SERVIÇO PÚBLICO FEDERAL
UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Avaliação mutagênica, genotóxica, carcinogênica e *in silico* da miotoxina BthTX-I isolada da peçonha da serpente *Bothrops jararacussu*

Discente: MSc. Maria Paula Carvalho Naves

Orientador: Prof. Dr. Mário Antônio Spanó

UBERLÂNDIA - MG

2022

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

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ATA DE DEFESA - PÓS-GRADUAÇÃO

Programa de Pós-Graduação em:	Genética e Bioquímica				
Defesa de:	Doutorado Acadêmico - Nº 01/2022/PPGGB.				
Data:	Vinte e seis de janeiro de dois mil e vinte e dois.	Hora de início:	13:30h	Hora de encerramento:	16:55h.
Matrícula do Discente:	11723GBI007				
Nome do Discente:	Maria Paula Carvalho Naves				
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Área de concentração:	Genética				
Linha de pesquisa:	Genética, Epigenética, Biologia e Melhoramento de Plantas e Animais.				
Projeto de Pesquisa de vinculação:	Avaliação do potencial antitumoral e mutagênico *in vivo* e *in vitro* da PLA_2 e BthTX-I isolada da peçonha *Bothrops jararacussu*.				

Aos vinte e seis dias do mês de janeiro de dois mil e vinte e dois, às 13:30 horas, reuniu-se via web conferência pela Plataforma *Zoom*, em conformidade com a Portaria nº 36, de 19 de março de 2020 da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES e Resolução de nº 06/2020 do Conselho de Pesquisa e Pós-graduação pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Genética e Bioquímica, assim composta: Prof. Dr. Regildo Márcio Gonçalves da Silva, Profª. Drª. Priscila Capelari Orsolin, Profª. Drª. Sandra Morelli, Profª. Drª. Renata Santos Rodrigues e Prof. Dr. Mário Antônio Spanó, orientador (a) do (a) candidato (a) e demais convidados presentes conforme lista de presença. Iniciando os trabalhos o (a) presidente da mesa, Prof. Dr. Mário Antônio Spanó, apresentou a Comissão Examinadora e o (a) candidato (a), agradeceu a presença do público, e concedeu o (à) Discente a palavra para a exposição do seu trabalho. A duração da apresentação do (a) Discente e o tempo de arguição e resposta foram conforme as normas do Programa de Pós-graduação em Genética e Bioquímica. A seguir o (a) senhor (a) presidente concedeu a palavra, pela ordem sucessivamente, aos examinadores, que passaram a arguir o (a) candidato (a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais. Em face do resultado obtido, a Banca Examinadora considerou o candidato (a):

APROVADO (A).

Esta defesa de Tese de Doutorado é parte dos requisitos necessários à obtenção do título de Doutor. O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU. Nada mais havendo a tratar foram

encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

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COMISSÃO EXAMINADORA

Presidente: Prof. Dr. Mário Antônio Spanó (Orientador)

Examinadores: Profa. Dra. Priscila Capelari Orsolin
Prof. Dr. Regildo Márcio Gonçalves da Silva
Profa. Dra. Renata Santos Rodrigues
Profa. Dra. Sandra Morelli

Data da Defesa: 26 /01 /2022

As sugestões da comissão examinadora e as normas do PPGGB para o formato da Tese foram contempladas

Prof. Dr. Mário Antônio Spanó

Dedico esta Tese aos que acreditam na ciência!

À minha família, aos que não estão mais presentes fisicamente neste mundo e a todos que contribuíram de alguma forma para a realização da mesma!

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Este trabalho foi realizado no Laboratório de Mutagênese (LABMUT) do Instituto de Biotecnologia (IBTEC) da Universidade Federal de Uberlândia (UFU, Uberlândia-MG), com o apoio estrutural, técnico e financeiro das seguintes Agências de Fomento e Instituições:

- Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq);

- Coordenação de Aperfeiçoamento de Pessoal do Ensino Superior (CAPES);

- Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG);

- Universidade de São Paulo (USP);

- Universidade Federal de Uberlândia (UFU).

Lista de Abreviaturas

AAPH - 2,2'-azobis (2-amidinopropane) dihydrochloride.

AMBIC - ammonium bicarbonate.

ATCC - american type culture collection.

B16F10 - linhagem celular de melanoma murino.

BH - balanced-heterozygous.

BthTX-I - bothropstoxina-I.

BthTX-II - bothropstoxina-II.

CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

CDK - ciclin dependent kinase.

CETA - Centro de Extração de Toxinas Animais.

CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico.

COS7 - linhagens de células semelhantes a fibroblastos, derivadas do tecido de rim de macaco.

cPLA$_2$ - fosfolipase A$_2$ citosólicas.

CYP450 - complexo citocromo P450.

DDT - dicloro-difenil-tricloroetano.

DNA - deoxyribonucleic acid.

DSBs - double-strand breaks.

DU-145 - linhagem celular de carcinoma de próstata humana.

DXR - doxorrubicina.

EDTA - ethylenediamine tetraacetic acid.

ETT - epitelial tumor test.

FAPEMIG - Fundação de Amparo a Pesquisa do Estado de Minas Gerais.

flr^3 - flare-3.

FM - frequency of mutation.

FR - frequency of recombination.

FRAP - ferric reducing antioxidant power.

FT - frequencies of total spots.

H_2O_2 - peróxido de hidrogênio.

HB - high bioactivation cross.

HepG2 - linhagem de células de câncer de fígado humano.

HL-60 - linhagem celular de leucemia humana.

HR - homologous recombination.

HUVEC - human umbilical vein endothelial cells.

IARC - International Agency for Research on Cancer.

IBAMA - Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis.

iPLA$_2$ - phospholipase A$_2$ independente de Ca^{2+}.

kDa - kilodaltons.

LMP - low melting point.

MCF7 - linhagem celular de câncer de mama humano.

MDA-MB-231 - linhagem celular de câncer de mama humano.

MH - marked trans-heterozygous.

MMR repair - mismatch repair.

MTT - 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide.

mwh - multiple wing hairs.

NC - negative control.

NIH 3T3 - linhagens celulares de fibroblastos embrionários de camundongo.

ORAC - oxygen radical absorbance capacity.

PAF - fator de ativação plaquetária.

PBS - phosphate buffered saline.

PC - positive control.

PC-12 - linhagem celular de feocromocitoma da medula adrenal de rato.

PDB - protein data bank.

PLA$_1$ - fosfolipase A$_1$ que hidrolisa o éster 1-acílico.

PLA$_2$ - fosfolipase A$_2$ que hidrolisa o éster 2-acílico.

PLB - fosfolipase que hidrolisa ambos os grupos acila.

PLC - fosfolipase que cliva a ligação glicerofosfato.

PLD - fosfolipase que remove o grupo de base dos fosfolipídios.

PLs - fosfolipases.

RNS - reactive nitrogen species.

ROS - reactive oxygen species.

RP-HPLC - reverse-phase chromatography.

RPMI - Roswell Park Memorial Institute.

S180 - células tumorais de sarcoma 180.

SD - standard deviation.

SDS - dodecilsulfato de sódio.

SDS-PAGE - eletroforese em gel de poliacrilamida.

SK-BR-3 - linhagem celular de câncer de mama humano.

SMART - somatic mutation and recombination test.

SOD - superóxido dismutase.

$sPLA_2$ - fosfolipase A_2 secretada.

ST - standard cross.

TPTZ - tripiridiltriazina.

URE - urethane.

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Sumário

Apresentação

Apresentação

A bothropstoxina-I (BthTX-I) é uma proteína miotóxica de cadeia simples semelhante à fosfolipase A_2, isolada da peçonha de *Bothrops jararacussu*. É composta por 121 resíduos de aminoácidos, com massa molecular de aproximadamente 13 kDa, um subgrupo com resíduo de aminoácido na 49ª posição, Lys-49 PLA_2. Apesar dessa proteína não possuir atividade catalítica, é a principal responsável pela atividade miotóxica causada pela peçonha de *B. jararacussu*. Além disso, induz inflamação muscular, mionecrose e liberação de creatina-quinase e lactato desidrogenase, que causa dano celular. Tem sido relatado citotoxicidade e redução da viabilidade celular em diferentes linhagens de células tumorais, além de indução de morte celular por apoptose, modulação do ciclo celular, promovendo retardo na fase G_0/G_1.

Alguns estudos destacam uma forte correlação das fosfolipases A_2 com danos genotóxicos em sistemas biológicos que induzem instabilidade genética. Outros estudos mostraram que danos significativos ao DNA foram observados em linfócitos humanos tratados com diferentes peçonhas de serpentes, bem como algumas moléculas isoladas, como a BthTX-I.

A peçonha bruta de *B. jararacussu*, utilizada no presente estudo, foi obtida do CETA Serpentarium - Centro de Extração de Toxinas Animais Ltda., Morungaba, SP, Brasil, registrada no Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis sob número 2087163.

Este trabalho teve como finalidade contribuir com informações sobre as atividades biológicas da BthTX-I, por meio das avaliações:

1] dos efeitos mutagênicos e recombinogênicos *in vivo* da BthTX-I em células somáticas de asas de *Drosophila melanogaster*, utilizando o teste para detecção de mutação e recombinação somática (SMART);

2] citotóxica e genotóxica da BthTX-I *in vitro*, realizando o ensaio de resazurina e do cometa com células HUVEC e DU-145;

3] do potencial carcinogênico e anticarcinogênico *in vivo* da BthTX-I, por meio do teste de detecção de tumores epiteliais (ETT) em *D. melanogaster*;

4] do potencial antioxidante da BthTX-I, por meio dos ensaios antioxidantes FRAP, ORAC e SOD;

5] da interação BthTX-I com DNA por meio de análises *in silico*.

Este trabalho está apresentado de acordo com os seguintes capítulos:

Capítulo I - Contém a Fundamentação Teórica, na qual é apresentada uma revisão literária a respeito de todo o trabalho, contemplando informações sobre a bothropstoxina-I, bem como sobre todos os testes que foram realizados nesse trabalho: teste para detecção de mutação e recombinação somática e teste para detecção de tumores epiteliais em *D. melanogaster*, ensaio de resazurina e do cometa, ensaios antioxidantes FRAP, ORAC e SOD e análises *in silico*.

Capítulo II - Apresenta o manuscrito intitulado "Mutagenic and genotoxic activities of Phospholipase A_2 Bothropstoxin-I from *Bothrops jararacussu* in *Drosophila melanogaster* and human cell lines". Esse manuscrito foi redigido na língua inglesa e foi publicado do periódico "International Journal of Biological Macromolecules" (fator de impacto: 6.953).

Capítulo III - Apresenta o manuscrito intitulado "Carcinogenic and anti-carcinogenic activities of Bothropstoxin-I from *Bothrops jararacussu* in *Drosophila melanogaster*, biochemical assays and protein-DNA molecular docking". Esse manuscrito foi redigido na língua inglesa e está de acordo com as normas da revista "Drug and Chemical Toxicology".

Capítulo I

Fundamentação Teórica

1. Peçonha de serpentes como agente terapêutico

A peçonha de serpentes é uma substância tóxica, secretada por glândulas especializadas, passível de inoculação em suas presas por meio de seu aparelho inoculador (dentes). As peçonhas compreendem uma mistura complexa de componentes que afetam vários sistemas biológicos. São compostos por proteínas, peptídeos, enzimas, toxinas e secreções não proteicas (Uzair *et al*. 2018).

Várias proteínas de peçonhas de serpentes, como desintegrinas, fosfolipases A_2, metaloproteases e L-aminoácido oxidases, devido à alta seletividade para seus alvos moleculares, têm mostrado potencial como agentes terapêuticos (**Fig. 1**), incluindo diagnóstico e tratamento de câncer (Zambelli *et al*. 2017).

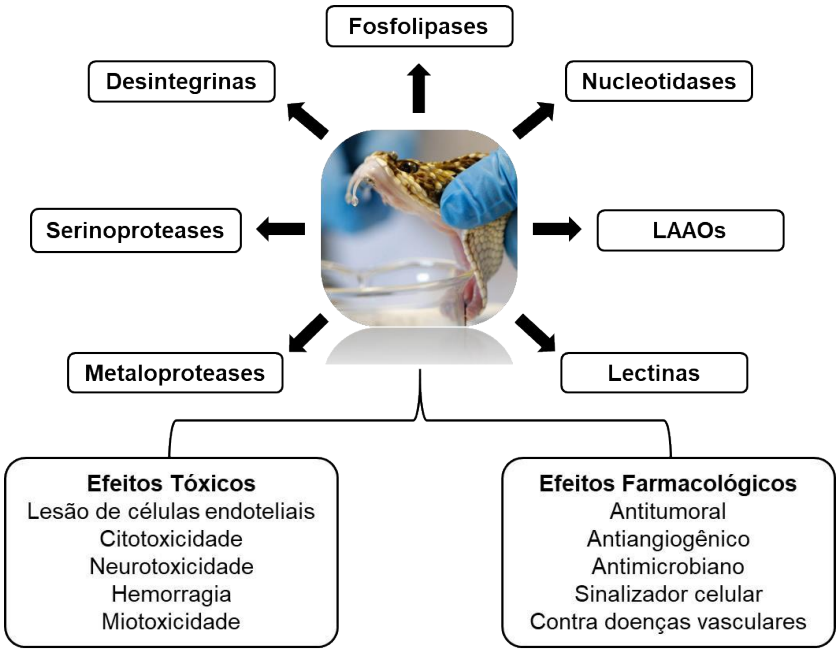

Figura 1. Componentes da peçonha de serpentes e seus possíveis efeitos tóxicos e farmacológicos (Adaptado de Calderon *et al*. 2014).

A utilização da peçonha de serpentes na terapia do câncer tem evoluído desde 1930, quando ainda se usava peçonhas brutas, até os dias atuais, onde têm-se isolado certos componentes biologicamente ativos, visando vias moleculares específicas. Além disso, a associação da peçonha com outras tecnologias ainda

está em seu estágio inicial de investigação para tratamentos combinatórios (Li *et al*. 2018).

Diversos estudos mostram resultados promissores de moléculas extraídas de peçonha de serpentes, como a leucurogina, uma desintegrina clonada da glândula da peçonha de *Bothrops leucurus*, que foi capaz de inibir mais de 50% do crescimento do tumor de Ehrlich implantado em camundongos, após 7 dias de administração de 10 µg/dia. O efeito anti-angiogênico da leucurogina foi avaliado pelo modelo de implante de esponja em camundongos, e inibiu de forma dose-dependente, o processo de vascularização da esponja (Higuchi *et al*. 2011). A bothropoidina, uma metaloprotease da peçonha da serpente *Bothrops pauloensis* também apresentou efeitos antitumorais e antiangiogênicos, em células de câncer de mama humano MDA-MB-231 (Guimarães *et al*. 2017).

1.1. Fosfolipase A_2

As fosfolipases (PLs) são enzimas que hidrolisam principalmente glicerofosfolipídios, embora algumas também possam degradar lipídios neutros. Dependendo do local em que as PLs atuam, elas são classificadas de diferentes formas: como fosfolipase A, B, C ou D (PLA, PLB, PLC e PLD, respectivamente). As enzimas da classe das PLAs hidrolisam o éster 1-acílico (PLA_1) ou o éster 2-acílico (PLA_2) dos fosfolípideos, liberando lisofosfolipídeos e ácidos graxos. As PLBs hidrolisam ambos os grupos acila, liberando da mesma forma lisofosfolipídeos e ácidos graxos. PLCs são fosfodiesterases que clivam a ligação glicerofosfato, formando diacilglicerol e inositol 1,4,5-trifosfato e as PLDs removem o grupo de base dos fosfolipídios, produzindo ácido fosfatídico, ácido lisofosfatídico, ceramida-1-fosfato, colina livre e outras moléculas hidrofílicas (**Fig. 2**) (Köhler *et al*. 2006, Magalhães 2017).

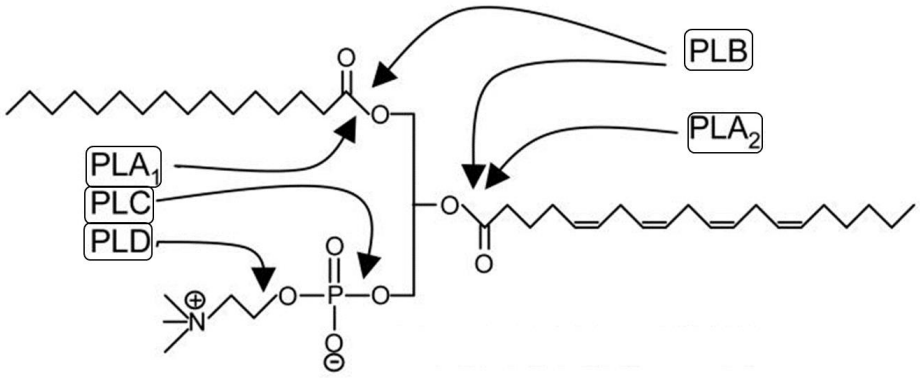

Figura 2. Representação de um fosfolipídeo e dos locais de hidrólise (indicados pelas setas) das diferentes fosfolipases (Adaptado de Köhler *et al*. 2006).

