid (0.02%, v/v). Glycoprotein staining was performed using the periodic acid-Schiff method (Zacharius et al., 1969).

2.8. Analysis of polypeptide chains

Polypeptide chain analyses were performed after reduction of disulfide bridges and alkylation. Lyophilized samples were reduced by the Friedman reaction (Friedman et al., 1970) with some modifications: BlL (0.5 mg) was dissolved in 250 μL of a solution containing 50 mM Tris–HCl, pH 8.6, 6 M urea, 10 mM EDTA and 179 mM DTT; the mixture was incubated for 3 h at 37 °C in the dark before N_2 purging. Free sulphydryl groups were then exposed to 100 μL of iodoacetate and the reaction continued for another 2 h under the same initial conditions. Iodoacetate derivative chains were desalted and separated by HPLC in a reverse-phase C4 column with the elution profile monitored at 280 nm.

2.9. Fluorescence spectroscopy

Intrinsic fluorescence emission of BlL in solution (0.07 mg/mL in 10 mM phosphate buffer pH 7.0) was measured at 25 °C using a spectrofluorimeter (JASCO FP-6300, Tokyo, Japan) and a cuvette (1-cm pathlength rectangular quartz). The excitation wavelengths were 280 and 295 nm; emission spectra were recorded at a range of 305 to 450 nm with band passes of 5 nm.

2.10. Circular dichroism (CD) measurements

CD measurements were carried out in a J-810 JASCO spectropolarimeter. The instrument was calibrated with D-10-camphorsulfonic acid. The measurement was carried out at 25 °C with a protein concentration of 0.250 mg/mL (8 μM) in a 1 mm pathlength cuvette. C spectrum was recorded in the 191–250 nm range as an average of

eight scans. The results were expressed as the mean residue ellipticity, [θ], defined as $[\theta]=\theta_{obs}/(10.C.l.n.)$, where θ_{obs} is the CD in millidegrees, C is the protein concentration (M), l is the pathlength of the cuvette (cm) and n is the number of amino acid residues assuming a mean number of 272 residues. The CDPro software was used to estimate the fractions of secondary structures (Sreerama and Woody, 2000) and the Cluster program was used to determine the tertiary structure class of BIL (Sreerama et al., 2001).

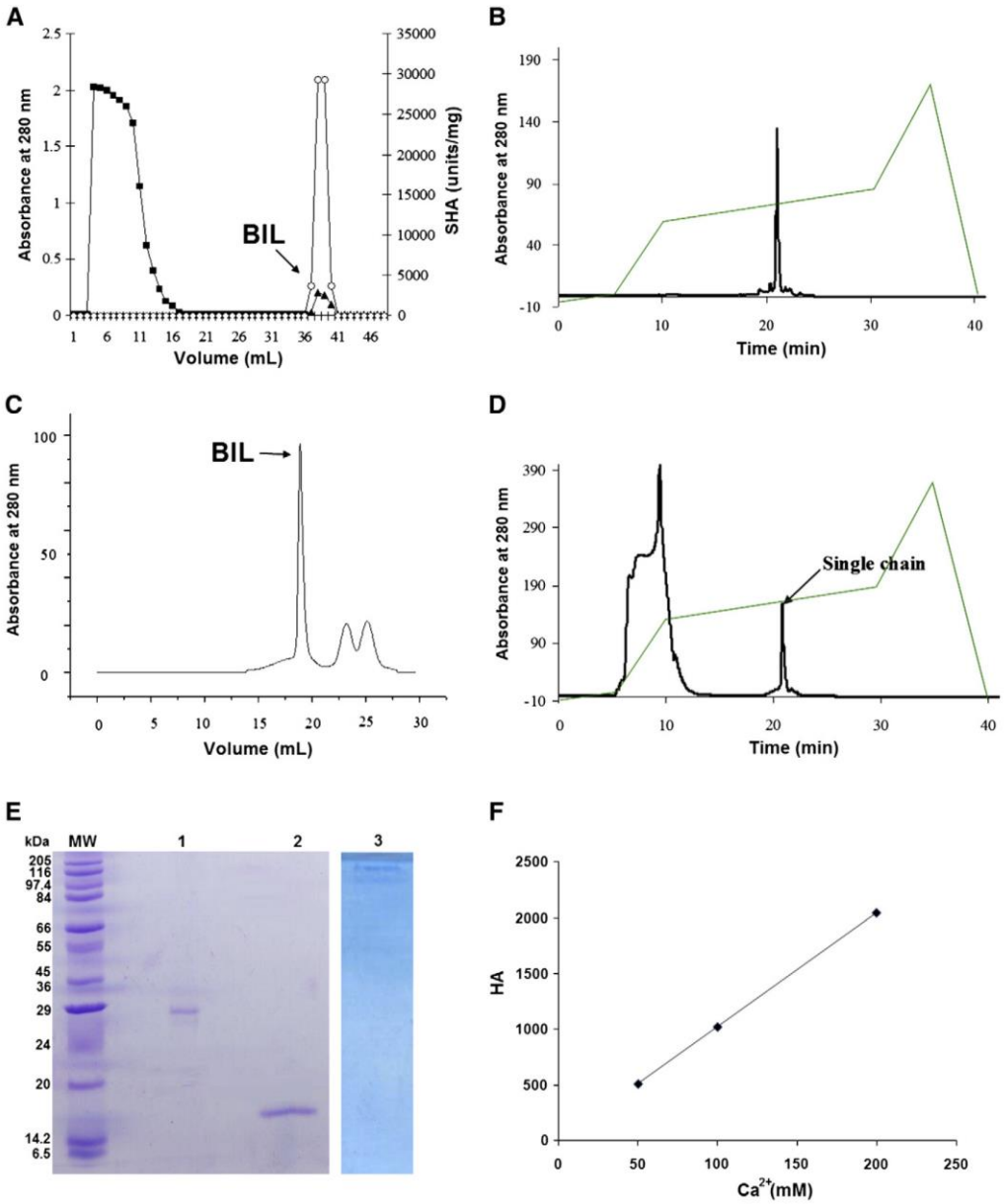

Fig. 1. (A) Purification of BIL by affinity chromatography of *B. leucurus* venom (30 mg of protein) in a guar gel column. Elution with CTBS buffer (—■—) followed by 200 mM D-galactose (—▲—; arrow). Specific hemagglutinating activity (SHA, -○-). (B) Purification of BIL by chromatography in Superdex 75 column coupled to an ÄKTA purifier system. (C) SDS-PAGE of BIL. (MW) molecular weight markers; BIL under non-reducing (lane 1) or reducing conditions (lane 2); electrophoresis under native conditions for acidic proteins (lane 3). (D) Reverse phase HPLC in a C4 column. (E) BIL chain separation after desalting in a C4 column. The column was equilibrated with 0.1% TFA (solvent A) and eluted using 90% acetonitrile/10% H_2O/0.1% TFA (solvent B) in a non-linear gradient, where B = 0% at t = 5 min, 45% at t = 10 min, 50% at t = 30 min and 100% at t = 35 min. (F) Hemagglutinating activity (HA) of EDTA-treated BIL after the addition of Ca^{2+} at different concentrations.

2.11. Antibacterial activity

Gram-positive (*Bacillus subtilis* ATCC-6633, *Staphylococcus aureus* ATCC-6538 and *Enterococcus faecalis* ATCC-6057) and Gram-negative (*Escherichia coli* ATCC-25922 and *Klebsiella pneumoniae* ATCC-29665) bacterial strains were provided by the Departamento de Antibióticos, Universidade Federal de Pernambuco, Brazil. Stationary cultures were maintained in nutrient agar and stored at 4 °C.

Bacteria were cultured in nutrient broth and incubated under continuous shaking at 37 °C overnight. The culture concentrations were turbidimetrically adjusted at 600 nm to 10^5–10^6 colony forming units (CFU)/mL. Purified lectin (BIL) was diluted (1:2048) in a microtiter plate containing nutrient broth (50 μL per well). Subsequently, 20 μL of bacterial suspension was applied to each well and the plate was incubated at 37 °C for 24 h. After incubation, the optical density at 490 nm (OD_{490}) was measured using a spectrophotometer for microplates. The assays were performed in triplicate. The minimal inhibitory concentration (MIC) corresponded to the lowest lectin concentration able to inhibit the growth of 50% or more of microorganisms relative to the negative control (Amsterdam 1996). Thereafter, aliquots (20 μL) of each well in which inhibitory activity was observed were transferred to petri plates containing nutrient agar. The plates were incubated at 37 °C for 24 h. The minimal bactericide concentration (MBC) corresponded to the lowest concentration of lectin able to reduce the number of CFU to 0.1% relative to the negative control. The antibacterial activity of BIL was also determined in the presence of 200 mM galactose.

3. Results and discussion

Crude extract of *B. leucurus* venom showed high lectin activity (SHA 136.5 units/mg) towards rabbit erythrocytes. The inhibition of HA by galactose suggested the presence of a galactoside-binding lectin; this result encouraged us to evaluate the use of affinity chromatography on guar gel matrix to purify lectin.

Lectin activity from crude venom adsorbed on guar gel column and only one active (SHA of 29,257) peak was detected after elution with 200 mM galactose (Fig. 1A). The use of guar gel was an inexpensive and innovative protocol for isolation of a snake venom lectin. Guar gum consists of straight chains of mannose substituted with α(1–6) galactose residues and is a versatile and viable matrix for the isolation of D-galactopyranosyl- and N-acetyl-galactosaminyl-binding lectins (Lonngren and Goldstein, 1976; Gupta et al., 1979). Guar gum has been used as an efficient and inexpensive affinity support for the purification of lectins from *Bauhinia monandra* leaves (Coelho and Silva, 2000) and *Moringa oleifera* seeds (Santos et al., 2009) as well as from the alga *Vidalia obtusiloba* (Melo et al., 2004).

The adsorbed fractions from guar gel affinity chromatography were loaded into gel filtration columns (Fig. 1B); three peaks can be seen in the chromatographic profile. The lectin (*B. leucurus* lectin; BIL) was eluted at 18 mL, corresponding to an apparent molecular weight around 8 kDa; however, this major peak (SHA 10,240) showed a molecular mass of 30 kDa in SDS–PAGE. In the presence of reducing agent β-mercaptoethanol, BIL was revealed to be a dimeric protein composed of two subunits with a molecular mass of 15 kDa (Fig. 1C). Because lectins may interact in undesirable ways with Superdex beads, a possible interaction of BIL with the stationary phase may have affected the retention time and apparent molecular weight of BIL in gel filtration chromatography as well as may be responsible for the reduction of SHA observed (Table 1).

Evaluation of BIL by native electrophoresis showed a single polypeptide band in PAGE for acidic proteins (Fig. 1C). No polypeptide band was detected in native PAGE for basic proteins. As shown by Lomonte et al. (1990), using isoelectric focusing, other snake venom lectins are characterized as acidic proteins. BIL was eluted from the C-4 column with about 50% acetonitrile (Fig. 1D). The reduction and alkylation reactions of BIL were performed using DTT. After desalting in the C-4 column, only one peak was obtained (Fig. 1E), suggesting the presence of homodimeric chains covalently linked by disulfide bridges; this result agrees with that observed by electrophoresis. Several lectins from snake venoms are constituted of disulfide-linked homodimers, such as the lectins from *Agkistrodon piscivorus piscivorus* and *Crotalus ruber* (Komori et al., 1999; Hamako et al., 2007). However, the crystal structure of a galactoside-binding lectin from *Crotalus atrox* revealed a decameric structure composed of two 5-fold symmetric pentamers (Walker et al., 2004).

Table 1 summarizes the BIL purification. The amount of protein recovered after gel filtration chromatography was less than 1%. The content of BIL in snake venom (< 1%) was similar to those found for other lectins isolated from snake venoms (Ogilvie et al., 1986; Lomonte et al., 1990; Carvalho et al., 1998; Nikai et al., 2000; Guimarães-Gomes et al., 2004).

The HA of BIL was abolished after treatment with the chelating agent EDTA (5 mM); Mn^{2+} and Mg^{2+} did not restore BIL HA but the activity was gradually increased by the addition of Ca^{2+} and completely restored when this ion was added at 200 mM (Fig. 1F); this result indicates that Ca^{2+} is essential for the carbohydrate-recognizing property of BIL. The homodimeric structure and calcium dependence indicate that BIL may be included in the group of true galactoside-binding lectins from snake venoms and does not belong to the group of snaclecs described by Clemetson et al. (2009). Several other lectins isolated from snake venoms are dependent on calcium (Ozeki et al., 1994; Kassab et al., 2001; Guimarães-Gomes et al., 2004; Clemetson, 2010). However, this statement can only be confirmed after N-terminal sequencing and homology studies.

Table 1
Purification of *B. leucurus* lectin (BIL).