A superfamília das PLA_2s consiste em alguns grupos diferentes de enzimas que têm papéis fisiológicos importantes. Existem cinco tipos principais de PLA_2s: $sPLA_2$s secretadas, caracterizadas pela baixa massa molecular (13 a 18 kDa), contém de 5 a 8 pontes dissulfeto, necessidade de histidina no sítio ativo e do íon Ca^{2+} para catálise; as $cPLA_2$s citosólicas, são proteínas grandes (61 a 114 kDa) funcionam por meio da ação de uma díade serina/ácido aspártico no sítio catalítico; $iPLA_2$s independente de Ca^{2+}, não requerem Ca^{2+} para exercer sua atividade e funcionam por meio de uma serina catalítica no seu sítio ativo; PAF acetil-hidrolases, são PLA_2s que hidrolisam o grupo acetil da posição sn-2 do fator de ativação plaquetária (PAF) e também funcionam por meio da ação de uma serina catalítica; e PLA_2 lisossomal, possui uma tríade Ser-His-Asp conservada e tem quatro resíduos de cisteína que são necessários para a atividade catalítica (Schaloske e Dennis 2006, Burke e Dennis 2009).

As fosfolipases A_2 são componentes encontrados em grandes quantidades em algumas peçonhas de serpentes. Essas moléculas podem induzir citotoxicidade em células tumorais por autofagia e apoptose, bem como bloqueio da migração celular (Calderon *et al*. 2014, da Silva *et al*. 2015, Azevedo *et al*. 2016). A BthTX-II, uma Asp-49 PLA_2 da peçonha de *Bothrops jararacussu*, mostrou efeitos antitumorais e antimetastáticos em células de câncer de mama humano MDA-MB-231. Além disso, apresentou citotoxicidade dose dependente, diminuição da proliferação celular e inibição da progressão do ciclo celular de células tumorais (Azevedo *et al*. 2019).

As PLA_2s da peçonha de serpentes são divididas em subgrupos de acordo com o resíduo de aminoácido na 49ª posição. Esses subgrupos contêm Asp-49, Lys-49, Arg-49 ou Ser-49, entre os quais Asp-49 e Lys-49 são os mais comuns. Embora os subgrupos contendo Lys-49 não exibam atividade catalítica de fosfolipase, são considerados homólogos de PLA_2 (ou semelhantes a PLA_2) (Matsui *et al*. 2019).

BnSP-6, uma PLA_2 Lys-49 isolada da peçonha da serpente *Bothrops pauloensis*, induziu citotoxicidade dose dependente em células de câncer de mama humano, MDA-MB-231, e inibiu a adesão e migração celular (Azevedo *et al*. 2016). Outro estudo revela que a BnSP-6 foi capaz de induzir maior atividade citotóxica e genotóxica em células tumorais quando comparada a células não tumorigênicas, sugerindo que essa proteína tem uma possível preferência por células cancerosas, mas não é totalmente exclusiva delas (Silva *et al*. 2018).

As enzimas da classe PLA_2 atuam diretamente no metabolismo das membranas fosfolipídicas, causando alterações na biossíntese lipídica e na lipogênese de células normais e tumorais (Mashima *et al*. 2009). Isso pode ser devido à presença de uma região C-terminal que é descrita como capaz de romper a matriz hidrofílica da membrana celular, abrindo os poros e permitindo a entrada de toxinas no ambiente intracelular (Montecucco *et al*. 2008). Vários estudos sugerem que os efeitos biológicos das miotoxinas estão relacionados a essa interação (Costa *et al*. 2008, Lomonte *et al*. 2010).

1.2. Bothropstoxina-I

A bothropstoxina-I (BthTX-I), uma proteína semelhante à fosfolipase A_2, isolada da peçonha de *Bothrops jararacussu*, é uma proteína miotóxica de cadeia simples, composta por 121 resíduos de aminoácidos, com massa molecular de aproximadamente 13 kDa, uma contraparte básica de Lys-49 PLA_2 (Homsi-Brandenburgo *et al*. 1988). Apesar dessa proteína não possuir atividade catalítica, é a principal responsável pela atividade miotóxica causada pela peçonha de *B. jararacussu*. Além disso, induz inflamação muscular, mionecrose e liberação de creatina-quinase e lactato desidrogenase, que causa dano celular (Boeno *et al*. 2020).

BthTX-I e seu peptídeo sintético catiônico, derivado da região C-terminal 115-129, induziu citotoxicidade em diferentes linhagens de células tumorais e em camundongos transplantados com células tumorais S180, reduzindo, respectivamente, 30 e 36% do tamanho do tumor após 14 dias e 76 e 79% após 60 dias (Gebrim *et al*. 2009). A BthTX-I reduziu a viabilidade celular de 40 a 50% em todas as linhagens tumorais avaliadas (humanas HL-60 e HepG2, e murinas PC-12 e B16F10), causando principalmente morte celular por apoptose. Essa miotoxina modulou o ciclo celular das células PC-12 e B16F10, promovendo retardo na fase G_0/G_1 do ciclo celular (da Silva *et al*. 2015).

BthTX-I na concentração de 102 µg/mL induziu morte celular por apoptose nas linhagens de células MCF7, SK-BR-3 e MDA-MB-231 de maneira dose dependente. Além disso, aumentou a expressão de receptores CD24, que medeiam a morte celular e induziu a expressão de proteínas pró-apoptóticas e pró-autofágicas em células MCF7 (Bezerra *et al*. 2019).

Estudos destacam uma forte correlação das fosfolipases A_2 com danos genotóxicos em sistemas biológicos que induzem instabilidade genética (Silva *et al*. 2018). Alguns outros estudos mostraram que danos significativos ao DNA foram observados em linfócitos humanos tratados com diferentes peçonhas de serpentes, bem como algumas moléculas isoladas, como a BthTX-I, por meio do teste de micronúcleo com bloqueio de citocinese e ensaio de cometa, indicando alto potencial genotóxico dessas enzimas (Marcussi *et al*. 2013).

2. Drosophotoxicologia

Nos últimos anos, tem-se buscado desenvolver modelos alternativos *in vivo* e *in vitro,* para reduzir a utilização de mamíferos nas pesquisas toxicológicas e genéticas*.* A *Drosophila melanogaster* é um inseto utilizado como modelo experimental na genética e na biologia do desenvolvimento há cerca de um século (Siddique *et al*. 2005, Roberts 2006).

Diversas propriedades biológicas, fisiológicas e neurológicas são conservadas entre os mamíferos e a *D. melanogaster*. Por esse motivo observa-se um crescente potencial da *Drosophila* na neurotoxicologia (Rand 2010).

A drosophotoxicologia engloba as abordagens metodológicas relacionadas a utilização da *D. melanogaster* como organismo de escolha em estudos de

toxicologia. Assim sendo, é muito utilizada no meio científico para avaliação da toxicidade, mutagenicidade, antimutagenicidade, carcinogenicidade e anticarcinogenicidade de diferentes compostos (Morais *et al*. 2018, Naves *et al*. 2019, Oliveira *et al*. 2020, Vasconcelos *et al*. 2020, Naves *et al*. 2021). Este modelo experimental oferece várias vantagens importantes, tais como dimorfismo sexual, que permite fácil distinção entre machos e fêmeas (**Fig. 3**), estrutura genômica relativamente simples e compacta, ciclo de vida curto e bem caracterizado (**Fig. 4**), baixo custo de manutenção das linhagens em laboratório, simplicidade de manipulação experimental, homologia de genes relevantes com organismos superiores, como os humanos e facilidade de obtenção de genótipos/fenótipos mutantes (Chifiriuc *et al*. 2016, Affleck e Walker 2019, Contestabile *et al*. 2020, Younes *et al*. 2020).

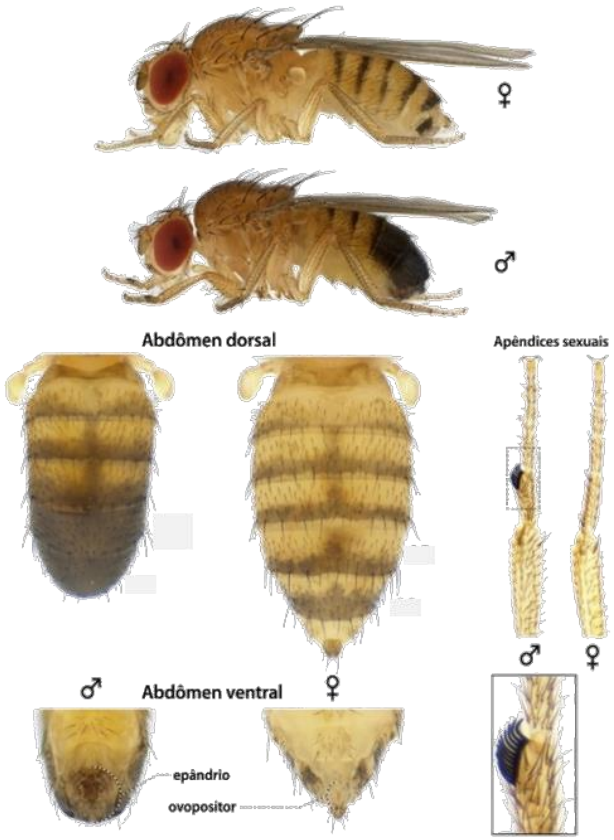

Figura 3. Dimorfismo sexual de *D. melanogaster* (Adaptado de Chyb e Gompel, 2013).

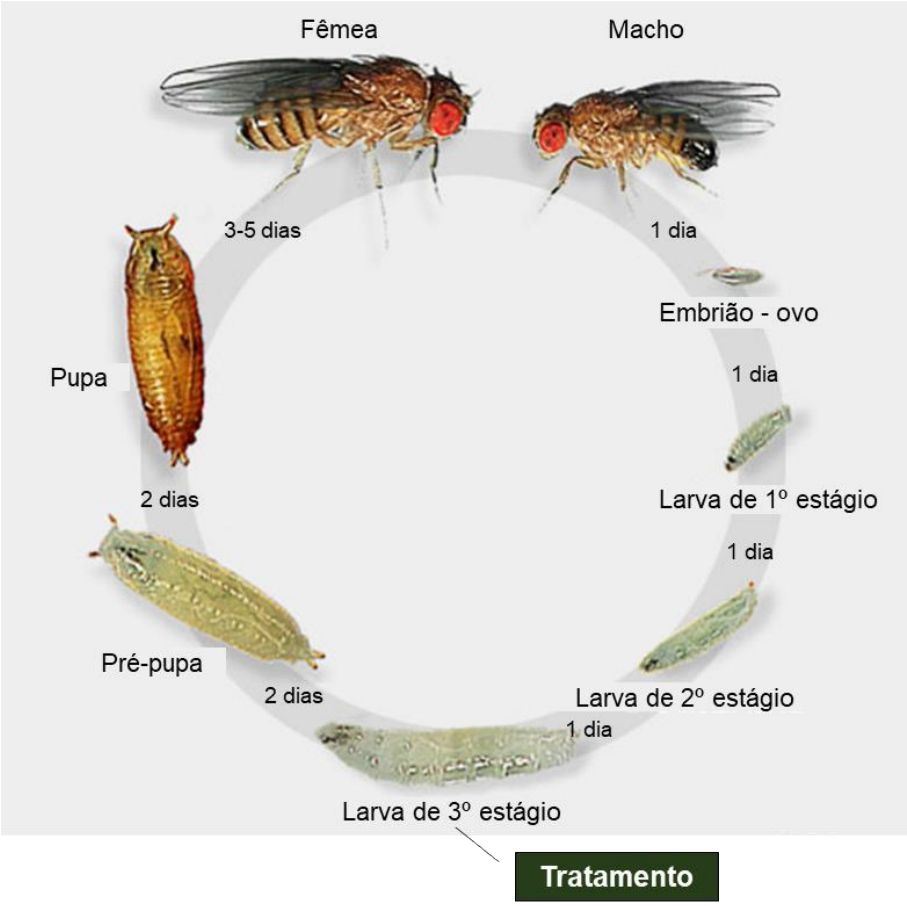

Figura 4. Ciclo de vida da *D. melanogaster* (Adaptado de http://flymove.uni-muenster.de/).

Dentre os testes realizados com *D. melanogaster* como organismo modelo, destacam-se o Teste de Mutação e Recombinação Somática (Somatic Mutation and Recombination Test - SMART), e o Teste para Detecção de Tumores Epiteliais (ETT).

2.1. Somatic Mutation and Recombination Test - SMART

O Teste de Mutação e Recombinação Somática em células de asas de *D. melanogaster*, também conhecido como SMART de asa, foi desenvolvido por Graf *et al*. (1984) e aprimorado por Graf e van Schaik (1992). Esse teste permite detectar um amplo espectro de eventos genotóxicos e antigenotóxicos, sejam eles

mutagênicos ou recombinogênicos. Baseia-se no fato de que no início do desenvolvimento embrionário da *D. melanogaster,* grupos de células se proliferam mitoticamente até se diferenciarem nas estruturas do corpo do indivíduo adulto. Caso ocorra alguma alteração em uma célula dos discos imaginais das asas, um clone de células mutantes será formado e, posteriormente, será detectado como uma mancha de pelos mutantes nas asas da mosca adulta, demonstrando fenotipicamente os genes marcadores *flr*^{3} ou *mwh* (Graf *et al*. 1984).

No SMART são utilizadas três linhagens de moscas mutantes: linhagem "*multiple wing hairs*" (*mwh/mwh*) com marcador *mwh*, presente no cromossomo 3 (3-0,3). Linhagem "*flare-3*" (*flr*^{3}), com constituição genética *flr*^{3} */ In(3LR)*TM3*, ri p*^{p} *sep l(3)89Aa bx*^{34e} e *Bd*^{S}*,* onde o marcador *flr*^{3} está presente no cromossomo 3 (3-38,8). E por último, a linhagem "*ORR; flare-3*" (*ORR; flare-3*), com constituição genética *ORR; flr*^{3} */ In(3LR)*TM3*, ri p*^{p} *sep l(3)89Aa bx*^{34e} e *Bd*^{S}. Os indivíduos pertencentes à essa linhagem possuem os mesmos marcadores que os da linhagem "*flare-3"*. Entretanto, essa linhagem é portadora de genes de expressão elevada de enzimas do complexo citocromo P450 (CYP450), localizados nos cromossomos 1 e 2, provenientes da linhagem *Oregon R*, naturalmente resistentes ao DDT (Graf *et al*. 1984, Graf e van Schaik 1992).

Com estas linhagens são realizados dois diferentes cruzamentos: o cruzamento padrão (ST - s*tandard cross*) entre machos "*mwh*" e fêmeas virgens "*flr*^{3}" (Graf *et al*. 1984, 1989); e o cruzamento de alta bioativação metabólica (HB - *high bioactivation cross*) entre machos "*mwh*" e fêmeas virgens "*ORR*; *flr*^{3}" (**Fig. 5**) (Graf e van Schaik 1992). Dois tipos de descendentes podem ser obtidos com esses cruzamentos: 1) Trans-heterozigotos marcados (MH - *marked trans-heterozygous*), que possuem a borda da asa normal ou lisa; 2) Heterozigotos balanceados (BH - *balanced-heterozygous*), caracterizados pela borda da asa serrilhada, devido à presença do marcador *Bd*^{S}.

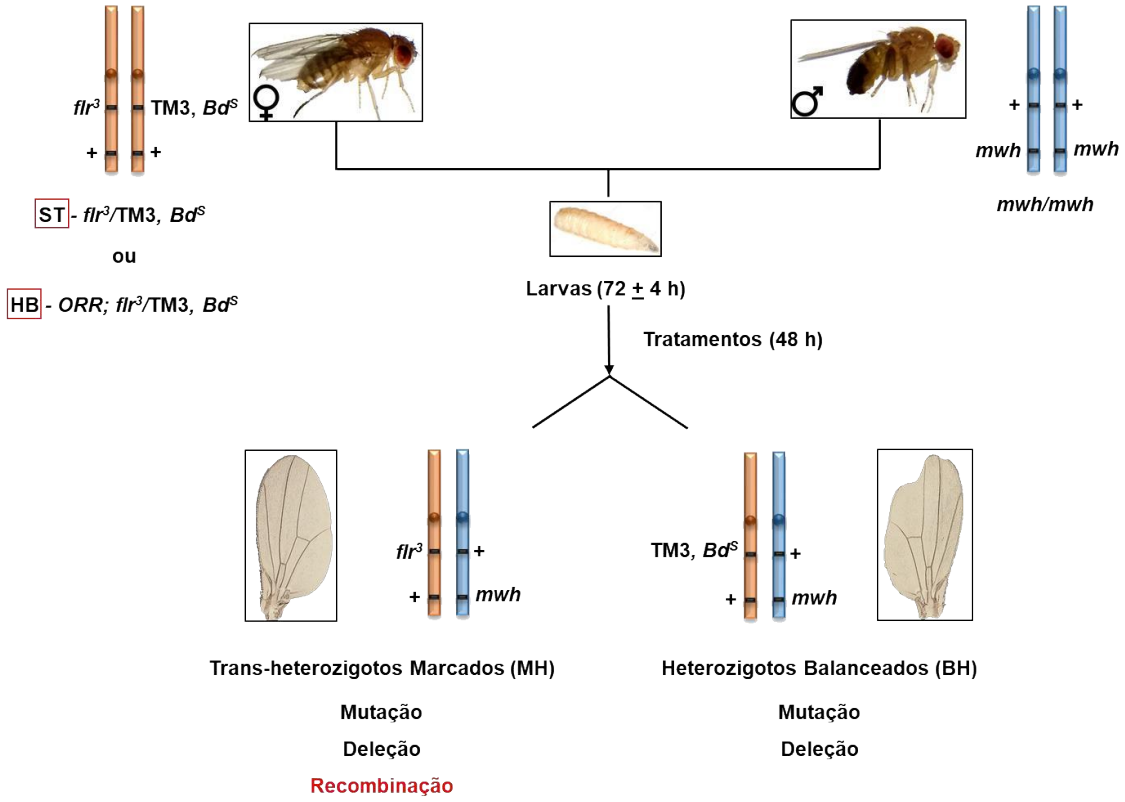

Figura 5. Representação esquemática do SMART de asa, com as diferentes linhagens de *D. melanogaster*.