Sample	Protein (mg)	Total HA[a]	SHA (HA/mg)	Purification (fold)[b]
Crude venom	30	4096	136.5	1
Affinity chromatography	0.21	6144	29,257	214.3
Gel filtration	0.1	1024	10,240	75

[a] Hemagglutinating activity (HA) with rabbit erythrocytes. SHA: specific HA (ratio between HA and protein content).
[b] Purification fold corresponds to the ratio between SHA of BIL and SHA of crude venom. The data correspond to one purification process.

Table 2
Inhibition of hemagglutinating activity of BIL by carbohydrates and glycoproteins.

Inhibitor	Minimal inhibitory concentration[a]
D-galactose	0.78
D-lactose	1.56
L-raffinose	1.56
D-glucose	12.5
N-acetyl- D-glucosamine	NI
D-arabinose	NI
D-mannose	NI
D-fructose	NI
D-xylose	NI
L-rhamnose	NI
Methyl-α-D-mannopyranoside	NI
Methyl-α-D-glucopyranoside	NI
Asialofetuin	NI
Fetuin	NI
Casein	0.25
Fetal bovine serum	0.25

Assays were performed with rabbit erythrocytes and in triplicate.
[a] Minimal inhibitory concentration corresponds to lowest carbohydrate and glycoprotein concentrations able to inhibit HA of BIL. Highest carbohydrate and glycoprotein concentrations used were 200 mM and 0.5 mg/mL, respectively. NI indicates that no inhibition was detected. SHA of BIL in absence of sugars or glycoproteins: 10,240.

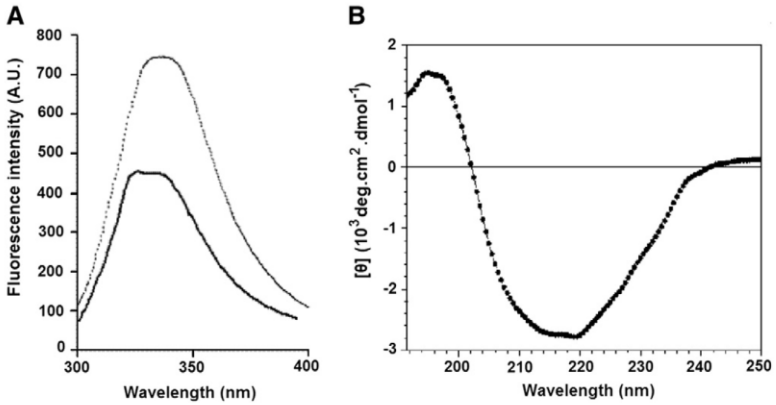

Fig. 2. (A) Intrinsic fluorescence emission of BlL excited at 280 nm (- - -) and 295 nm (—). (B) CD spectrum of BlL in 50 mM phosphate buffer, pH 7.2, at 25 °C. Measurements are the average of eight scans using a solution containing 0.25 mg of protein/mL. CD spectrum deconvolution using CDPro software calculated 1% α-helix, 44% β-sheet, 24% β-turn, 31% unordered structures and an *RMS* of 2%.

BlL was not detected by glycoprotein staining using periodic acid-Schiff's reagent and no carbohydrate was detected using the phenol-sulphuric acid method. The absence of carbohydrate moiety was also reported for lectins from the snakes *Bothrops atrox*, *Lachesis muta*, *Dendroaspis jamesonii*, and *Bothrops jararacussu* (Gartner et al., 1980; Gartner and Ogilvie, 1984; Ogilvie et al. 1986; Carvalho et al., 2002). BlL recognized the structure of saccharides comprising the surface of erythrocyte membranes since it agglutinated glutaraldehyde-treated erythrocytes from rabbits (SHA 10,240) and human types A, B and O (SHA of 320, 320 and 160, respectively). The difference in erythrocyte agglutination, depending on cell type (A, B, AB and O) and species of origin, may be due to the presence of different glycoproteins on the erythrocyte surface. Similar to other lectins from snakes, HA of BlL was abolished in the presence of galactose, lactose and raffinose (Table 2), indicating that BlL is a galactoside-binding protein. Inhibition assays revealed that BlL was partially inhibited by fetal bovine serum and casein, but not by fetuin and asialofetuin (Table 2). Casein is a protein with a small carbohydrate fraction and contains phosphorus in its structure (Roman and Sgarbieri, 2005). Two N-glycosidic carbohydrate complex-types rich in galactose are present in the structure of α-fetoprotein, a protein found in fetal bovine serum (Krusius and Ruoslahti, 1982); the presence of galactose may be responsible for the inhibition of BlL in the presence of fetal bovine serum. The results indicate that BlL recognizes complex glycoproteins.

BlL HA was heat-stable up to 70 °C, with total loss of activity after heating to 80 °C indicating that HA depends on BlL native conformation. The HA of BlL was not affected at a pH range of 4.0 to 7.0, unlike *B. jararacussu* lectin, which was more active in neutral pH (Elífio-Esposito et al., 2007). The intrinsic protein fluorescence spectra of BlL (Fig. 2A) revealed a single major peak at 344 nm. Tryptophan residues exposed to water show a maximal fluorescence emission at wavelengths around 340–350 nm whereas completely buried residues fluoresce at about 330 nm. The displacement of the mass center of the aromatic residues indicates partially buried hydrophobic domains within the lectin structure. C-type lectins are usually a dimer of two identical polypeptides, each containing two tryptophan residues and one tyrosine residue (Morita, 2005). The individual subunits are able to bind carbohydrates but for the lectin-like function they need at least bivalency, which is achieved through a simple interchain disulfide linkage. Although dimerization is essential, the two tryptophan residues and the tyrosin are essential to stabilize intra-subunit contacts and for biological activities in C-type lectins (Doyle and Kini, 2009).

The CD spectrum of BlL (Fig. 2B) indicated that BlL possessed a large amount of β-sheet structure, characterized by a maximum at approximately 195 nm and a minimum in the range 216–220 nm (Venyaminov and Yang, 1996). Analysis of the secondary structure content using CDPro software yielded the following results: 1.0% α-helix, 44% β-sheet, 24% β-turn, 31% unordered structures and an *RMS* (root-mean-square) of 2.0%. Cluster analysis classified BlL as a β-class protein (proteins containing mainly β structure), corroborated by the results of CDPro analysis (68% β structures). The secondary structure content of BlL was similar to that determined for *L. muta* snake venom lectin (Aragón-Ortíz et al., 1989); however, the majority of lectins from snake venoms belong to the α + β class, such as the C-type lectin from *B. jararacussu* venom which possesses 18.8% α-helix and 32.2% β-sheet (Silva Jr. et al., 2008).

In the present study, antibacterial assays demonstrated that BlL exhibited antibacterial effects against the human pathogenic Gram-positive bacteria *S. aureus*, *E. faecalis* and *B. subtilis*. Minimal inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined for BlL (Table 3). The lectin was not effective against Gram-negative bacteria *E. coli* and *K. pneumoniae*. A possible reason for the difference in susceptibility is the difficulty that BlL encounters in crossing the outer cell wall of Gram-negative bacteria to reach the periplasmic space. BlL may interact with the peptidoglycan present in the Gram-positive bacteria cell wall while the lectin may not be able to bind peptidoglycans of Gram-negative bacteria whether it does not enter in the periplasmic space. BlL showed an absence of antimicrobial activity in the presence of 200 mM galactose, thereby assuring that the antibacterial effect involves the carbohydrate-binding property of lectin. MIC values of BlL against *S. aureus* and *E. faecalis* show the clinical relevance of lectin since these concentrations are below the range of 64–100 μg/mL (Gibbons, 2004). The MBC of BlL

Table 3
Minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of BlL.

Bacteria	MIC[a]	MBC[a]
Staphylococcus aureus (+)	31.5	500
Enterococcus faecalis (+)	62.5	330
Bacillus subtilis (+)	125	250
Escherichia coli (−)	ND	ND
Klebsiella pneumoniae (−)	ND	ND

[a] MIC and MBC expressed as μg/mL of lectin. ND: antibacterial activity not detected at 1000 μg/mL of BlL. Gram-positive (+) and Gram-negative (−) bacteria.

against *B. subtilis* (250 μg/mL) was lower than that (500 μg/mL) described for *Phthirusa pyrifolia* leaf lectin (Costa et al., 2010).

The MBC for bactericidal drugs is generally the same or not more than four-fold higher than the MIC. In contrast, the MBC of bacteriostatic drugs are many-fold higher than their MIC (Levison, 2004). The term tolerant is applied to bacterial strains whose growth stops in the presence of an antimicrobial concentration but which do not rapidly die leading to high values of MBC (Charpentier and Tuomanen, 2000). On the basis of MBC/MIC ratio, *S. aureus* was found to be tolerant to BlL since the MBC was 15.8-fold greater than MIC (Ishida et al., 1982). On the other hand, Canillac and Mourey (2001) reported that if the MBC/MIC ratio was found to be less than or equal to 4, the bacteria were considered to be susceptible. Therefore, *B. subtilis* was susceptible to BlL. Recently, Torres et al. (2010) showed that *Bothrops leucurus* total venom (BleuTV) inhibited the growth of *S. aureus*. Therefore, according to our results we can suggest that BlL is involved in the antibacterial activity of the venom.

In conclusion, a new galactoside-binding lectin was isolated from *B. leucurus* venom. BlL showed antibacterial activity against human pathogenic Gram-positive bacteria. Further studies are required to determine the mechanisms involved in this bactericidal activity.

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Capítulo II

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Inactivation and fragmentation of lectin from *Bothrops leucurus* snake venom by gamma irradiation

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ABSTRACT

Gamma radiation alters the molecular structure of biomolecules and is able to mitigate the action of snake venoms and their isolated toxins. The effect of γ-radiation on the folding of *Bothrops lecurus* venom lectin was measured by a hemagglutinating assay, intrinsic and bis-ANS fluorescence. Intrinsic and bis-ANS fluorescence analyses indicated that irradiation caused unfolding followed by aggregation of the lectin. Our results suggest that irradiation can lead to significant changes in the protein structure, which may promote the loss of its binding property and toxic action.

1. Introduction

Snake venoms are complex mixtures of bioactive proteins and polypeptides (Koh et al., 2006). These toxins are enzymatic and non-enzymatic proteins and synergistic interactions between venom proteins enhanced the lethal potency of the snake venom. Complexes of protein families, such as metalloproteases, serine proteases, C-type lectins (CTLs), C-type lectin-related proteins (CLRPs) and three-finger toxins (3FTxs), have also been reported in venoms (Doley and Kini, 2009). Lectins are proteins or glycoproteins that are ubiquitous in nature and bind reversibly to carbohydrates (Sharon and Lis, 2004).

Ionizing radiation causes changes in the function and integrity of biomolecules, including proteins, by two different means: first, by interacting directly on proteins (Kempner, 2001) and second, by the formation of major products from radiolysis of water (Wang and Wang, 2007). However, the effect induced after water radiolysis represents 90% of the molecular radiation-induced damage. The exposure of proteins to low doses of radiation produces chemical and physical damage that may result in changes to the protein's primary, secondary or tertiary structure, yet keeping their immunological properties intact (Nascimento et al., 1996).

The intimate relationship existing between the structure and activity of proteins has received attention from researchers. Many studies have shown the effects of gamma radiation at the molecular level, including on the biological activity of snake venom, where radiation results in a decrease or loss of enzymatic and toxic actions (Casare et al., 2006). BlL is a galactoside-binding lectin from *Bothrops leucurus* snake venom. The lectin, a protein of 30 kDa composed of two subunits of 15 kDa, has antibacterial activity against Gram-positive bacteria (Nunes et al., 2011). Here, the interest lies in studying the effects of ionizing radiation on a lectin isolated from the venom of *Bothrops leucurus*, in order to employ these results in the explanation of how radiation affects the structure-activity relationship of snake venom lectin.

2. Experimental

2.1. Purification of Bothrops leucurus lectin (BlL) and lectin irradiation

Bothrops leucurus lectin (BlL) was purified according to Nunes et al. (2011). The lectin aliquots (0.07 mg/mL), in phosphate buffer (pH 7.0) and borosilicate glass vials (16–125 mm), were frozen and irradiated under atmospheric O_2 using a Gammacell

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220 Excel ^{60}Co gamma ray irradiator (Ontario, Canada) at doses of 1 and 2 kGy at a rate of 7.2 kGy/h.

2.2. Hemagglutination activity and protein concentration

Hemagglutination activity (HA), which was defined as the lowest sample dilution that showed hemagglutination, was evaluated as described by Correia and Coelho (1995). Specific HA (SHA) corresponded to the relationship between HA and protein concentration measured according to Bradford (1976), using bovine serum albumin (BSA) as a standard. The percentage of the remaining SHA (%SHA_{REM}) was calculated according to the equation: $\%SHA_{REM} = (SHA)_{GM}/(SHA)_{GO} \times 100$, where G_M is the SHA of the lectin at each radiation dose (1 and 2 kGy) and G_0 is the SHA of non-irradiated lectin (control).