Os descendentes MH (*mwh*+/+*flr*^{3}) podem apresentar pelos mutantes nas asas originados por mutações, deleções ou recombinações ocorridas nos loci gênicos *mwh* e *flr*^{3}. Já os descendentes BH (*mwh*+/+TM3,*Bd*^{s}) apresentam um cromossomo balanceador com múltiplas inversões (TM3, *Bd*^{s}), o que inviabiliza a recombinação gênica, permitindo apenas a detecção de eventos causados por mutação ou deleção. Por isso, faz-se necessária a análise de ambos os indivíduos (MH e BH) para que se tenha a frequência de mutação e recombinação do composto avaliado.

Nos adultos emergentes MH, cada célula das asas deverá possuir um único pelo (ou tricoma). No entanto, de acordo com Graf et al. (1984), eventos genotóxicos, tais como aberração cromossômica (deleção), mutação de ponto, recombinação mitótica e/ou não disjunção cromossômica, ocorridos nas células em proliferação mitótica nos discos imaginais de asas, durante o desenvolvimento embrionário, levam à perda de heterozigose e, consequentemente, após a

metamorfose, à alteração do fenótipo, que se manifesta como uma mancha mutante (clone de células mutantes) nas asas da mosca adulta. As manchas mutantes aparecem como manchas simples, apresentando o fenótipo “mwh” ou “flare”, devido à ocorrência de mutação de ponto, deleção, recombinação homóloga ou não disjunção, enquanto manchas gêmeas (formadas por células com pelos múltiplos e flare, adjacentes) são originadas exclusivamente por recombinação mitótica. Durante a análise das asas, são considerados o número e o tipo de manchas encontradas, fornecendo dados quantitativos e qualitativos, para a elucidação do evento genotóxico.

A **Fig. 6** apresenta fotomicrografias de manchas de pelos mutantes em asas de *D. melanogaster*, observadas em microscópico óptico de luz.

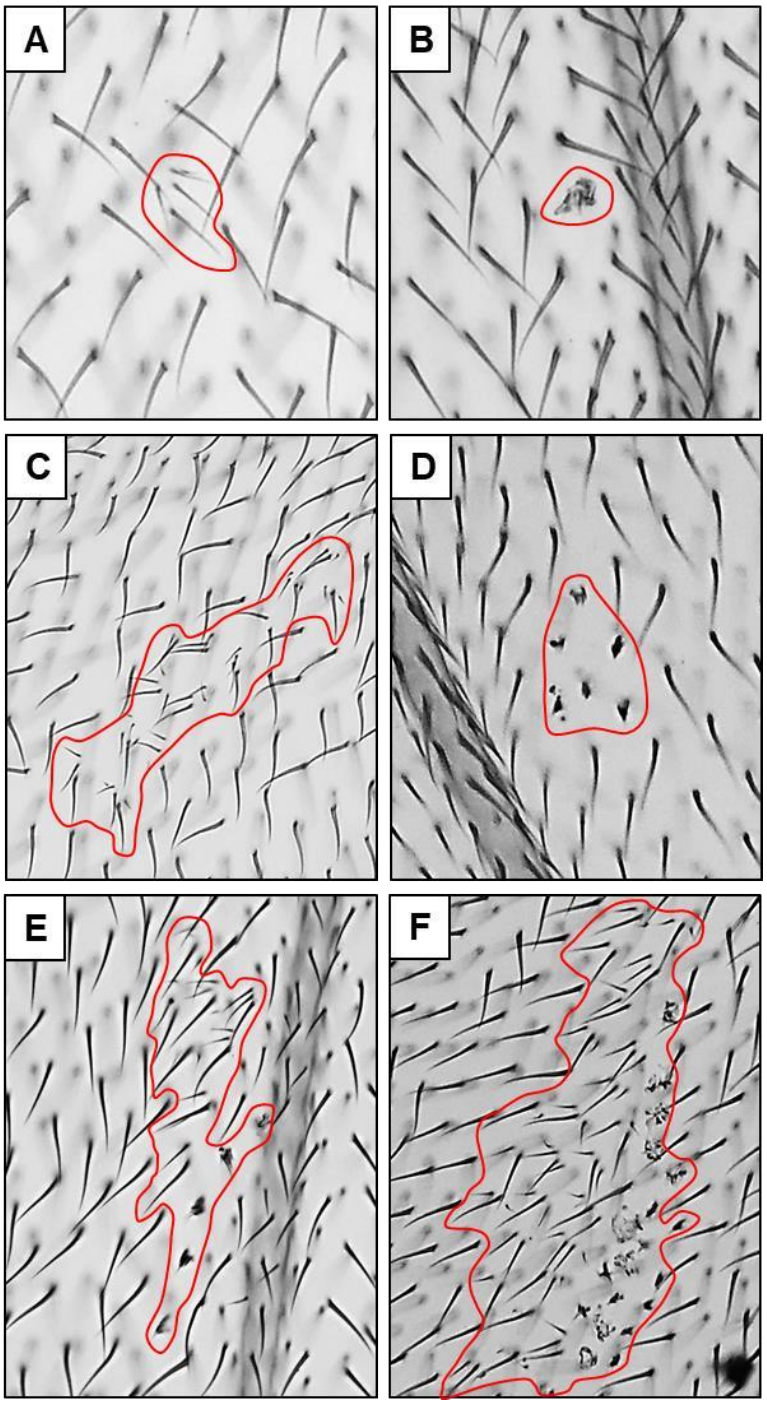

Figura 6. Fotomicrografias de manchas de pelos mutantes em asas de *D. melanogaster*, observadas em microscópico óptico de luz (aumento de 400X). (A) Mancha simples pequena com pelos múltiplos (mwh). (B) Mancha simples pequena com pelo flare (flr). (C) Mancha simples grande com pelos mwh. (D) Mancha simples grande com pelos flr. (E) e (F) Manchas gêmeas com pelos mwh e flr adjacentes.

As frequências de cada tipo de mancha (simples pequena, simples grande ou gêmea) e a frequência total de manchas por mosca, para cada tratamento, são comparadas aos pares (tratados com os respectivos controles), de acordo com Kastenbaum e Bowman (1970) com $p = 0,05$ (Frei e Würgler 1988, 1995). Os dados são inicialmente avaliados de acordo com o procedimento de decisão múltipla de Frei e Würgler (1988). Posteriormente, para excluir resultados falso-positivos e inconclusivos, devido à superdispersão dos dados, pode ser utilizado o teste U de Wilcoxon, Mann e Whitney, resultando em dois diagnósticos diferentes: positivo e negativo (Frei e Würgler 1995).

2.2. Epithelial Tumor Test - ETT

O teste para detecção de tumores epiteliais, em *D. melanogaster*, produz resultados confiáveis e altamente reproduzíveis. Tem sido amplamente utilizado para determinar os efeitos carcinogênicos e anticarcinogênicos de diferentes agentes xenobióticos (Nepomuceno 2015, Vasconcelos *et al*. 2020).

A semelhança e conservação evolutiva de genes supressores de tumor entre *D. melanogaster* e mamíferos têm estimulado os estudos relacionados à indução e desenvolvimento de tumores nesse inseto, podendo contribuir diretamente para o entendimento de processos carcinogênicos em humanos (Justice *et al*. 1995, Eeken *et al*. 2002). As proteínas quinases e CDK (quinase dependente de ciclinas) formam um complexo responsável pelo controle da regulação do ciclo celular em *D. melanogaster.* Diversos proto-oncogenes e genes supressores de tumor participam desse controle. Um dos genes envolvidos é o gene recessivo *wts* (*warts)*, que tem homologia ao gene supressor de tumor *LATS1* de mamíferos (Eeken *et al*. 2002).

O ETT utiliza uma linhagem que possui o gene marcador *wts* (*warts*), que está localizado no cromossomo 3 da mosca e é letal para o zigoto quando em homozigose. Por isso, o alelo *warts* é mantido nessa linhagem com a presença de um balanceador cromossômico (TM3). Caso ocorra a perda da heterozigose nas células do disco imaginal, haverá formação de clones homozigotos, que são viáveis em conjuntos de células isoladas da larva e se manifestam como tumores em diferentes partes da mosca adulta (Sidorov *et al*. 2001).

O ETT é realizado por meio do cruzamento de machos da linhagem "*multiple wing hairs"* (*mwh*/*mwh*), com constituição genética *Y*; *mwh JV* (3-0,3) e fêmeas virgens da linhagem *warts* (*wts*/TM3,*sb*[1]), com constituição genética ST [1] *in* [1]*kni*[*ri*-1]*wts*[3-17]/TM3, *sb*[1] (**Fig. 7**). Dois descendentes são possíveis com esse cruzamento: descendentes trans-heterozigotos marcados - MH (*wts* +/+ *mwh*), que apresentam pelos longos e finos do tórax e cabeça da mosca; e descendentes heterozigotos balanceados - BH (TM3,*sb*[1] +/+ *mwh*), que são caracterizados por possuirem pelos curtos e grossos na cabeça e tórax, causados pela presença do marcador cromossômico TM3*, Sb*[1]. Os indivíduos BH são descartados e os indivíduos MH são analisados, visto que são os únicos que possuem o gene de interesse no estudo (*wts*).

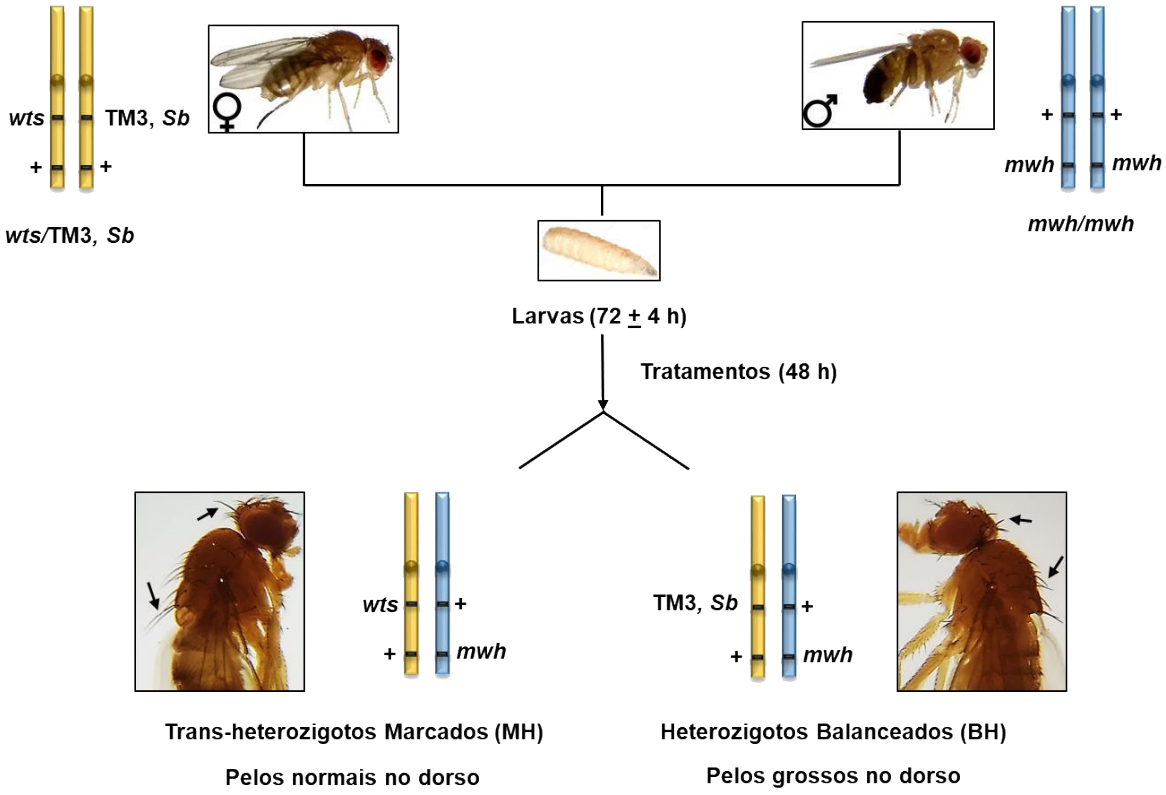

Figura 7. Representação esquemática do ETT em *D. melanogaster*.

Os possíveis efeitos cancinogênicos e anticarcinogênicos são validados pelo teste U não paramétrico de Mann, Whitney e Wilcoxon, utilizando α = 0,05 como nível de significância.

A **Fig. 8** apresenta imagens de tumores epiteliais encontrados em diversas partes do corpo (cabeça, olhos, tórax, asas, halteres e pernas) de descendentes MH de *D. melanogaster*, observados ao microscópio estereoscópico (aumento de 25X).

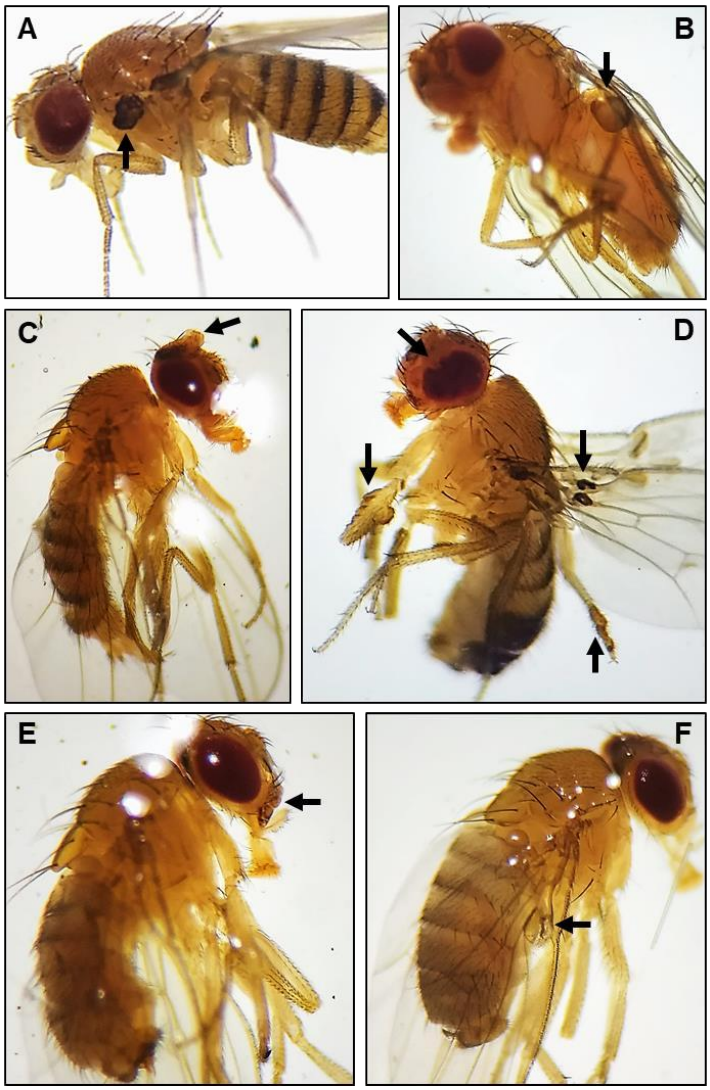

Figura 8. Imagens de tumores epiteliais (setas) encontrados em diversas partes do corpo de descendentes MH de *D. melanogaster*, observados ao microscópio estereoscópico (aumento de 25X): (A) tórax; (B) halter; (C) parte superior da cabeça; (D) olho, pernas e asas; (E) parte frontal da cabeça; (F) asa.

3. Ensaios *in vitro*

Os ensaios *in vitro* surgiram como uma alternativa à utilização de animais nas pesquisas científicas. Um ensaio *in vitro* (expressão latina que significa “em

vidro") é um ensaio realizado fora de um organismo vivo, que é feito com células que são colhidas de um ser vivo e multiplicadas em laboratório. Esses testes podem ser ferramentas complementares importantes para entender o possível mecanismo de ação de determinada molécula (Anadón *et al*. 2014).

As linhagens celulares podem crescer indefinidamente em cultura, fornecendo uma fonte "inesgotável" de material biológico para fins experimentais. Uma vez que todas as células de uma linhagem são clonais, elas representam um tipo de célula uniforme, o que tem implicações importantes para sua utilização como material experimental. Por isso, as linhagens celulares são sistemas modelo *in vitro* que têm sido amplamente utilizados em ensaios de genotoxicidade (Mirabelli *et al*. 2019, dos Santos *et al*. 2020).

Nos ensaios que utilizam células em cultura como sistema modelo, é importante saber quantas células vivas e mortas estão presentes durante e/ou depois do experimento, principalmente quando as células são incubadas por um período de tempo que permite o crescimento e a divisão celular. Relacionar o número de células viáveis existentes melhora a robustez estatística do ensaio (Riss *et al*. 2019).

3.1. Teste de resazurina

A resazurina é um corante azul, utilizado como indicador redox, que é permeável às células e pode ser usado para monitorar o número de células viáveis. A mesma pode ser dissolvida em tampões fisiológicos (resultando em uma solução de cor azul intensa) e adicionada diretamente às células em cultura em um formato homogêneo. As células viáveis, com metabolismo ativo, podem reduzir a resazurina no produto resorufina que é rosa e fluorescente. A quantidade de resorufina produzida é proporcional ao número de células viáveis, que podem ser quantificadas usando um fluorômetro de microplaca, equipado com um conjunto de filtros de excitação 560 nm / emissão 590 nm (Riss *et al*. 2016).