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli (1970). Protein samples were mixed with loading buffer (60 mM Tris–HCl, pH 6.8, with 2% SDS, 25% glycerol and 0.1% bromophenol blue), resolved on a 15% separating gel and stained using Coomassie Brilliant Blue (Sigma). The following standard molecular weight markers were used: rabbit muscle myosin (205 kDa), *E. coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase b (97.4 kDa), rabbit muscle fructose-6-phosphate kinase (84 kDa), bovine serum albumin (66 kDa), bovine liver glutamic dehydrogenase (55 kDa), egg albumin (45 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), bovine milk α-lactalbumin (14.2 kDa) and bovine lung aprotinin (6.5 kDa).

2.4. Reverse phase chromatography analysis

BIL (0.07 mg/mL) was irradiated (1 and 2 kGy) and submitted to reverse phase chromatography in a C-4 column (Vydac-protein peptide ultrasphere), performed on an HPLC system (Shimadzu LC-10AD, Kyto, Japan) and monitored at 280 nm. The column was equilibrated with solvent A (0.1% TFA in H_2O) at a flow rate of 0.7 mL/min, and a non-linear gradient elution was used with solvent B (90% acetonitrile, 10% H_2O, 0.1% TFA) in A, with 0% B at $t=5$ min; 45% B at $t=10$ min; 50% B at $t=30$ min and 100% B at $t=35$ min.

2.5. Fluorescence spectroscopy

The fluorescence emission intensity of the irradiated 0.07 mg/mL BIL solution in phosphate buffer at pH 7.0 was measured at 25 °C using a spectrofluorometer (JASCO FP-6300, Tokyo, Japan) in a rectangular quartz cuvette with a 1 cm path length. The excitation wavelengths were 295; emission spectra were recorded in the range of 305–450 nm, and band passes were 5 nm. Light scattering was measured at 90° for the aggregation assays; light scattering values at 320 nm were monitored (300–340 nm). The spectra displayed in the figures are the average of three scans that were corrected for the solution signal by subtracting the solution spectrum.

2.6. Hydrophobic surface analysis

The lectin's hydrophobic surface was measured using the same conditions employed for the intrinsic fluorescence experiment. Samples were transferred to a quartz cuvette and then mixed with 5 μM bis-ANS; fluorescence was measured in a JASCO spectrofluorometer. The fluorescence emission was obtained at 400–600 nm with excitation at 360 nm (Bhattachryya et al., 2000).

3. Results and discussion

Ionizing radiation has been widely employed to attenuate venoms and isolated toxins, preserving their immunogenic properties. However, little is known about the molecular changes that irradiated proteins undergo. Thus, we compared native and irradiated *Bothrops leucurus* snake venom lectin (BIL) to characterize the structural modifications that radiation produces. BIL activity was determined after irradiation. At a dose of 1 kGy, no change was observed. However, at a dose of 2 kGy, BIL shows loss of SHA (Fig. 1a). The loss of HA suggests modification in the interaction of dimers which can result in pentamer dissociation and loss of interaction sites on the surface of cells (Walker et al., 2004).

The maintenance of activity without structural fragmentation was observed at a dose of 1 kGy. Degradation of the main band was detected (2 kGy), indicating that the aggregate formed was composed of fragmented polypeptides (Fig. 1b). The reverse phase chromatography analysis showed the loss of the peak area (Fig. 1c). Aggregation can be evaluated by light scattering (Fig. 1d). According to Puchala and Schuessler (1995), fragile bonds in the polypeptide chain break as a result of radiation. Because of the generation of inter-protein cross-linking reactions, formation of disulfide bonds, as well as hydrophobic and electrostatic interactions, proteins can be converted to higher molecular weight aggregates (Moon and Song, 2001). The analysis of molecular weight pattern and RP-HPLC shows denaturation, with subsequent precipitation into insoluble amorphous aggregates.

C-type lectins (CTLs) are usually dimers of two identical polypeptides, containing two tryptophan residues and one tyrosine residue (Morita, 2005). Individual subunits are able to bind to carbohydrates, but for lectin-like function they need at least bivalency, which is achieved through a simple interchain disulfide linkage. Although dimerization is essential, two Trp residues (one at each end) in the middle of the interaction interface are essential to stabilize intra-subunit contacts and for the expression of their biological activities (Doyle and Kini, 2009). The intrinsic fluorescence of BIL was used to study association into dimers after irradiation. The emission spectrum of the BIL shows tryptophan emission at 295 nm excitation. The intrinsic fluorescence emission decreased (1 and 2 kGy), while changing the λ_{max} at approximately 345 nm (Fig. 2a). Tryptophan residues that are exposed to water have a maximal fluorescence at wavelengths around 340–350 nm, whereas completely buried residues fluoresce at about 330 nm. The displacement of the mass center of the aromatic residues indicates possible protein denaturation and exposure of hydrophobic domains to the solvent.

Oxidative damage may distort the intra-subunit contacts and the hydrophobic core of proteins, allowing dissociation of chains and denaturation. Bis-ANS have proved to be sensitive probes for partially folded intermediates in protein-folding pathways. Compared with the buffer, bis-ANS was weakly detected in the non-irradiated BIL. Bis-ANS fluorescence, after irradiation, increased, with a maximum to 500 nm at a dose above 1 kGy (Fig. 2b). Hydroxyl radicals may attack hydrophobic surfaces in proteins, causing changes in protein hydrophobicity, which is considered the determining factor for structural collapse. Molten globule intermediates and insoluble amorphous aggregates are characterized by high bis-ANS fluorescence intensities due to the exposure of hydrophobic core regions that are inaccessible to the dye in the native structure (Semisotnov et al., 1991).