Testes de citotoxicidade *in vitro* fornecem um meio crucial de classificação de compostos para serem utilizados na descoberta de novas drogas (Niles *et al*. 2008). O ensaio baseado na redução da resazurina tem sido amplamente utilizado como um método eficaz para avaliar a viabilidade celular (Musa e Cidre-Aranaz 2021, Varçin *et al*. 2021).

3.2. Ensaio do cometa

A eletroforese em gel de célula única, também conhecida como ensaio do cometa, é um ensaio simples, sensível e versátil para avaliar danos e reparos de DNA em células individuais. Esse teste é baseado na lise de membranas celulares, seguida pela possível fragmentação do DNA e, depois, indução da migração eletroforética do DNA liberado em matriz de agarose em resposta a um campo elétrico (Møller *et al*. 2020).

Quando vista ao microscópio, a célula migrada adquire a forma semelhante a um “cometa”, com cabeça - região nuclear, e cauda - que contém fragmentos ou fitas de DNA que migraram na direção do ânodo. A análise dos cometas formados, baseia-se no grau de fragmentação do DNA e sua migração pela microeletroforese. Medidas, como o comprimento total da "cauda" e a densidade de DNA nos diferentes locais do cometa, fornecem dados indiretos sobre o estado do DNA da amostra (Brianezi *et al*. 2009).

Existem dois tipos de ensaio do cometa: a eletroforese neutra, que é considerada menos sensível, porque detecta apenas lesão de fita dupla de DNA (Olive 1989); e a alcalina, que é adotada pela maioria dos laboratórios, por detectar maior variedade de lesões ao DNA, como quebra de fita simples, lesões de sítios alcalinos lábeis, locais de reparos incompletos e crosslinks (Singh *et al*. 1988).

O ensaio do cometa tem sido amplamente utilizado como uma ferramenta para avaliar a genotoxicidade de novas moléculas, produtos químicos ou farmacêuticos (Azqueta *et al*. 2020, Bonassi *et al*. 2021).

4. Ensaios antioxidantes

O estresse oxidativo é definido como um desequilíbrio entre a produção de espécies reativas de oxigênio (ROS) e a capacidade antioxidante da célula. As ROS são consideradas subprodutos do processo normal do metabolismo aeróbio das mitocôndrias, implicados em uma grande variedade de doenças (Migdal e Serres 2011). Uma propriedade comum de todos os tipos de ROS é que eles podem causar danos oxidativos a quase todas as biomoléculas, mas principalmente às proteínas, DNA e lipídios (Pitzschke *et al*. 2006).

A avaliação do estresse oxidativo pode fornecer dados importantes sobre o mecanismo de ação de várias substâncias, pois é um processo que pode desempenhar papel fundamental na progressão tumoral, alterando o potencial mutacional do DNA, vias de sinalização intracelular, proliferação celular, motilidade e invasividade (Fiaschi e Chiarugi 2012).

Manter o equilíbrio entre radicais livres e antioxidantes é essencial para o bom funcionamento do organismo. O controle dos processos de estresse oxidativo é fundamental tanto na prevenção quanto no tratamento de diversas doenças, como diabetes, aterosclerose, câncer, inflamação, doenças hepáticas, cardiovasculares, neurodegenerativas e várias outras (Poznyak *et al*. 2020). O estresse oxidativo pode ocorrer devido a um aumento na taxa de produção de ROS, deficiências de antioxidantes de baixa massa molecular e/ou inativação de enzimas com atividade antioxidante (Pham-Huy *et al*. 2008).

Os antioxidantes são tipicamente átomos e moléculas quimicamente estáveis, que possuem elétrons livres em seu envelope de elétrons. São produzidos pelo sistema protetor de vários organismos para responder aos efeitos destrutivos dos radicais livres. Os antioxidantes são capazes de reduzir os danos causados por ROS ou espécies reativas de nitrogênio (RNS), e até mesmo pelo cloro. A ação do sistema protetor pode limitar ou interromper os efeitos negativos dos radicais livres, prevenindo a formação de radicais reativos (Valko *et al*. 2007, Flieger *et al*. 2021).

4.1. Ferric Reducing Antioxidant Power - FRAP

O ensaio do poder antioxidante redutor férrico é um método direto, relativamente simples, rápido e barato, utilizado para medir a atividade antioxidante de uma amostra e utiliza Trolox como padrão. Foi realizado pela primeira vez por Benzie e Strain (1999).

Esse método é baseado na habilidade dos antioxidantes em reduzir íons férricos (Fe^{3+}) em íons ferrosos (Fe^{2+}) na presença de tripiridiltriazina (TPTZ), formando o complexo azul ferro $^{2+}$ – TPTZ, com uma absorção máxima a 593nm. O aumento na absorbância é proporcional ao conteúdo de antioxidantes, provocando uma mudança na coloração de verde para azul escuro. Neste método é avaliada a

capacidade da amostra em agir como redutor, antioxidante (Benzie e Devaki 2018, Spiegel *et al*. 2020).

4.2. Oxygen Radical Absorbance Capacity - ORAC

A capacidade antioxidante total pode ser avaliada pelo ensaio da capacidade de absorção de radicais de oxigênio, que é um método relativamente simples, sensível e confiável (Rodríguez-Bonilla *et al*. 2017).

A singularidade deste ensaio é que a capacidade antioxidante total de uma amostra é estimada levando-se a reação de oxidação até o fim. Consiste na medida do decaimento da fluorescência das proteínas, como consequência da perda de sua conformidade ao sofrer dano oxidativo. Utiliza como molécula alvo dos radicais livres de oxigênio as ß-ficoeritrinas (ß-PE), altamente fluorescentes, que contêm um pigmento vermelho fotorreceptor. Os resultados são quantificados medindo a proteção produzida pelos antioxidantes (Cao *et al*. 1993).

Inúmeros trabalhos têm utilizado o ORAC para avaliar a capacidade antioxidante total de diferentes amostras (Abraham *et al*. 2021, Ivy *et al*. 2021, Quek *et al*. 2021).

4.3. Ensaio da superóxido dismutase - SOD

Os sistemas de defesa intracelulares, que protegem as células de danos induzidos por espécies reativas de oxigênio (ROS), incluem enzimas como a superóxido dismutase (SOD), que catalisa alternadamente a dismutação do radical superóxido (O_2^-) em qualquer oxigênio molecular comum (O_2) ou peróxido de hidrogênio (H_2O_2) (Missirlis *et al*. 2001, Ancuelo *et al*. 2021).

O superóxido é produzido como um subproduto do metabolismo do oxigênio e, se não for controlado, causa muitos tipos de danos às células. O peróxido de hidrogênio também é prejudicial e é degradado por outras enzimas, como a catalase. Por esse motivo, a SOD é uma importante defesa antioxidante em quase todas as células vivas expostas ao oxigênio. A alta atividade da SOD reduz os níveis de ROS e, consequentemente, os danos ao DNA (Kirby *et al*. 2002).

5. Docking molecular proteína-DNA

O docking molecular proteína-DNA *in silico*, é uma ferramenta fundamental para prever a ligação de moléculas no local de ligação de uma proteína alvo. É uma estratégia que envolve a utilização de simulações, realizadas *in silico*, para modelar interações de ácido nucleico com um alvo, objetivando determinar a força de ligação de cada uma das sequências do ácido nucleico, em relação ao alvo (Yuriev e Ramsland 2013, Navien *et al*. 2021).

Esta metodologia pode ser realizada com proteínas-alvo conhecidas ou desconhecidas, por meio da ancoragem contra alvos modelados por homologia. Os programas de docking molecular realizam um algoritmo de busca, no qual a conformação do ligante é avaliada recursivamente até que a convergência para a energia mínima seja alcançada (Pagadala *et al*. 2017).

A metodologia *in silico* de identificação de moléculas principais contra uma proteína alvo tem sido amplamente utilizada na descoberta de novas abordagens terapêuticas (Singh *et al*. 2021; Hajji *et al*. 2022).

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Capítulo II

Mutagenic and genotoxic activities of Phospholipase A_2 Bothropstoxin-I from *Bothrops jararacussu* in *Drosophila melanogaster* and human cell lines

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Resumo

A Fosfolipase A_2 Bothropstoxin-I (PLA_2 BthTX-I) é uma Lys49-PLA_2 miotóxica extraída da peçonha da serpente *Bothrops jararacussu*. Para avaliar o dano ao DNA causado pela BthTX-I, utilizamos o Teste de Mutação e Recombinação Somática (SMART) em *Drosophila melanogaster* e o Ensaio Cometa em células HUVEC e DU-145. Para o SMART, diferentes concentrações de BthTX-I foram usadas (6,72 a 430 µg/mL) e nenhuma alteração significativa na taxa de sobrevivência foi observada. Aumento significativo na frequência de manchas mutantes foi observado no cruzamento ST na concentração mais alta de BthTX-I devido à atividade recombinogênica. No cruzamento HB, a BthTX-I aumentou o número de manchas mutantes em concentrações intermediárias, sendo 53,75 µg/mL altamente mutagênica e 107,5 µg/mL predominantemente recombinogênica. As maiores concentrações não foram mutagênicas nem recombinogênicas, o que pode indicar citotoxicidade nessas concentrações, nas células da asa de *D. melanogaster*. *In vitro*, todas as concentrações de BthTX-I (1 a 50 µg/mL) induziram diminuição na viabilidade das células HUVEC, bem como nas células DU-145 nas concentrações de 10, 25 e 50 µg/mL. O ensaio do cometa mostrou que em células HUVEC e DU-145, todas as concentrações de BthTX-I promoveram aumento do dano ao DNA. Novos estudos devem ser realizados para elucidar o mecanismo de ação da PLA_2 BthTX-I e sua possível utilização em estratégias terapêuticas contra o câncer.

Palavras-chave: Ensaio cometa; Lys49-PLA_2; Miotoxina; SMART; Teste de mutação e recombinação somática.

Mutagenic and genotoxic activities of Phospholipase A_2 Bothropstoxin-I from *Bothrops jararacussu* in *Drosophila melanogaster* and human cell lines

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Highlights:

- BthTX-I did not change the survival rate of *D. melanogaster*.
- BthTX-I was recombinogenic at the highest concentration (ST cross).
- BthTX-I was mutagenic/recombinogenic at intermediate concentrations (HB cross).
- BthTx-I promoted the decrease in HUVEC and DU-145 cell viability.
- BthTx-I was genotoxic to HUVEC and DU-145 cells, *in vitro*.

Abstract

Phospholipase A_2 Bothropstoxin-I (PLA_2 BthTX-I) is a myotoxic Lys49-PLA_2 from *Bothrops jararacussu* snake venom. In order to evaluate the DNA damage caused by BthTX-I, we used the Somatic Mutation and Recombination Test (SMART) in *Drosophila melanogaster* and Comet assay in HUVEC and DU-145 cells. For SMART, different concentrations of BthTX-I (6.72 to 430 µg/mL) were used and no significant changes in the survival rate were observed. Significant frequency of mutant spots was observed for the ST cross at the highest concentration of BthTX-I due to recombinogenic activity. In the HB cross, BthTX-I increased the number of mutant spots at intermediate concentrations, being 53.75 µg/mL highly mutagenic and 107.5 µg/mL predominantly recombinogenic. The highest concentrations were neither mutagenic nor recombinogenic, which could indicate cytotoxicity in the wing cells of *D. melanogaster*. *In vitro*, all BthTX-I concentrations (1 to 50 µg/mL) induced decrease in HUVEC cell viability, as well as in DU-145 cells at concentrations of 10, 25, and 50 µg/mL. The comet assay showed that in HUVEC and DU-145 cells, all BthTX-I concentrations promoted increase of DNA damage. Further studies should be performed to elucidate the mechanism of action of PLA_2 BthTX-I and its possible use in therapeutic strategies against cancer.

Keywords: Comet assay; Lys49-PLA_2; Myotoxin; SMART; Somatic mutation and recombination test.

1. Introduction

Phospholipases A_2 (PLA_2s) are enzymes found in large quantities in snake venom. These compounds are able to hydrolyze the 2-acyl ester bond of phospholipids by releasing fatty acids and lysophosphatides [1]. PLA_2s have a remarkable medical-scientific interest and biotechnological applications due to their involvement in a wide variety of physiological activities, such as anticoagulant, bactericidal, cardiotoxic, hemolytic, inflammatory, myotoxic, neurotoxic, oedematogenic, as well as the ability of inducing or inhibiting platelet aggregation [1,2].

Phospholipase A_2 Bothropstoxin-I (PLA_2 BthTX-I) is a Lys49-PLA_2 (a variant containing lysine on carbon 49), which is enzymatically inactive, with molecular weight of 13.8 kDa, isolated from *Bothrops jararacussu* snake venom [3]. PLA_2 BthTX-I has shown to possess antimicrobial and antitumoral activities, besides being an inducer of apoptosis, cell damage, cytotoxicity, muscle inflammation and myonecrosis. Furthermore, studies have demonstrated modulation of the cell cycle with delay in the G_0/G_1 phase and reduction of cell viability [2,4-7]. Some studies highlight a strong correlation of PLA_2 with genetic instability in biological systems, thus indicating the high genotoxic potential of these substances [8,9].

Considering the lack of information related to genetic toxicology of PLA_2 BthTX-I, it is important to evaluate its mutagenicity, recombinogenicity and genotoxicity. Owing to the need to reduce the use of mammals in scientific research, efforts have been made to develop *in vivo* and *in vitro* alternative models [10].

Drosophila melanogaster has been successfully considered as an alternative experimental *in vivo* model because various basic biological, physiological and neurological properties are conserved between mammals and fly, and the signaling pathways involved in cancer development were also conserved in the species [11]. Besides that, *D. melanogaster* develops faster, requires few infrastructures and is easy to manipulate genetically [12,13]. In this context, *Drosophila* has stand out in the scientific research as a promising organism to evaluate the toxicity, mutagenicity and carcinogenicity of different compounds [14-17].

The Somatic Mutation and Recombination Test (SMART) is among the tests performed with *D. melanogaster* as a *in vivo* model organism [18,19]. The SMART is based on the fact that, at the beginning of the embryonic development of *D.*

melanogaster, groups of cells proliferate mitotically until they differentiate into the body structures of the adult fly. In case of an alteration in some cell of the wing imaginal disk, a clone of mutant cells will be formed, and later it will be detected as a spot of mutant hair on the wings of the adult fly, phenotypically expressing the marker genes *flr*^{3} or *mwh* [18]. Indeed, SMART has been successfully used to detect the mutagenic/recombinogenic properties of several chemical compounds [16,17,20,21].

Unlike primary cells, cell lines can grow indefinitely in cell culture, providing an indefinite source of biological material for experimental purposes. Once all cells in a row are clonal, they represent a uniform cell type, which has important implications for their use as experimental material [22]. Therefore, cell lines are *in vitro* model systems that have been widely used in genotoxicity assays [23,24].

The single cell gel electrophoresis, also known as comet assay, is a simple, sensitive and versatile assay to assess DNA damage (strand breaks and alkali-labile sites) in individual cells, based on the ability of DNA fragments to be pulled through an agarose gel in response to an electric field, appearing as a “comet”. The comet assay has been extensively used as a tool to evaluate the genotoxicity of new molecules, chemicals or pharmaceuticals [25-27].

In the present study, we investigated the mutagenic and recombinogenic activities of a PLA_2 BthTX-I in *D. melanogaster* using the wing SMART, which was performed through two crosses: 1. standard cross - ST, with basal levels of cytochromes P450 (CYP450) enzymes, specially CYP6A2; and 2. high bioactivation cross - HB, with high levels of CYP450. We found that PLA_2 BthTX-I was mutagenic and recombinogenic. Moreover, we investigated the genotoxic activities of PLA_2 BthTX-I in human umbilical vein endothelial cell line (HUVEC) and in DU-145 tumor cell line isolated from a metastatic site in the brain, prostate cancer-derived, using the comet assay. We noticed that PLA_2 BthTX-I promoted a decrease in cell viability and induced significant DNA damage in both cell lines. We believe that these results can be useful for the development of new therapeutic strategies for the treatment of various human diseases, including cancer.

2. Material and Methods

2.1. Crude venom

The crude venom was collected from *B. jararacussu* snake kept at the Ceta serpentarium, Animal Toxin Extraction Center, Ltda. – CNPJ: 08.972.260/0001-30, Morungaba, SP, Brazil. This serpentarium has obtained proof of IBAMA registration and use of renewable natural resources (n° 2087163). This work is registered in the National Management System of Genetic Heritage and Associated Traditional Knowledge under the number A2C2534.

2.2. Isolation of PLA_2 BthTX-I

PLA_2 BthTX-I was purified from *B. jararacussu* venom according to the methodology described by Homsi-Brandenburgo et al. (1988) [3], with minor modifications. *B. jararacussu* crude venom (200 mg) was dispersed in 2.0 mL ammonium bicarbonate buffer (AMBIC) 0.05 M, pH 8.0 and centrifuged at 100 x*g* for 10 min at 4 °C. The clear supernatant was removed and applied to CM-Sepharose Fast Flow (GE Healthcare) column previously equilibrated with 0.05 M AMBIC, pH 8.0. Protein elution was initiated with equilibration buffer (0.05 M AMBIC), followed by a convex concentration gradient of 0.05 M AMBIC to 0.5 M. The fractions were collected at 1.0 mL/tube under a constant flow of 6.5 mL/h in the Amersham Biosciences Red Frac collector. The absorbance of each fraction was read at 280 nm through a spectrophotometer (Ultrospec 1000 Pharmacia Biotech). After, the fraction containing PLA_2 BthTX-I was rechromatographed by reverse-phase chromatography (RP-HPLC) in a C18 column (GE Healthcare). The column was equilibrated with solvent A (0.1% trifluoroacetic acid, 5% acetonitrile) and eluted with a concentration gradient of solvent B (0.1% trifluoroacetic acid, 80% acetonitrile) from 0% to 100%, with a flow rate of 0.5 mL/min for 33 min. The single peak was lyophilized and analyzed on 12.5% SDS PAGE (v/v). Thermo Scientific molecular weight standard was used, where 116.0 kDa corresponds to β-galactosidase, 66.2 kDa: bovine serum albumin, 45.0 kDa: ovalbumin, 35.0 kDa: lactate dehydrogenase, 25.0 kDa: REase Bsp98I, 18.4 kDa: β-lactoglobulin and 14.4 kDa: lysozyme. The samples were stored at -20 °C.

2.3. Somatic Mutation and Recombination Test - SMART

Three strains were used: “Multiple wing hairs” (*mwh*), 3 (3-0.3), with genetic constitution *y; mwh jv*; “flare-3” (*flr*^{3}), 3 (3-38.8), with genetic constitution *flr*^{3}*/In (3LR)*TM3*, ri p*^{p} *sep l (3) 89Aa bx*^{34e} and *Bd*^{S}; and “ORR; flare-3” (*ORR; flr*^{3}), with genetic constitution *ORR; flr*^{3}*/In(3LR)*TM3*, ri p*^{p} *sep I(3)89Aa bx*^{34e} and *Bd*^{S}. This strain carries genes for the high expression of cytochrome P450 complex enzymes, located on chromosomes 1 and 2, derived from the naturally occurring DDT-resistant “Oregon R” strain [18,19].

2.3.1. Crossings and treatments

Two different crosses were performed: standard cross (ST) between “*mwh*” males and “*flr*^{3}” virgin females [18,28]; and high bioactivation cross (HB) between “*mwh*” males and “*ORR; flr*^{3}” virgin females [19]. Both crosses (ST and HB) produced two progeny types: marker trans-heterozygous (MH) (*mwh+/+flr*^{3}) and balancer heterozygous (BH) (*mwh+/+*TM3*, Bd*^{S}).

Eggs were collected over 8 h in vials containing a solid agar base (4% agar in water) and a layer of yeast (*Saccharomyces cerevisiae*) supplemented with sucrose. PLA_2 BthTX-I concentrations (6.72, 13.44, 26.88, 53.75, 107.5, 215, 430 µg/mL) evaluated in the SMART were selected after performing a survival test, where 100 larvae of 72 ± 4 h were counted before the distribution in treatment vials, containing different concentrations of PLA_2 BthTX-I. The hatched flies were counted and stored in 70% ethanol. The survival tests were performed only once, without replicates.

Third instar larvae (72 ± 4 h), from both crosses, were submitted to chronic treatment (approximately 48 h) with different concentrations of PLA_2 BthTX-I: 6.72, 26.88, 53.75, 107.5, 215 and 430 µg/mL. Treatments were performed in vials containing 1.5 g of alternative culture medium (instant mashed potato, Yoki Hikari®) [29], hydrated with 5 mL of each concentration tested. Ultrapure water (18.2 MΩ) obtained from the MilliQ system was used as a negative control, and as a positive control, 10 mM urethane (ethyl carbamate - CAS: 51-79-6, Buchs, Switzerland). All treatments were performed in duplicate.

2.3.2. Slide preparation and analysis

Emerging adults after treatment were collected and stored in flasks containing 70% ethanol. The wings were removed with the aid of entomological forceps on a stereomicroscope, and aligned in a glass slide. For adhesion of the wings and the coverslip to the slide, Faure's solution (30 g of gum arabic, 20 mL of glycerol, 50 g of chloral hydrate and 50 mL of water) and metal weights were used. The wings (both the dorsal and ventral surface) were analyzed under a light microscope with final magnification of 400x. According to Graf et al. (1984) [18], cell lineages derived from genotypically altered cells form a clone, recognized as a mutant spot, when the appropriate markers *flr*^{3} or *mwh* are phenotypically expressed. The mutant spots can be classified as small single spot (1 or 2 cells in size, mwh or flr^3 type); large single spot (> 2 cells, mwh or flr^3 type), or twin spot (with mwh and flr^3 hairs together in a same spot) (**Fig. 2**).

2.3.3. Statistical Analysis

From both crosses (ST and HB), two types of progeny were obtained: 1] marker-heterozygous (MH) (*mwh+/+flr*^{3}); and 2] balancer-heterozygous (BH) (*mwh+/*+TM3*, Bd*^{S}). The wings of both genotypes (MH and BH) were analyzed for the occurrence of mutant phenotypes. In the MH genotype, the single spots (mwh or flare) can be due to either mutational events (deletions, point mutations) or mitotic recombination, if mitotic crossing-over takes place between the two marker genes. Twin spots (composed of a mwh and a flr^3 area) can be produced by mitotic recombination between the proximal marker *flr*^{3} and the centromere of chromosome 3. In contrast, in the BH genotype, only mutational events lead to spot formation, because all recombinational events are eliminated. Therefore, only mwh single spots can be recovered [18].

The frequency of each spot type (small single, large single or twin) and the total frequency of wing spots for each treatment were compared pair-wise (i.e. PLA_2 BthTX-I-treated *vs.* negative control) according to Kastenbaum and Bowman (1970) [30], with $p < 0.05$ [31,32].

Based on the control corrected clone induction frequency per 10^5 cells, the recombinogenic activity was calculated. Frequency of mutation (FM) = frequency of clones in BH flies / frequency of clones in MH flies. Frequency of recombination (FR)

= 1 – frequency of mutation (FM). Frequencies of total spots (FT) = total spots observed in MH flies (considering mwh and flr^3 spots) / number of flies [33-35]. Chi-squared test was performed for statistical comparisons of the survival rate ratios for independent samples [36].

2.4. Cell lines and culture conditions

Cell culture procedures were performed according to Bal-Price and Coecke (2011) [37]. Human umbilical vein endothelial cells (HUVEC) were obtained from ATCC (American Type Culture Collection, Cat. Nº. CRL-4053). DU-145 tumor cell line was isolated from a metastatic site in the brain, prostate cancer-derived, of a 69-year-old male and was obtained from ATCC (Cat. Nº. HTB-81TM). HUVEC and DU-145 cells were maintained in RPMI 1640 culture medium (Roswell Park Memorial Institute 1640 Medium; Gibco, Carlsbad, CA, USA) with 10% Bovine Fetal Serum (SBF; Gibco, Carlsbad, CA, USA), 1% antibiotic/antimycotic mix (10,000 units/mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin B; Gibco, Carlsbad, CA, USA) and 0.024% sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA). In culture, the cells were kept in a CO_2 incubator with 5% atmosphere at 37 °C and 95% relative humidity. All cell culture procedures were performed in Class II and Type 1A laminar flow hoods of the Bioprotector VSFL-09 model (VecoFlow Ltda.; Campinas, SP, Brazil). All the experiments were conducted between the third and the eighth cell passage.

2.5. Resazurin test

The cell viability assessment using the resazurin reduction assay (Sigma-Aldrich, St. Louis, LO, USA) was carried out according to the protocol described by Riss et al. (2016) [38]. Briefly, HUVEC and DU-145 human cells (1×10^4) were seeded in 96-well culture plates (Greiner Bio-One; Kremsmünster, Austria) and incubated for 24 h before treatment with different PLA_2 BthTX-I concentrations (1, 5, 10, 25 and 50 µg/mL), positive (10 µM Cisplatin) and negative (RPMI 1640) controls for further 24 h. After this, 20 µL of resazurin working solution (0.15 mg/mL in PBS) was added to each well. The plates were then incubated for 2 h in a 37 ºC oven, and the fluorescence was analyzed on the Synergy™ HTX Multiplate Reader (BioTek; Agilent Technologies, Santa Clara, CA, USA) with an excitation of 530 nm

and an emission of 590 nm. The fluorescence intensity was used to determine cell viability by comparing treatment groups with negative control, which was assigned a value of 100% cell viability. The experiments were carried out in triplicate.

2.6. Comet assay

The alkaline comet assay was performed according to the protocol reported by Tice et al. (2000) [39]. HUVEC and DU-145 cells ($5x10^4$) were seeded in 24-well culture plates and grown in the complete culture medium, under standard conditions, for 24 h. Posteriorly, the cells were treated with different PLA_2 BthTX-I concentrations (10, 25, and 50 µg/mL), positive (10 µM Cisplatin), and negative (RPMI 1640) controls for a further 24 h. Then, treatments were removed from each well, washed with PBS (1x), and TrypLE™ Express Enzyme (1x; without red phenol) was added. The plate was kept in an incubator at 37 °C for 3 min. After this period, complete culture medium was added to each well, the content was homogenized, and the cell suspension was centrifuged for 5 min at 300 x*g*. Cell viability was determined by the trypan blue technique using the Countess® Automated Cell Counter (Life Technologies; Carlsbad, CA, USA). Samples with viability > 70% were subjected to the comet assay, as follows. The pellet resulting from each treatment was suspended in 0.5% Low Melting Point (LMP) agarose (Invitrogen) in the proportion of 1:4 (1 µL homogenate: 4 µL LMP agarose). This mixture was transferred for a conventional slide pre-coated with 1.5% normal melting point agarose (Invitrogen), the slides were covered with coverslips (24 x 60 mm) and cooled at 4 °C for 20 min. Afterwards, the coverslips were removed, and the slides were immersed in freshly prepared lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10) for 1 h, at 4 °C. Then, the slides were transferred to an electrophoresis chamber filled with buffer (300 mM NaOH and 1 mM EDTA pH > 13) for 20 min and electrophoresis was conducted under standard conditions (25 V; 300 mA; 1.25 Vcm^{-1}) for 20 min. The slides were neutralized (0.4 M Tris pH 7.5) for 15 min, air-dried, and fixed in absolute ethanol for 5 min. Before the analysis, the slides were stained with GelRed® (Biotium Inc., Fremont, CA, USA - GelRed™/PBS; 1: 10.000 µL; v/v). The slides were analyzed using a fluorescence microscope (Carl Zeiss, Axiostar Plus; Jena, Germany) with λ = 515-560 nm excitation filter and λ = 590 nm barrier filter, with the aid of the comet assay IV™

software (Perceptive Instruments; Haverhill, UK). The tail intensity (% of DNA in the tail) was used as the analysis parameter. The experiments were carried out in triplicate.

2.6.1. Statistical Analysis

The results were expressed as the mean ± standard deviation (SD). The Shapiro-Wilk test was used to analyze data normality distribution. Statistical analysis was performed using ANOVA followed by Dunnett's post-test ($p < 0.05$) and Two-Way ANOVA followed by Bonferroni's test. All analysis were obtained using GraphPad Prism 7.00 software for Windows (La Jolla, CA, USA).

3. Results and Discussion

PLA_2 BthTX-I was obtained from *B. jararacussu* venom after one chromatographic step on CM-Sepharose (**Fig. 1A**), and its homogeneity was confirmed by reverse-phase chromatography (RP-HPLC) C18 (**Fig. 1B**) and SDS-PAGE (**Fig. 1C**).

The Somatic Mutation and Recombination Test (SMART) in wing somatic cells of *D. melanogaster* was performed to assess the mutagenic and recombinogenic potential of PLA_2 BthTX-I.

The different concentrations of PLA_2 BthTX-I used in the SMART were selected based on survival rates (%) of individuals from both crosses (ST and HB) upon exposure to different concentrations of PLA_2 BthTX-I (6.72, 13.44, 26.88, 53.75, 107.5, 215, 430 µg/mL). The results indicated that there was no reduction in the survival rate ($p > 0.05$) when compared to the negative control (water) (**Fig. 3**). Thus, these survival data validated the use of six concentrations (6.72, 26.88, 53.75, 107.5, 215, 430 µg/mL) of PLA_2 BthTX-I, which were tested in two independent experiments. The data were pooled after verifying that there were no significant differences between repetitions.

Ethyl carbamate (urethane - URE) is an organic compound classified by the International Agency for Research on Cancer (IARC) as a Group 2A agent, which can be carcinogenic to humans due to the production of reactive oxygen species (ROS), DNA depurination and mitochondrial dysfunction [40-42]. URE has been successfully used as positive control in SMART assays [14-17] because it has a

mutagenic/recombinogenic potential in *Drosophila*, with a clear dose response and dependence on metabolic activation, which involves cytochrome P450-dependent enzyme activities [43].

Both progenies (MH and BH) were analyzed in both crosses (ST and HB) when positive results were found in the MH progeny.

The results for the MH and BH descendants derived from the ST cross treated with different concentrations of PLA_2 BthTX-I are shown in **Table 1**. In the ST cross, only the highest concentration (430 µg/mL) of PLA_2 BthTX-I caused a significant increase in the frequency of mutant spots, which was essentially recombinogenic (64.0%).

The results for the MH and BH descendants, derived from the HB cross, treated with different concentrations of PLA_2 BthTX-I are shown in **Table 2**. PLA_2 BthTX-I induced an increase in the number of mutant spots at intermediate concentrations (53.75 and 107.5 µg/mL), being 53.75 µg/mL highly mutagenic (94.45%) and 107.5 µg/mL recombinogenic (59.03%). These rates were calculated according described in item 2.3.3. Statistical Analysis. In order to know the real influence of PLA_2 BthTX-I in the induction of mutant spots, the rates of mutation and recombination induced by these both concentrations were calculated from corrected values (values observed in the treated ones - value observed in negative control). Therefore, very low values of frequency of clone formation / 10^5 cells per division were obtained in both types of descendants (MH and BH), which meant that small differences in the frequencies of mutant spots caused great differences regarding the mutation and recombination rates.

The highest concentrations tested were not associated with mutagenic or recombinogenic events, which could be due to the toxic/cytotoxic effects of PLA_2 BthTX-I, owing to the constitutively high levels of cytochromes P450, especially CYP6A2 existing in HB individuals, which may lead to increased production of toxic metabolites. Nevertheless, these concentrations did not alter survival rates in *D. melanogaster*.

In previous studies carried out on human (HepG2 and HL-60) and murine (PC-12 and B16F10) tumor cell lines, PLA_2 BthTX-I at high concentrations was cytotoxic for inducing cell death mechanisms of apoptosis and/or necrosis, besides promoting delay in the G0/G1 phase of the cell cycle of murine tumor cells [7].

In addition, the greater appearance of recombinogenic effects observed in individuals treated with 430 µg/mL of the ST cross and 107.5 µg/mL of the HB cross may be due to DNA repair mechanisms. This is probably because PLA_2 BthTX-I produces primary DNA damage that is later processed by homologous recombination (HR), which is one of the main events detected by the somatic mutation and recombination test in *Drosophila* [29].

We used the resazurin reduction assay to determine the extent of PLA_2 BthTX-I cytotoxicity after 24 h of treatment. This toxin significantly decreased cell viability ($p < 0.05$) of HUVEC cells treated with concentrations ranging from 1 to 50 µg/mL, and of DU-145 cells ($p < 0.05$) treated with 10, 25 and 50 µg/mL (**Fig. 4**). Cisplatin at 10 µM (positive control) effectively reduced cell viability of both cell lines.

Cisplatin is a well-known chemotherapeutic agent which belongs to the DNA alkylating family [44], used for the treatment of numerous human cancers, including bladder, head, neck, lung, ovarian and testicular cancers [45]. Cisplatin binds to nucleophilic groups in DNA, introducing intra- and interstrand DNA cross-links which lead to growth inhibition and apoptosis [46,47]. Cisplatin is known for its cytotoxic and genotoxic effects, both *in vitro* and *in vivo*, mainly due to the production of radical oxygen species (ROS) [48,49]. Therefore, it has been used as a positive control in different *in vitro* assays [49,50].

Hence, PLA_2 BthTX-I decreased cell viability of both HUVEC and DU-145 cells, corroborating reports in the literature on the cell death-inducing capacity of snake venom PLA_2 [51,52]. PLA_2 BthTX-I and PLA_2 BthTX-II, two PLA_2s purified from *Bothrops jararacussu* snake venom and separated in fractions 6-1 and 6-2, respectively, are cytotoxic to NIH 3T3 cells (mouse embryonic fibroblasts) and COS7 cells (fibroblast-like cell lines derived from monkey kidney), as assessed using the MTT assay [51]. PLA_2 BthTX-II induces concentration-dependent cell death in MDA-MB-231 cells (triple negative human breast cancer) and inhibits cell proliferation by inducing apoptosis [52].

Some studies have been conducted to elucidate the mechanisms by which PLA_2s induce cell death. PLA_2 BthTX-I weakly induces cell death of the human tumor cell lines HL-60 (promyelocytic leukemia) and HepG2 (hepatocellular carcinoma) via apoptosis, as assessed using flow cytometry. HepG2 cells are more susceptible to apoptosis than HL-60 cells, with cell death rates of 35% and 4%,

respectively [7]. PLA_2 BthTX-I at concentrations higher than 100 µg/mL induces cell death in the breast cancer cell lines MCF7, SKBR3 and MDA-MB-231, as determined using the MTT assay, as well as it elicits apoptosis in MCF7, SKBR3 and MDA-MB-231 cells in a concentration-dependent manner [6]. The pro-caspase 3 and pro-caspase 8 protein expression is upregulated but Bcl-2 protein expression is downregulated in MCF7 cells. In this study, PLA_2 BthTX-I probably reduced cell viability by inducing apoptosis.