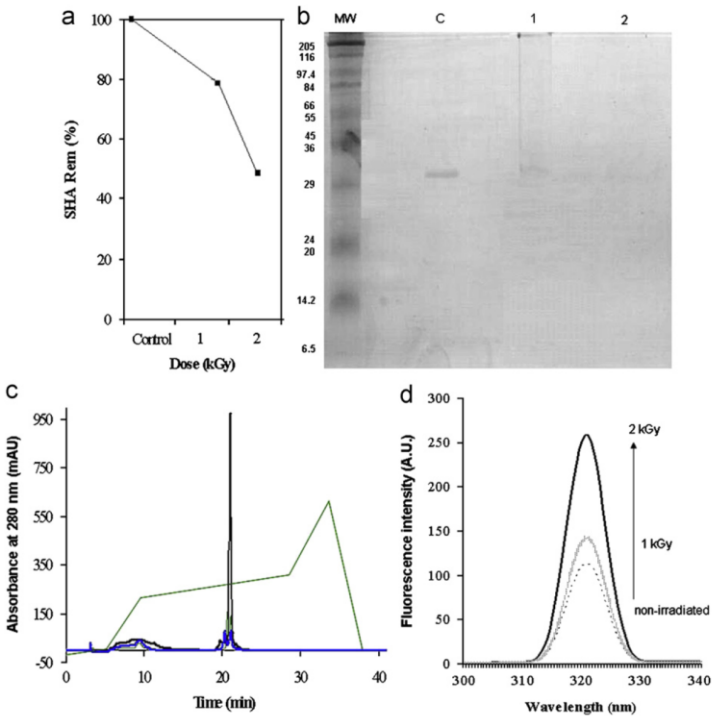

Fig. 1. Effect of γ-radiation on lectin activity and molecular weight. (a) The percentage of remaining specific hemagglutination activity, $\%SHA_{REM}$ is represented after irradiation. (b) SDS-PAGE was performed in a discontinuous system with 15% separating and 5% stacking gels. (MW) Molecular weight; (C) non-irradiated control; (1) 1 kGy and (2) 2 kGy. (c) Reverse phase chromatography in an HPLC system: (— control and irradiated lectins at (▬) 1 kGy and (▬) 2 kGy. (d) Light scattering was measured at 90° for the aggregation assays.

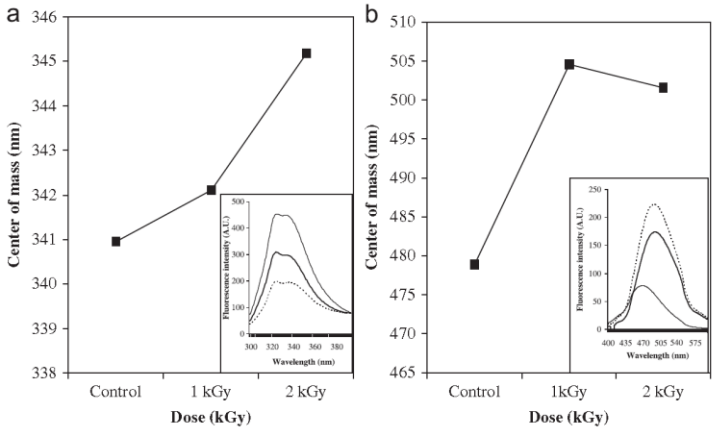

Fig. 2. BIL intrinsic fluorescence. (a) Mass center tryptophan; lectin excitation (295 nm) and emission (305–450 nm). (b) BIL bis-ANS fluorescence. Mass center; lectin excitation (360 nm) and emission (400–600 nm).

Nascimento et al. (1996) observed that crotoxin, the main neurotoxin isolated from snake venom *Crotalus durissus terrificus*, when irradiated with a 2 kGy dose of gamma rays produced protein aggregation and generation of lower molecular weight breakdown products. Such clusters showed less myotoxic, devoid of phospholipase activity and are virtually non-toxic in mice when compared with native crotoxin. Irradiation of bothropstoxin-1, the main myotoxic component of *Bothrops jararacussu* snake venom, promoted structural modifications of the toxin, characterized as aggregates and oligomers (Caproni et al., 2009).

4. Conclusion

Based on these considerations, we suggest that the irreversible loss in activity, induced by gamma radiation, may be ascribed to structural changes in BlL. These changes may have affected the interaction of the toxin with glycoconjugates exposed on the cell surface. In conclusion, our results indicate that gamma irradiation of BlL can induce significant modifications in its structure, as well as promote effective loss of hemagglutinating activity.

Acknowledgments

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ANEXO I

Aranda-Souza, M. A., Souza, R. M., Silva-Júnior, C. A., Silva, L. C. N., Pereira, D.S.T., Silva, V. M., Silva, M. V., Correia, M.T.S.

Antioxidant Potential and Total Phenolic content of leaves extracts from Parkinsonia aculeata l. cultivated in Brazilian Caatinga Biome In: I São Paulo Advanced School (ESPCA) on Redox Processes in Biomedicine / VII Meeting of the SFRBM-South American Group

Free Radicals Brazil, 2011, São Pedro – SP, Brazil.

Antioxidant potential and total phenolic content of leaves extracts from *Parkinsonia aculeata* l. cultivated in brazilian caatinga biome

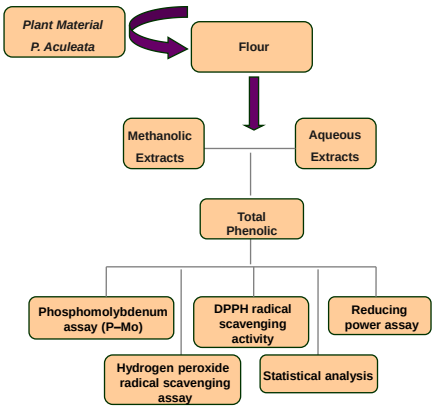
Aranda-Souza, MA[a *]; Souza, Rm[a]; Silva-Júnior, CA[a] ; Silva, LCN[a]; Pereira, DST[a]; Silva, MV[a]; Correia, MTS[a]

[a]Departamento de Bioquímica; Centro de Ciências Biológicas, Universidade Federal de Pernambuco, Recife, Brazil

Introduction & Objective

***Parkinsonia aculeata* L., is a specie of Brazilian Caatinga used in folk medicine as antihyperglycemic, antimalarial and amebicidal agent. This work evaluated the antioxidant and polyphenol content of leaves extracts from *Parkinsonia aculeata* cultivated in Brazilian Caatinga. *P. aculeata* Aqueous (PAWE) and Methanolic (PAME) leaves extracts were investigated for their antioxidant capacities by four *in vitro* methods: DPPH radical scavenging, hydrogen peroxide quenching phosphomolybdenum and reducing power assays.**

Methodology



Results & Discussion

Table I
Phenolic contents, antioxidant and free radical scavenging activity of *P. aculeata* extracts.

Extracts	Total phenol	% TAC	DPPH IC_{50} [2]	Reducing Power IC_{50} [2]	H_2O_2 IC_{50} [2]
PAME	288 ± 5.67	46.94 ± 3.73	35.29 ± 4.95	162.66 ± 0.49	32.11 ± 4.98
PAWE	196 ± 7.2	26.52 ± 2.57	49.38 ± 5.12	200.74 ± 0.68	46.13 ± 0.02
Gallic acid	-	-	10.83± 3.12	74.97 ± 1.09	16.43 ± 1.22

[1]Total phenol content expressed as mg GAE g^{-1}
[2] IC50 expressed as µg/ml

Figure 1.