Compared with the negative control, cisplatin induced stronger DNA damage in HUVEC cells than in DU-145 cells (**Fig. 5**), in agreement with literature reports. The DNA mismatch repair (MMR) proteins participate in cisplatin-induced cytotoxicity through binding to cisplatin-DNA adducts and triggering MMR protein-dependent cell death, as well as they mediate the DNA damage response after treatment with cisplatin. Defects in the MMR repair system or loss of this protein cause resistance to cisplatin and poor prognosis in cancer [53,54]. MMR proteins are required to maintain the cisplatin interstrand cross-links formed during DNA damage, which in turn increases cell sensitivity to this drug [55]. Thus, it is expected that DU-145 cells from brain metastasis of prostate tumor with deficient MMR repair system present less cisplatin-induced damage when compared with HUVEC cells with proficient DNA repair.

All the PLA_2 BthTX-I concentrations tested significantly increased DNA damage in HUVEC cells ($p < 0.05$). We also examined the extent of PLA_2 BthTX-I-induced DNA damage in DU-145 cells after 24 h of treatment, and found that the toxin increased DNA damage in a concentration-dependent manner in the concentration range tested (10 - 50 µg/mL) (**Fig. 5**).

PLA_2s from snake venom exhibit a variety of physiological activities, but most of them are toxic and induce a wide spectrum of pharmacological effects, such as neurotoxicity, cardiotoxicity, and myotoxicity. The mechanisms by which PLA_2s damage tissues and cells are not yet fully understood [56]. The combination of transcriptomic and established proteomic methods developed in the last decade has made possible to identify and quantify protein families in snake venoms. More than 400 PLA_2s isolated from snake venom are reported in the UniProtKB database for the collection of functional information on proteins [57]. Snake venom PLA_2s display many toxic properties that range from inhibition of coagulation to blockage of nerve

transmission and induction of muscle necrosis [58]. The biological properties of snake venom PLA_2s can be attributed to protein-protein interactions that remain to be studied. These properties can be useful for the application of snake venom PLA_2s in pharmacological and biotechnological products.

The mechanisms by which snake venom toxins, including PLA_2s, cause genotoxicity have not been elucidated yet and there are no literature data on possible genotoxic effects of PLA_2 BthTX-I to human tumor cells. But, studies have shown that the treatment with the PLA_2s BthTX-I and BthTX-II, isolated from *Bothrops jararacussu* venom, increases micronuclei formation and DNA damage in human lymphocytes *in vitro*, as assessed by the micronucleus test and comet assay, indicating a high genotoxic potential of these substances probably due to the formation of reactive oxygen species (ROS) [8]. Another recent study also reported that PLA_2 BthTX-I enhances ROS production in cell cultures [59]. In the present study, we suggest that the induction of ROS production by different PLA_2 BthTX-I concentrations is the main mechanism of genotoxicity that explains the statistically significant increase in DNA damage in the tests performed.

4. Conclusions

Under these experimental conditions, we concluded that PLA_2 BthTX-I was mutagenic and recombinogenic in somatic cells of *D. melanogaster*, and genotoxic in HUVEC and DU-145 cells. Further studies should be conducted to elucidate the mechanism of action of this PLA_2, thereby enabling the development of new therapeutic strategies for the treatment of various human diseases, including cancer.

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6. Conflict of Interests

There is no conflict of interest.

7. References

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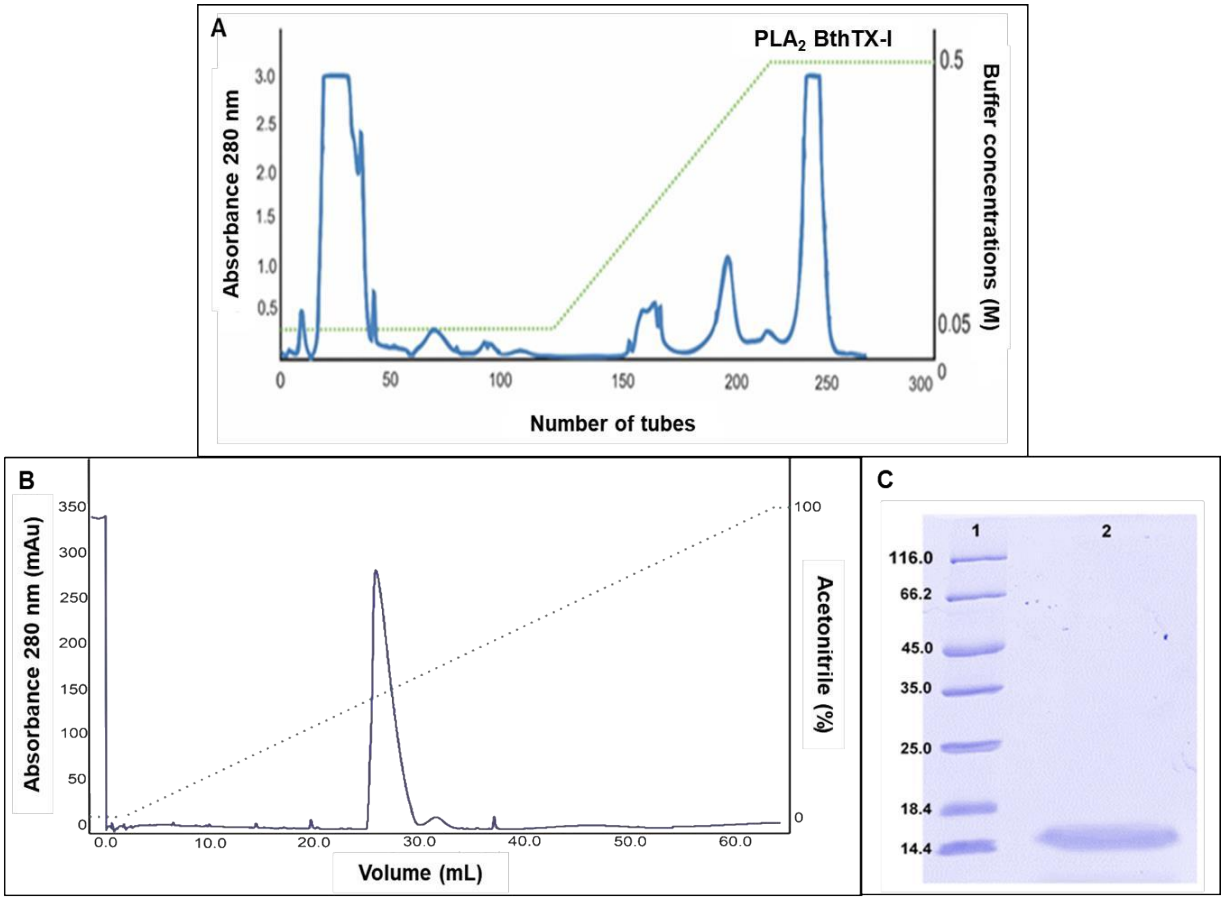

Fig. 1. Purification of PLA_2 BthTX-I from *Bothrops jararacussu* venom. (A) Chromatographic profile of 200 mg of *B. jararacussu* venom, applied to the CM-Sepharose Fast Flow column with convex gradient AMBIC buffer (0.05 M to 0.5 M), pH 8.0. (B) Samples of 200 µg of BthTX-I were dissolved in 500 µL of solvent A and applied to RP-HPLC C18. The sample was eluted with a linear gradient of solvent B from 0 to 100% at a flow rate of 1 mL/minute. (C) Polyacrylamide gel electrophoresis (12.5%) under denaturing conditions. (1) Molecular weight standard (kDa) and (2) PLA_2 BthTX-I. Running time 90 min at 12 mA.

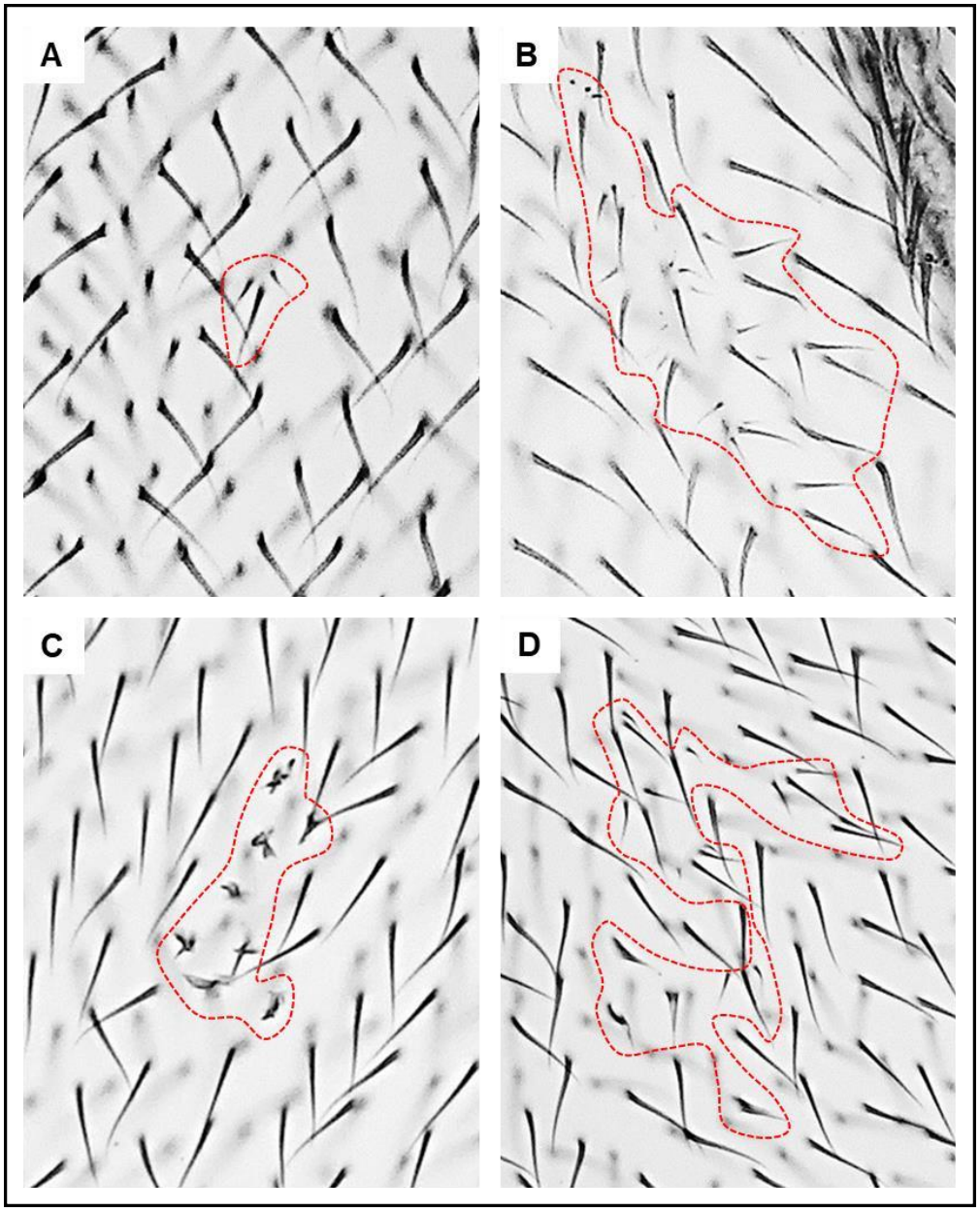

Fig. 2. Photomicrographs (400x magnification) of *D. melanogaster* wings obtained by light microscopy. (A) Small single spot (with one cell), "mwh" type; (B) Large single spot (> 2 cells), "mwh" type; (C) Large single spot, “flare” type; (D) Twin spot with "mwh" and "flare" hairs.

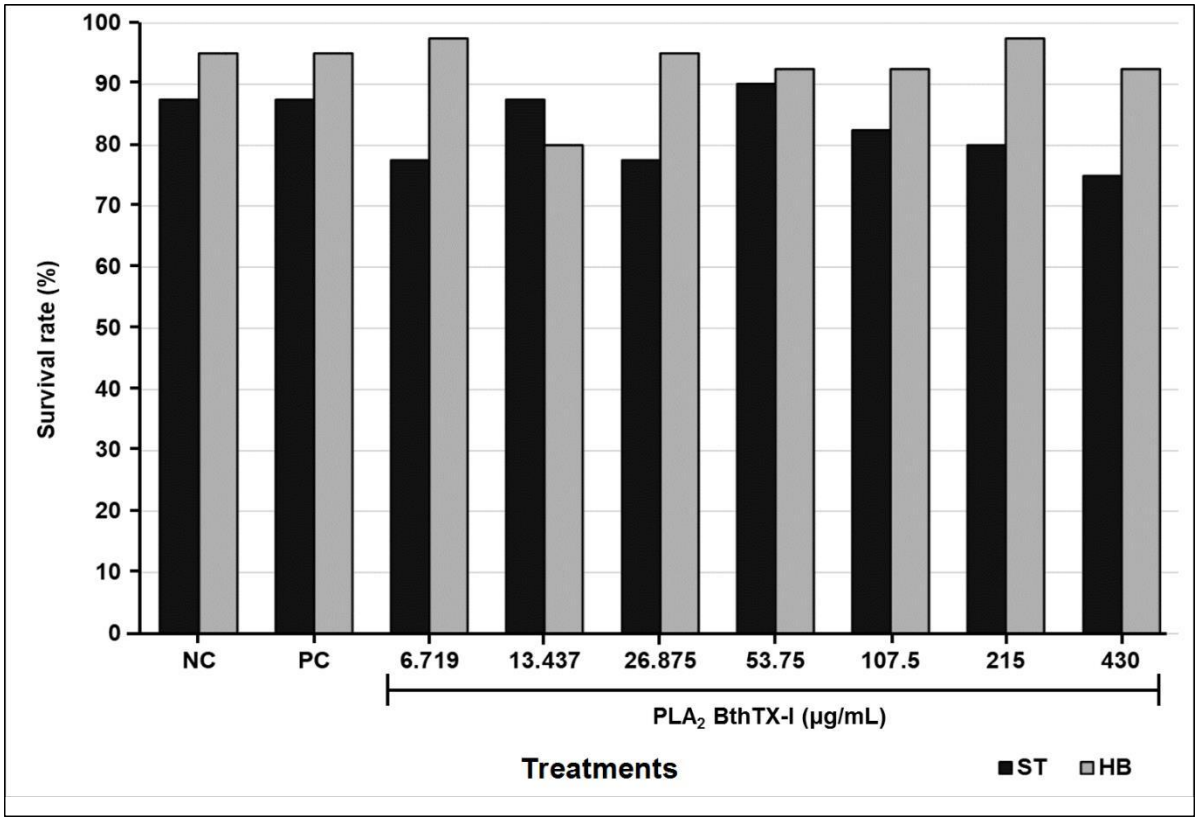

Fig. 3. Survival rates (%) of individuals from ST and HB crosses upon exposure to different concentrations of PLA_2 BthTX-I (6.719 - 430 µg/mL), ultrapure water as negative control (NC) and Urethane (URE - 10 mM) as positive control (PC); $*p < 0.05$. Data are representative of survival tests performed only once, without replicate.

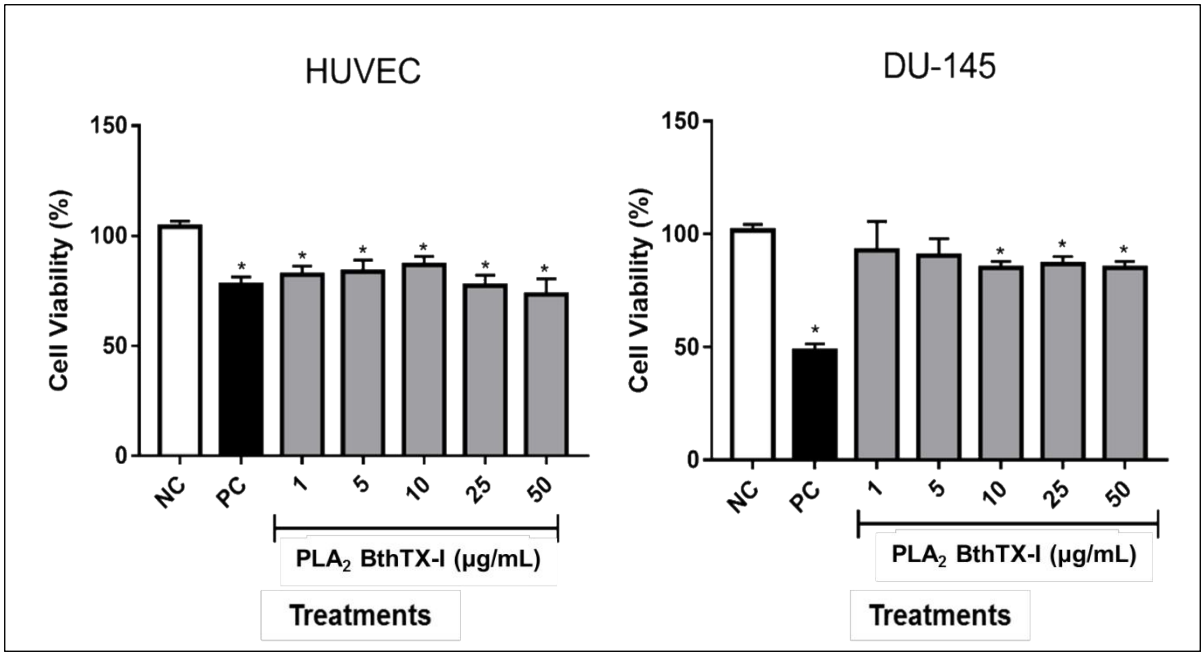

Fig. 4. Cell viability in HUVEC and prostate tumor DU-145 cells treated with PLA_2 BthTX-I (1 - 50 µg/mL) for 24 h, assessed by resazurin test. Data expressed as mean ± standard deviation of the percentage of cell viability in relation to NC (100% cell viability). NC: Negative Control (RPMI 1640); PC: Positive Control (10 µM Cisplatin). *$p < 0.05$ *vs.* NC (ANOVA followed by Dunnett's test).

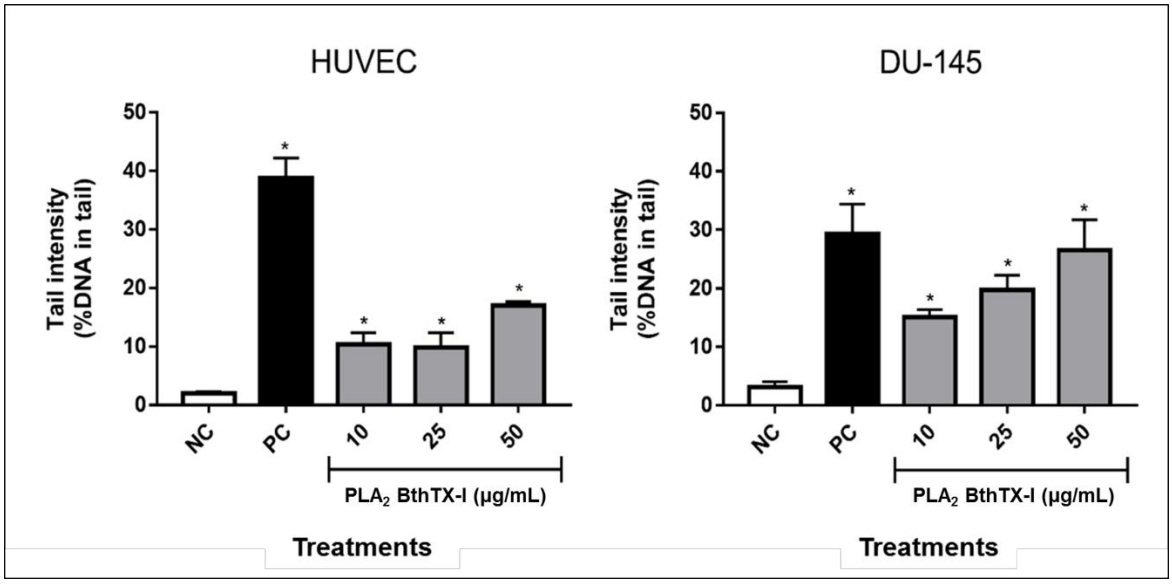

Fig. 5. Relative Tail Intensity (% of DNA in the tail) in HUVEC and DU-145 cells treated with PLA_2 BthTX-I (10 - 50 µg/mL) for 24 h. Each bar represents the mean ± standard deviation of three parallel cultures (100 nucleoids per replicate). NC: Negative Control (PBS); PC: Positive Control (10 µM Cisplatin); *$p < 0.05$ *vs*. NC (ANOVA followed by Dunnett's test).

Table 1. Summary of results obtained with the *Drosophila* wing spot test (SMART) in the marker-heterozygous (MH) and balancer-heterozygous (BH) progeny of the standard cross (ST) after chronic treatment of larvae with ultrapure water (negative control), urethane 10 mM (positive control) and different concentrations of PLA_2 BthTX-I (µg/mL)

Genotypes and Treatments	Number of flies	Spots per fly (number of spots); statistical diagnoses[a]				Spots with *mwh* clone[c]	Frequency of clone formation/10^5 cells per division[d]		Recombination (%)
		Small single spots (1-2 cells)[b]	Large single spots (>2 cells)[b]	Twin spots	Total spots		Observed	Control corrected	
***mwh*/*flr*³**									
Negative control	60	0.57 (34)	0.03 (02)	0.03 (02)	0.63 (38)	37	1.26		
Positive control	60	2.47 (148) +	0.18 (11) +	0.05 (03) i	2.70 (162) +	150	5.12	3.86	31.86
PLA_2 BthTX-I									
6.72	60	0.72 (43) -	0.10 (06) i	0.02 (01) i	0.83 (50) -	49	1.67	0.41	
26.88	60	0.48 (29) -	0.03 (02) i	0.03 (02) i	0.55 (33) -	30	1.02	-0.24	
53.75	60	0.65 (39) -	0.03 (02) i	0.05 (03) i	0.73 (44) -	42	1.43	0.17	
107.5	60	0.58 (35) -	0.02 (01) i	0.05 (03) i	0.65 (39) -	38	1.30	0.04	
215	60	0.77 (46) i	0.08 (05) i	0.03 (02) i	0.88 (53) i	50	1.71	0.45	
430	60	0.78 (47) i	0.12 (07) i	0.08 (05) i	0.98 (59) +	59	2.01	0.75	64.00
***mwh*/TM3**									
Negative control	60	0.43 (26)	0.02 (01)		0.45 (27)	27	0.92		
Positive control	60	1.72 (103) +	0.02 (01) i		1.73 (104) +	104	3.55	2.63	
PLA_2 BthTX-I									
430	60	0.58 (35) i	0.00 (00) i		0.58 (35) i	35	1.19	0.27	

Marker-trans-heterozygous flies (*mwh*/*flr*³) and balancer-heterozygous flies (*mwh*/TM3) were evaluated.
[a] Statistical diagnoses according to Frei and Würgler (1988, 1995). Probability levels: -, negative; +, positive; i, inconclusive; $p < 0.05$ *vs*. negative control.
[b] Including rare flr single spots.
[c] Considering mwh clones from mwh single and twin spots.
[d] Frequency of clone formation: clones/flies/48,800 cells (without size correction).

Table 2. Summary of results obtained with the *Drosophila* wing spot test (SMART) in the marker-heterozygous (MH) and balancer-heterozygous (BH) progeny of the high bioactivation cross (HB) after chronic treatment of larvae with ultrapure water (negative control), urethane 10 mM (positive control) and different concentrations of PLA_2 BthTX-I (µg/mL)

Genotypes and Treatments	Number of flies	Spots per fly (number of spots); statistical diagnoses[a]				Spots with *mwh* clone[c]	Frequency of clone formation/10^5 cells per division[d]		Recombination (%)
		Small single spots (1-2 cells)[b]	Large single spots (>2 cells)[b]	Twin spots	Total spots		Observed	Control corrected	
***mwh*/*flr*³**									
Negative control	60	0.62 (37)	0.15 (09)	0.05 (03)	0.82 (49)	45	1.53		
Positive control	60	10.98 (659) +	1.22 (73) +	1.55 (93) +	13.75 (825) +	742	25.34	23.81	22.68
PLA_2 BthTX-I									
6.72	60	0.80 (48) -	0.05 (03) -	0.12 (07) i	0.97 (58) -	55	1.88	0.35	
26.88	60	0.82 (49) -	0.07 (04) -	0.12 (07) i	1.00 (60) -	58	1.98	0.45	
53.75	60	0.93 (56) +	0.13 (08) i	0.08 (05) i	1.15 (69) +	66	2.25	0.72	5.55
107.5	60	0.98 (59) +	0.13 (08) i	0.07 (04) i	1.18 (71) +	69	2.36	0.83	59.03
215	60	0.80 (48) -	0.05 (03) -	0.08 (05) i	0.93 (56) -	53	1.81	0.28	
430	60	0.77 (46) -	0.05(03) -	0.07 (04) i	0.88 (53) -	48	1.67	0.14	
***mwh*/TM3**									
Negative control	60	0.68 (41)	0.03 (02)		0.72 (43)	43	1.47		
Positive control	60	9.40 (564) +	0.30 (18) +		9.70 (582) +	582	19.88	18.41	
PLA_2 BthTX-I									
53.75	60	0.93 (56) -	0.12 (07) i		1.05 (63) -	63	2.15	0.68	
107.5		0.82 (49) -	0.07 (04) i		0.88 (53) -	53	1.81	0.34	

Marker-trans-heterozygous flies (*mwh*/*flr*³) and balancer-heterozygous flies (*mwh*/TM3) were evaluated.
[a] Statistical diagnoses according to Frei and Würgler (1988, 1995). Probability levels: -, negative; +, positive; i, inconclusive; $p < 0.05$ *vs.* negative control.
[b] Including rare flr single spots.
[c] Considering mwh clones from mwh single and twin spots.
[d] Frequency of clone formation: clones/flies/48,800 cells (without size correction).

Capítulo III

Carcinogenic and anti-carcinogenic activities of Bothropstoxin-I from *Bothrops jararacussu* in *Drosophila melanogaster*, antioxidant assays and protein-DNA molecular docking

Resumo

A Bothropstoxina-I (BthTX-I), uma proteína semelhante à fosfolipase A_2, isolada da peçonha da serpente *Bothrops jararacussu*, possui efeitos mutagênicos, genotóxicos, antitumorais e apoptóticos. O objetivo deste estudo foi avaliar a carcinogenicidade/anticarcinogenicidade da BthTX-I sozinha ou em combinação com doxorrubicina (DXR 0,4 mM) utilizando o Teste de Tumor Epitelial (ETT) em *Drosophila melanogaster*. A BthTX-I sozinha não alterou a taxa de sobrevivência das moscas, mas foi carcinogênica em todas as concentrações testadas (6,7 - 107,5 µg/mL). O pré-tratamento de larvas com DXR seguido por tratamento com água ultrapura ou BthTX-I demonstrou que as maiores concentrações de BthTX-I (13,4, 53,7 e 107,5 µg/mL) reduziram significativamente a frequência de tumores previamente induzidos por DXR. A frequência de tumores observada em larvas pré-tratadas com água ultrapura ou diferentes concentrações de BthTX-I seguida de tratamento com DXR não foi significativamente diferente do controle. Ensaios bioquímicos (FRAP, ORAC e SOD) revelaram que a BthTX-I aumentou a defesa antioxidante das moscas. A análise *in silico* com docking molecular proteína-DNA mostrou uma interação estável entre BthTX-I e DNA. Em conclusão, esses resultados sugerem que os efeitos modulatórios de BthTX-I na carcinogenicidade de DXR podem ser devidos ao reparo por recombinação homóloga, por sua capacidade de induzir apoptose e/ou de reduzir o estresse oxidativo induzido por DXR.

Palavras-chave: Teste de tumor epitelial; ETT; análise *in silico*; Lys49-PLA_2; Miotoxina; Estresse oxidativo.

Carcinogenic and anti-carcinogenic activities of Bothropstoxin-I from *Bothrops jararacussu* in *Drosophila melanogaster*, biochemical assays and protein-DNA molecular docking

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Abstract

Bothropstoxin-I (BthTX-I), a phospholipase A_2-like protein isolated from *Bothrops jararacussu* snake venom has mutagenic, genotoxic, antitumor and apoptotic effects. The aim of this study was to evaluate the carcinogenicity/anticarcinogenicity of BthTX-I alone or in combination with doxorubicin (DXR 0.4 mM) using the Epithelial Tumor Test (ETT) in *Drosophila melanogaster*. BthTX-I alone did not change the survival rate of flies, but it was carcinogenic at all tested concentrations (6.7 - 107.5 µg/mL). Pre-treatment of larvae with DXR followed by treatment with ultrapure water or BthTX-I demonstrated that higher concentrations of BthTX-I (13.4, 53.7 and 107.5 µg/mL) significantly reduced the frequency of tumors previously induced by DXR. The frequency of tumors observed in larvae pre-treated with ultrapure water or different concentrations of BthTX-I followed by treatment with DXR was not significantly different from the control group. Biochemical assays (FRAP, ORAC and SOD) revealed that BthTX-I increased the antioxidant defense of flies. *In silico* analysis with protein-DNA molecular docking showed a stable interaction between BthTX-I and DNA. In conclusion, these results suggest that modulatory effects of BthTX-I on the carcinogenicity of DXR may be due to homologous recombination repair, its capacity to induce apoptosis or/and to reduce DXR-induced oxidative stress.

Keywords: Epithelial Tumor Test; ETT; *in silico* analysis; Lys49-PLA_2; Myotoxin; Oxidative stress.

1. Introduction

Snake venoms are complex mixtures formed mainly of proteins and polypeptides that have different pharmacological activities. Bothropstoxin-I (BthTX-I) is a PLA_2-like protein, a variant containing lysine on carbon 49, isolated from *Bothrops jararacussu* snake venom, which is enzymatically inactive (Homsi-Brandeburgo *et al*. 1998). BthTX-I found in snake venoms has demonstrated cytotoxic effects on cancer cells through induction of apoptosis, cell cycle arrest, suppression of proliferation and antiangiogenic effect (Benati *et al*. 2018, Silva *et al*. 2018, Polloni *et al*. 2021). Due to the greater cytotoxic effects on cancer cells than on normal cells, they can be considered potential chemotherapeutic agents (Hiu and Yap 2020).

Prior studies reported that BthTX-I has mutagenic and genotoxic activities in *Drosophila melanogaster* and human cell lines (Naves *et al*. 2021). This BthTX-I has shown antitumor effects, causing apoptosis, necrosis and cytotoxicity in different cell lines and, consequently, suppression of cell proliferation (Bezerra *et al*. 2019). The BthTX-I also induces cell cycle arrest between the G_0 and G_1 phases and a significant reduction in the size of induced-tumors in rats (Gebrim *et al*. 2009, da Silva *et al*. 2015).

Doxorubicin (DXR), considered as positive control in this study, is an anthracycline antibiotic used in the chemotherapy against numerous solid tumors (Bhagat and Kleinerman 2020, Bagheri *et al*. 2021). DXR binds to double-stranded DNA through intercalation; inhibits the enzyme topoisomerase II, which plays a pivotal role in DNA replication, causing DNA damage resulting in cell death (Lohlamoh *et al*. 2021); inhibits the PI3K/Akt signaling pathway, important in regulating the cell cycle (Fabi *et al*. 2021) and induces severe oxidative stress by mitochondrial ROS generation (Antonucci *et al*. 2021).

Several methods and model organisms have been suggested to reduce the use of animals in scientific research (Doke and Dhawale 2015). Notably, *D. melanogaster* has been recognized as an alternative experimental model for drug and chemical testing, owing to the many basic biological properties that are conserved between mammals and flies, as well as signaling pathways involved in cancer progression. *D. melanogaster* develops very quickly, requires few infrastructures and is easy to be genetically manipulated (Staats *et al*. 2018,

Mirzoyan *et al*. 2019). For these reasons, *D. melanogaster* has been well accepted and widely used as a model organism to assess the toxicity, mutagenicity and carcinogenicity of different compounds (Oliveira *et al*. 2020, Vasconcelos *et al*. 2020, Naves *et al*. 2021).

The Epithelial Tumor Test (ETT) is based on the conservation of tumor suppressor genes between *D. melanogaster* and mammals. One of these genes is the *warts* (*wts*) recessive gene, which has homology to the *LATS1* tumor suppressor gene in mammals. The ETT uses a strain that has the *wts* marker gene, which is located on chromosome 3 of the fly and is lethal to the zygote when in homozygosis (Eeken *et al*. 2002). Therefore, the warts allele is maintained in the stock strain with the presence of a chromosomal balancer (TM3). If heterozygous loss occurs in imaginal disc cells, there will be formation of homozygous clones, which are viable in clusters of cells isolated from the larvae that manifest as epithelial tumors in the adult fly. Indeed, ETT has been successfully used to detect the carcinogenic/anticarcinogenic properties of several chemical compounds (Abou-Eisha and El-Din 2018, de Morais *et al*. 2018, Lima *et al*. 2018, Naves *et al*. 2018, Saturnino *et al*. 2018, Vasconcelos *et al*. 2020, da Silva *et al*. 2021).

The assessment of oxidative stress can provide important data on the mechanism of action of various substances, because it is a process that can play a fundamental role in tumor progression, changing the DNA mutational potential, intracellular signaling pathways, cell proliferation, motility and invasiveness (Fiaschi and Chiarugi 2012). The total antioxidant capacity can be evaluated by the ferric reducing antioxidant power (FRAP) assay, that measures the ability to reduce Fe^{3+} to Fe^{2+} or oxygen radical absorbance capacity (ORAC), that evaluates the absorption capacity of oxygen free radicals (Rodríguez-Bonilla *et al*. 2017). Intracellular defense systems that protect cells from damage induced by reactive oxygen species (ROS) include enzymes such as superoxide dismutase (SOD), which converts superoxide anions into H_2O_2 (Missirlis *et al*. 2001).

For a better understanding of the possibility of binding BthTX-I to DNA, we used the *in silico* protein-DNA molecular docking. The procedure is a key tool to predict the binding of molecules at the binding site of a target protein, therefore, it has been increasingly used as a strategy in drug discovery (Yuriev and Ramsland 2013). This methodology can be carried out with either known or unknown target

proteins, by anchoring against homology-modeled targets. Molecular docking programs perform a search algorithm in which the conformation of the ligand is recursively evaluated until convergence for the minimum energy is reached (Pagadala *et al*. 2017).

The aim of the present study was to evaluate the carcinogenicity of BthTX-I when administered alone or its anticarcinogenicity when administered before or after DXR treatment, through ETT. Additionally, we performed analyzes of an oxidative stress biomarker and total antioxidant capacity, as well as *in silico* investigations with protein-DNA molecular docking.

2. Material and Methods

2.1. Chemical agents

BthTX-I was isolated from *Bothrops jararacussu* snake venom according to the methodology described by Naves *et al*. (2021) and stored at -20 °C. The snakes are kept at the CETA serpentarium, Animal Toxin Extraction Center, Ltda. - CNPJ: 08.972.260/0001-30, Morungaba, SP, Brazil. This serpentarium has obtained proof of IBAMA registration and use of renewable natural resources (number 2087163). This work is registered in the National Management System of Genetic Heritage and Associated Traditional Knowledge under the number A2C2534.