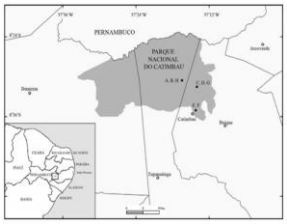


Fig. 1. Localization of PARNA do Catimbau (Buíque, Tupanatinga and Ibimirim municipalities), Pernambuco state, Brazil.

Figure 2.

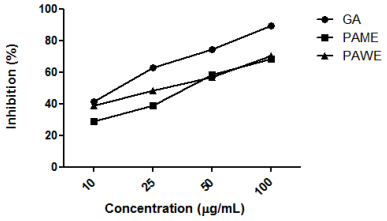


Fig. 2. DPPH radical scavenging of extracts, gallic acid was used as reference. Absorbance of the reaction was measured at 230 nm. Values are means ± S.D. (n = 3).

Figure 3.

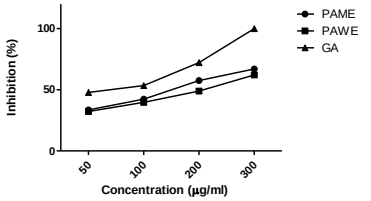


Fig. 3. Reducing power activity of extracts, gallic acid was used as reference. Absorbance of the reaction was measured at 230 nm. Values are means ± S.D. (n = 3).

Figure 4.

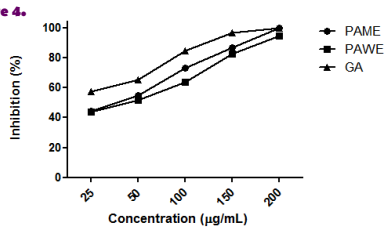


Fig. 4. Hydrogen peroxide quenching ability of extracts, gallic acid was used as reference. Absorbance of the reaction was measured at 230 nm. Values are means ± S.D. (n = 3).

Conclusion

Our results showed that *Parkinsonia aculeata* extracts displayed potent antioxidant and radical scavenging properties, supporting the ethnomedical use given to this plant for treatment of diseases.

Acknowledgements

ANEXO II

Nunes, E. S., **Aranda-Souza, M. A.**, Vaz, F.M., Paula, R. A., Melo, M.S., Oliva, M. L. V., Silva, T. G., Melo, A. M. M. A., Guarnieri, M. C., Correia, M.T.S.
Gamma irradiation abolish in vitro cytotoxicity of lectin of Bothrops leucurus snake venom In:
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Gamma irradiation abolish in vitro cytotoxicity of lectin of *Bothrops leucurus* snake venom

Nunes, E. S. [1]; Aranda-Souza, M. A. [1]; Vaz, A. F. M. [1]; Paula, R. A.[1]; Melo, M. S. [1]; Oliva, M. L. V. [2]; Silva, T. G. [3]; Guarnieri, M. C. [4]; Melo, A. M. M. A. [5]; Correia, M. T. S. [1]

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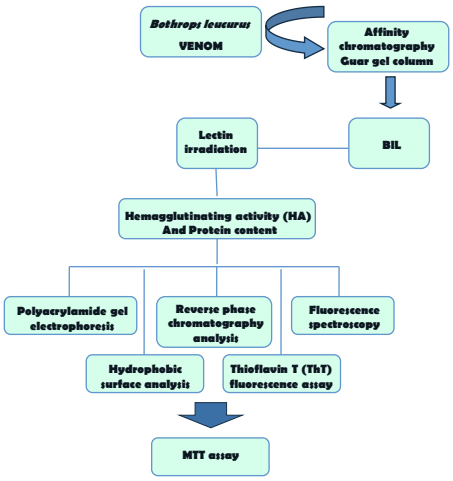
INTRODUCTION

The viper, *Bothrops leucurus*, is a common venomous snake in northeastern Brazil. Lectins are polyvalent carbohydrate-binding proteins of non-immune origin. Identified in plants and animals, they have also been found in snake venoms. Lectins bind to cells promoting hemagglutination and antimicrobial effect.

OBJECTIVES

This work evaluated the effects of ionizing radiation in *Bothrops lecurus* venom lectin (BIL) by fluorescence spectroscopy and in vitro cytotoxicity.

METHODS



Supported by CAPES CNPq FACEPE Fundação de Apoio à Ciência e Tecnologia do Estado de Pernambuco

RESULTS

BIL has been structurally altered and hemagglutinating assay shows significant change after gamma irradiation and the results are presented in Figure 1.

1.

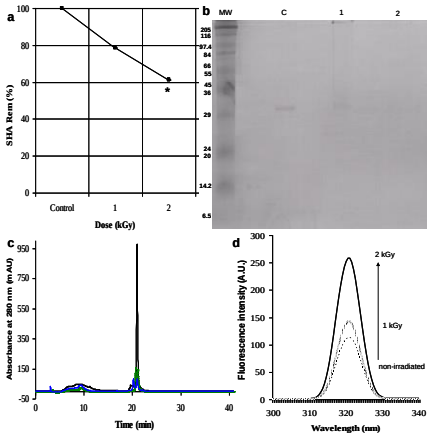


Fig. 1. Effect of γ-radiation on lectin activity and molecular weight. (a) The percentage of remaining specific hemagglutination activity, $\%SHA_{REM}$ is represented after irradiation. Error in the determination of $\%SHA_{REM}$ for the different doses was approximately ± 1%, which is less than the size of the symbols. * Significant difference ($p < 0.05$) compared to non-irradiated lectin. (b) SDS-PAGE from irradiated BIL. SDS-PAGE was performed in a discontinuous system with 15% separating and 5% stacking gels. (MW) Molecular weight; (C) non-irradiated control; (1) 1 kGy and (2) 2 kGy. (c) Reverse phase chromatography on an HPLC system: (—) control and irradiated lectins at (—) 1 kGy and (—) 2 kGy. (d) Light scattering was measured at 90° for the aggregation assays.

The effect of γ-radiation on the folding of the lectin was measured by intrinsic and bis-ANS fluorescence.

2.

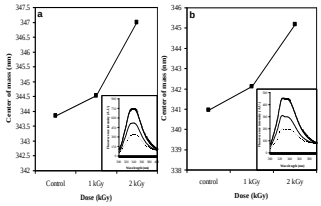


Fig. 2. BIL intrinsic fluorescence. (a) Mass center; Lectin excitation (280 nm) and emission (295–450 nm). (b) Mass center tryptophan; Lectin excitation (295 nm) and emission (305-450 nm).

CONCLUSION

Our results indicates that gamma irradiation of BIL can induce modifications in their structure and promote its effective detoxification.

ANEXO III

Aranda-Souza, M.A., Nunes, E.S., Rossato, F.A., Costa, R.A.P., Ferreira V.V.A.N., Coelho, L.C.B.B., Vercesi, A.E., Correia, M.T.S.

Mechanism of B16-F10 melanoma cells death induced by *Bothrops leucurus* venom lectin: correlation with mitochondrial permeability transition In:

XLI Annual Meeting of The Brazilian Biochemistry and Molecular Biology Society SBBq, 2012, Foz do Iguaçú-PR.

Mechanism of B16-F10 melanoma cells death induced by *Bothrops leucurus* venom lectin: correlation with mitochondrial permeability transition

Aranda-Souza, M. A. [1]; Nunes, E. S. [2] ; Rossato, F.A.[3] ; Costa, R.A.P.[3] ; Ferreira.V.V.A.N.[3] ; Coelho, L.C.B.B.[1], Vercesi, A.E.[3]; Correia, M.T.S.[1]

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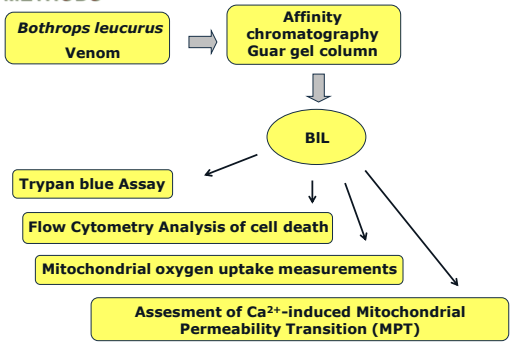
OBJECTIVES

The aims of this work is to clarify the mechanism of the cell death induced by *B. leucurus* lectin (BIL), and it's correlation with mitochondrial dysfunction.

METHODS



RESULTS

BIL effect was evaluated in B16-F10 using the trypan blue exclusion test and by flow cytometry after 24 h of exposure in culture. Morphological alterations also was verified by optical microscopy. It was observed characteristics as loss of plasmatic membrane integrity and cell lysis, but not formation of cytoplasmic vesicles, confirming cell death by necrosis.

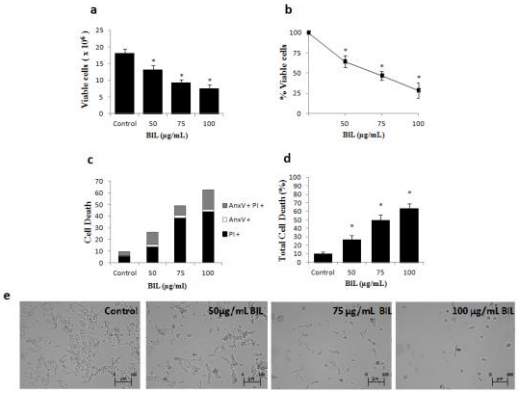


Figure 1. BIL reduce B16-F10 melanoma cell viability. B16-F10 cells were treated with increasing concentrations of BIL for 24h; the number of viable cells were determinate using trypan blue (a and b). BIL induce necrosis in B16-F10 melanoma cells. The percentages of necrotic cells were determinate by Flow cytometry (c and d). The cells BIL-treated were photographed under a microscope Leica DFC360 FX, using the LAS AF software (Leica Microsystems) (e). Values are mean ± s.e.m. of at least five independent experiments. * Significantly different from control at P<0.05 level, Student's *t*-test.

This lectin had no significant effect on fibroblasts viability under the same incubation conditions (Fig. 2).

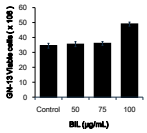


Figure 2. GN-13 fibroblast viability after BIL treatment. After trypsinization, fibroblasts (1 x 106 cells/ml) were incubated in DMEM medium with 1% fetal bovine serum in the presence of different BIL concentrations for 24 h. Data are the average ± s.e.m. of four independent experiments. In all the concentrations $p>0.05$.

We investigated the direct effect of BIL in isolated liver mitochondria For the analysis of alterations in the O_2 consumption, it was verified the effect of BIL on ADP-supported state 4. The mitochondrial treatment with BIL (10 µg/mL) demonstrate an increase of state 4 in 57.1% of the O_2 consumption and in state 3 no significant alterations were observed.

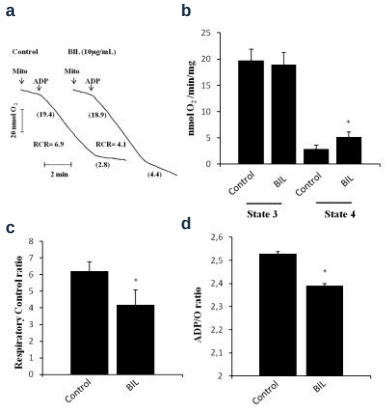


Figure 3. BIL (10 mg/ml) effect in the mitochondrial respiration. Mitochondria isolated from rat liver (0.5 mg / ml) were incubated at 30 ° C in assay medium (pH 7.2) containing 125 mM sucrose, 20 mM HEPES-K ⁺ and 200 µM EGTA, in the presence and absence of BIL. 300 mM ADP were added after 2 minutes of preincubation to initiate the oxidative phosphorylation. (a and b) BIL effect in the oxygen consumption during state 3 and 4. (c) Effect of BIL (10 mg/mL) on respiratory control ratio (RCR) (d) ADP/O ratio indicating the number of molecules of ADP phosphorylated to ATP when two electrons are transferred from a substrate through the respiratory chain to the reduce of the one oxygen ($^1/_2 O_2$). Data are the average ± SD of six independent experiments. *p<0,05

The figure 4 presents the experiments in which the Safranine O fluorescence detected the decrease of the mitochondrial transmembrane electric potential. BIL significantly decreased ΔΨm and stimulated PTP opening.

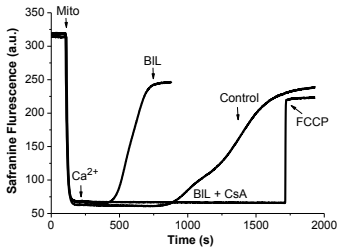


Figure 4. Ca^{2+}-induced dissipation of liver mitochondrial transmembrane potential (ΔΨ): Effect of the BIL (10 µg/mL) Mitochondrial suspensions (0.5 mg/mL) were added to reaction medium containing BIL and, where indicated, EGTA (200 µM) or cyclosporin A (1 µM) was present as an experimental control. The increase of the safranin fluorescence is related with the decrease in mitochondrial membrane potential. FCCP (2 µM) was added where indicated. Data are of six independent experiments.

CONCLUSION

These experiments provide evidence that BIL induced B16-F10 tumor cells necrotic death, mediated by mitochondrial membrane permeability transition.

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