Doxorubicin (DXR - CAS Number 25316-40-9), commercially known as Adriblastina® RD, Batch 5PL5111, obtained from Pfizer - Itapevi, SP, Brazil, was used as a positive control, and ultrapure water (18.2 MΩ), obtained from the MilliQ System (Millipore S.p.A.; Vimodrone (Milano), Italy), as the negative control. The flake-mashed potatoes, used as an alternative culture medium, were obtained from Yoki Hikari® Alimentos S.A. - São Bernardo do Campo, SP, Brazil.

2.2. Epithelial Tumor Test (ETT) of *Drosophila melanogaster*

The ETT was performed by mating multiple wing hairs (mwh) males (genetic constitution: *y; mwh jv*) with virgin females carrying the tumor suppressor gene warts (*wts*) (genetic constitution: *st[1] in[1] kni[ri-1] wts[3-17]/*TM3*, Sb[1]*). This cross produced two types of progeny: marked trans-heterozygous (MH - *wts+/+mwh*), phenotypically with long fine hairs on the chest and balanced heterozygous (BH - TM3, *sb1+/+mwh*) with short thick hairs on the chest (**Fig. 1**).

2.2.1. Treatments

Eggs were collected over 8 h in vials containing a solid agar base (4% agar in water) and a layer of yeast (*Saccharomyces cerevisiae*) supplemented with sucrose.

BthTX-I concentrations (6.7, 13.4, 26.8, 53.7 or 107.5 µg/mL) were selected after performing a survival test, where 100 larvae of 72 ± 4 h were counted before the distribution in treatment vials, containing different concentrations of BthTX-I. The hatched flies were counted and stored in 70% ethanol. The survival tests were carried out only once, without replicates.

Three different treatment groups (A-C) were performed, according to administration protocols (**Fig. 2**). All treatments were conducted in duplicate.

Group A. Third instar larvae (72 ± 4 h) were submitted to chronic treatment (approximately 48 h) with ultrapure water as negative control (NC); doxorubicin (DXR 0.4 mM) as positive control (PC) and different concentrations of BthTX-I (6.7 - 107.5 µg/mL). Larvae treatments were performed in vials containing 1.5 g of alternative culture medium (according to Spanó *et al*. 2001), hydrated with 5 mL of each tested substance.

Group B. Combined treatment 24 - 48 h: Second instar larvae (48 ± 4 h) were submitted to pre-treatment (24 h) with DXR 0.4 mM followed by treatment (approximately 48 h) with ultrapure water or different concentrations of BthTX-I (6.7 - 107.5 µg/mL).

Group C. Combined treatment 24 - 48 h: Second instar larvae (48 ± 4 h) were submitted to pre-treatment (24 h) with ultrapure water or different concentrations of BthTX-I (6.7 - 107.5 µg/mL) followed by treatment (approximately 48 h) with ultrapure water or DXR 0.4 mM.

Larvae pre-treatments were performed in vials containing 1.5 g of alternative culture medium hydrated with 5 mL of each concentration tested. After 24 h (end of pre-treatment period), larvae were resuspended and separated from the alternative culture medium by flotation in a glass beaker containing tap water at room temperature and 20% sucrose. Floating larvae were transferred to vials containing 1.5 g of alternative culture medium hydrated with 5 mL of each concentration tested, and were allowed to feed until the end of larval period (approximately 48 h).

Emerging adults after treatment were collected and stored in flasks containing 70% ethanol. The analysis of individuals was done in a petri dish, with the flies emerging in glycerin, to facilitate the manipulation and visualization of epithelial tumors under a light stereomicroscope. The epithelial tumors can appear in different parts of the fly, including eyes, head, wings, body, legs and halters (**Fig. 3**).

2.2.2. Statistical analysis

Statistical comparisons for survival rates were made by using Chisquare test for ratios for independent samples ($\alpha = 0.05$).

The frequency of each tumor type and the total frequency of tumors for each treatment group were compared pair-wise (i.e.: Group A: DXR *vs.* NC; BthTX-I *vs.* NC. Group B: DXR + water *vs*. water + water; DXR + BthTX-I *vs*. DXR + water. Group C: water + DXR *vs*. water + water; BthTX-I + DXR *vs*. water + DXR) and the possible carcinogenic/anticarcinogenic effects were validated by the nonparametric U test, Mann-Whitney and Wilcoxon, using $\alpha = 0.05$ as the significance level.

2.3. Antioxidant assays

After larval exposure to chronic treatment (approximately 48 h) with ultrapure water as negative control (NC); doxorubicin (DXR 0.4 mM) as positive control (PC) and different concentrations of BthTX-I (6.7 - 107.5 µg/mL), adult flies (50 per treatment group) were euthanized using liquid nitrogen and homogenized in 0.33 mL of potassium phosphate, buffer pH 7.4 and then centrifuged at 2000 × g for 20 min at 4 °C (Liu *et al*. 2015). The supernatant was used to evaluate the oxidative stress parameters.

2.3.1. Ferric-ion reducing antioxidant power (FRAP) assay

Total antioxidant capacity was measured by the ferric-ion reducing antioxidant power (FRAP) assay by using a modification of FRAP assay of Benzie and Strain (1996). The antioxidant molecules present in the homogenate reduce Fe^{3+} to Fe^{2+}, which is chelated by 2,4,6-tris(2-pyridyl)-striazine (TPTZ) to form an Fe^{2+}−TPTZ complex. The fly homogenates were incubated with the FRAP reagent

(10 volumes of 0.3 mol L^{-1} sodium acetate buffer, pH 3.6, 1 volume of 10 mmol L^{-1} TPTZ, and 1 volume of 20 mmol L^{-1} ferric chloride) at 37 °C for 6 min. The absorbance was measured at 593 nm (Molecular Devices, Menlo Park, CA, USA); a blank absorbance value (from wells without fly homogenate) was subtracted from all absorbance values. Antioxidant capacity was determined using an analytical curve, constructed with Trolox as standard.

2.3.2. Oxygen radical absorbance capacity (ORAC) assay

This test consists of fly homogenate and control mixed separately with fluorescein (0.085 nM) and incubated at room temperature for 15 min. After incubation, 153 mM 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was added and fluorescence (485 nmex/528 nmem) was measured at 37 °C during 90 min (Perkin-Elmer LS 55, Massachusetts, USA); the loss of fluorescence was measured by calculating the area under the curve. All reagents were prepared in 75 mM phosphate buffer (pH 7.4). Antioxidant capacity was determined using an analytical curve, constructed with Trolox as standard (Naguib 2000).

2.3.3. Superoxide dismutase (SOD) assay

Superoxide dismutase (SOD) activity was assessed by the inhibition of auto-oxidation of pyrogallol by SOD present in the samples. The fly homogenates were mixed with 50 mmol.L^{-1} Tris-HCl buffer (pH 8.2) containing 1 mmol.L^{-1} EDTA to deactivate metal-dependent enzymes such as metalloproteases, 80 U.mL^{-1} catalase and 24 mmol.L^{-1} pyrogallol. The kinetic assay was monitored during 10 min at 420 nm (Molecular Devices, Menlo Park, CA, USA) using an analytical curve constructed with SOD as standard (Marklund and Marklund 1974).

2.3.4. Statistical Analysis

Data were statistically evaluated by using one-way ANOVA analysis of variance followed by Dunnett's of multiple comparisons to control, with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

2.4. Docking Protein-DNA

The interaction of BthTX-I (PDB:4WTB) with DNA (PDB:1HCQ) was performed by blind docking obtained through the PatchDock server (Schneidman-Duhovny *et al*. 2005), using the parameters predefined by the program. The best protein-DNA complex based on the docking positions was selected by the geometric shape complementarity score defined by PatchDock. The post-docking 3D image of the complex was generated in the CHIMERA program (Pettersen *et al*. 2004), and a 2D diagram of the interface of the interactions between the molecules was generated using the NUCPLOT program (Luscombe *et al*. 1997).

3. Results

3.1. Epithelial Tumor Test (ETT) of *Drosophila melanogaster*

The ETT of *D. melanogaster* was performed to assess the carcinogenic potential of BthTX-I or its anticarcinogenic potential when associated to DXR.

BthTX-I did not cause significant alteration in the survival rate of individuals at any of the concentrations tested, thus indicating that this phospholipase is not toxic to *D. melanogaster*. The survival rates (%) are depicted in **Fig. 4**.

The survival data validated the use of five concentrations (6.7 - 107.5 µg/mL) of BthTX-I alone or in combination with DXR to carry out the ETT. Three different treatment groups (A-C) were performed, according to administration protocols (**Fig. 2**). Only the marked trans-heterozygous (MH - *wts* +/+ *mwh*) adult flies were analyzed because they have the gene of interest. The data of two independent experiments were pooled after verifying that there were no significant differences between repetitions.

Table 1 shows the frequency of tumors found in the different segments of the body of *D. melanogaster* treated with different concentrations of BthTX-I alone (Group A). The total frequency of tumors observed in the MH descendants treated with BthTX-I and DXR (0.4 mM) was statistically significant ($p < 0.05$) when compared to the frequencies observed in the NC.

Table 2 describes the frequency of tumors found in *D. melanogaster* submitted to treatment of Group B. BthTX-I (13.4, 53.7 and 107.5 µg/mL) significantly reduced ($p < 0.05$) the frequency of tumors induced previously by DXR 0.4 mM.

Table 3 exhibits the frequency of tumors found in *D. melanogaster* submitted to treatment of Group C. The total frequency of tumors observed in the MH descendants was not statistically significant ($p > 0.05$) when compared to the frequencies observed in those treated with water, followed by treatment with DXR.

3.2. Biochemical assays

All the biochemical assays were performed with *D. melanogaster* treated with ultrapure water (NC), DXR 0.4 mM (PC) and different concentrations of BthTX-I (6.7 - 107.5 µg/mL).

Total antioxidant capacity measured by the ferric-ion reducing antioxidant power (FRAP) assay in *D. melanogaster* showed that BthTX-I presents significant antioxidant activity (with $p < 0.01$ for BthTX-I 13.4 and 53.7 µg/mL; and $p < 0.05$ for BthTX-I 26.8 and 107.5 µg/mL) (**Fig. 5 A**).

Oxygen radical absorbance capacity (ORAC) assay in *D. melanogaster* revealed that all BthTX-I concentrations presented significant antioxidant activity ($p < 0.001$) (**Fig. 5 B**). The SOD activity in *D. melanogaster* showed that only BthTX-I 26.8 µg/mL presented a significant increase in the activity of SOD enzyme ($p < 0.05$) (**Fig. 5 C**).

3.3. Docking Protein-DNA

The highest PatchDock score of BthTX-I and DNA after blind docking was selected for further analysis (**Fig. 6**) (score 12326). DNA interacts with the two strands of PLA_2. Analysis of the 2D interaction between BthTX-I and DNA (**Fig. 7**) indicates non-covalent interactions between amino acids and nucleotides performed between the A and B chains of BthTX-I and the C and D chains of DNA.

4. Discussion

The toxicity and carcinogenicity of BthTX-I alone or combined with the chemotherapeutic agent DXR was assayed using the Epithelial Tumor Test (ETT) in *D. melanogaster.*

Survival test results demonstrated that BthTX-I was not toxic to *D. melanogaster* in any of the treatments performed, which is in accordance with data from the literature (Naves *et al*. 2021), using the same model organism.

DXR induced increased frequencies of tumors in all parts of the fly (eyes, head, wings, body, legs and halters). DXR induces oxidative stress (Keeney *et al*. 2018) and cell cytotoxicity through induction of DNA double-strand breaks (DSBs) (Fant *et al*. 2021). Recent studies have reported that DSBs are one of the most harmful lesions to a cell and can lead to genomic instability and cancer (Srivastava and Raghavan 2015). The two major repair systems for handling DNA DSBs are homologous recombination and non-homologous end joining repair (Helleday *et al*. 2007). Previous investigations proposed *D. melanogaster* C-terminal binding protein 1 as an end resection factor essential for efficient homology-directed repair (Yannuzzi *et al*. 2021).

BthTX-I alone induced an increase in the frequency of epithelial tumors in *D. melanogaster*, at all concentrations tested, revealing its carcinogenic potential. Combined treatment of DXR 0.4 mM, followed by treatment with BthTX-I indicated that this phospholipase-like protein has the ability to reduce the frequency of DXR-induced tumors, acting on pre-established damage. The effect of decreasing carcinogenic activity of DXR may be due to the antioxidant effect that BthTX-I causes in the fly's body. These data corroborate studies that demonstrate its greater action on tumor cells than on non-tumor cells. BthTX-I and its synthetic cationic peptide derived from the 115-129 C-terminal region exhibited antitumor activity *in vitro* and *in vivo*, being cytotoxic to different tumor cell lines and reducing tumor size when compared to the untreated control group (Araya and Lomonte 2007, Gebrim *et al*. 2009).

Combined treatment of different concentrations of BthTX-I followed by treatment with DXR 0.4 mM had no significant difference from the control, thereby indicating that this phospholipase does not have a preventive action in the formation of tumors in *D. melanogaster*.

Previous studies have reported the antitumor potential of various PLA_2, such as PLA_2 BnSP-6, which induce a higher cytotoxic and genotoxic effects in MDA-MB-231 breast cancer cells than in non-tumorigenic breast cell lines, in addition to inhibiting the proliferation and affecting the cell cycle progression (Silva *et al*. 2018). BthTX-II had antitumor and antimetastatic effects on MDA-MB-231 cells inducing dose-dependent cell death, by apoptosis and autophagy, and decreasing the proliferation and inhibiting cell cycle progression, invasion, migration and 3D cell

growth (Azevedo *et al*. 2019). BnSP-7, a Lys49 PLA_2 from *Bothrops pauloensis* snake venom, was able to inhibit endothelial cell proliferation, adhesion and migration of HUVECs and blocked *in vitro* angiogenic process (Polloni *et al*. 2021).

BthTX-I induced apoptosis and autophagy in MCF7, SKBR3 and MDA-MB-231 breast cancer cell lines in a dose-dependent manner (Bezerra *et al*. 2019). Da Silva *et al*. (2015) also reported antitumor properties of BthTX-I, which showed cytotoxicity by apoptosis in human (HL-60 and HepG2) and murine (PC-12 and B16F10) tumor cell lines; and modulating effects on the cell cycle of PC-12 and B16F10 cells, promoting delay in the G_0/G_1 phase.

In the present study, we found that homogenates of *D. melanogaster* treated with BthTX-I had higher antioxidant capacity, compared to those treated with ultrapure water (NC), as evidenced in FRAP and ORAC assays. These results may indicate that BthTX-I enhances the antioxidant mechanisms of *D. melanogaster* probably through a compensatory mechanism of DNA damage. Moreover, we also observed that high concentrations of BthTX-I can increase the activity of SOD in *D. melanogaster*. These results suggest a possible mechanism underlying the antitumor effect of BthTX-I. The high activity of SOD reduces ROS levels and, consequently, the DNA damage that has been associated with tumor promotion in *D. melanogaster*. In fact, SOD2 knockdown causes susceptibility to oxidative stress increasing adult mortality (Kirby *et al*. 2002), and the lack of SOD1 in *D. melanogaster* mutants leads to specific DNA repair-defective mutations (Woodruff *et al*. 2004). The role of reactive oxygen species (ROS) in tumor development and progression is currently undefined since contradictory effects have been reported in the scientific literature. Recent studies have suggested that ROS may contribute differently to cancer pathogenesis depending on the stage of tumor development (Assi 2017). Although the collaborative effects of ROS on cancer biology still remain unsolved, the oxidative damage to the genetic material of cells is accepted as part of cancer pathophysiology. Indeed, increased levels of 8-oxo-7,8-dihydroguanine (8-oxoGua), the major product resulting from oxidative damage to DNA, were found in the urine of patients with lung and colon cancer (Tudek *et al*. 2010). The activity of antioxidant enzymes is also the target of studies on tumor promotion. In this regard, changes in SOD2 expression have been described in several types of cancer (Hempel *et al*. 2011). However, the association between ROS and

mutagenesis/carcinogenesis in *D. melanogaster* has not been fully evidenced and reports on the subject are very scarce. It has been suggested that extracellular ROS activate hemocytes of *Drosophila* macrophages and in a pathway mediated by JNK and TNF ligand (Eiger), ROS could mediate tumor growth (Fogarty *et al*. 2016).

To better elucidate the interaction between BthTX-I and DNA, we performed a protein-DNA molecular docking, showing the most likely mode of interaction between them. A 2D diagram depicts the hydrogen bonds between Leu31 and Asp58 and the DNA, as well as multiple Van der Waals interactions that can be important in the complex stabilization. Previous researches have demonstrated that molecular docking is extremely efficient to know how a molecule binds to DNA and thus better understanding its mechanism of action (Hage-Melim *et al*. 2009, Gomes *et al*. 2020, Jiang *et al*. 2020, Vasconcelos *et al*. 2020).

5. Conclusions

Under these experimental conditions, we concluded that BthTX-I alone was carcinogenic to *D. melanogaster*. Nevertheless, this phospholipase-like protein showed protective effects when administered after treatment with DXR 0.4 mM, reducing the frequency of tumors induced by DXR, acting on pre-established damage. Biochemical assays demonstrated that BthTX-I increased the antioxidant defense of flies. *In silico* analysis with molecular docking between BthTX-I and DNA showed a stable interaction. Thus, based on the findings from our study and on literature data, we suggest that modulatory effects of BthTX-I may be related to homologous recombination-mediated double-strand break repair; its capacity to induce apoptosis, as well as its antioxidant effect, capable of reducing the oxidative stress induced by DXR. Further studies should be carried out to clarify the possible antitumor mechanism of action of BthTX-I.

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7. Conflict of Interests

No potential competing interest was reported by the authors.

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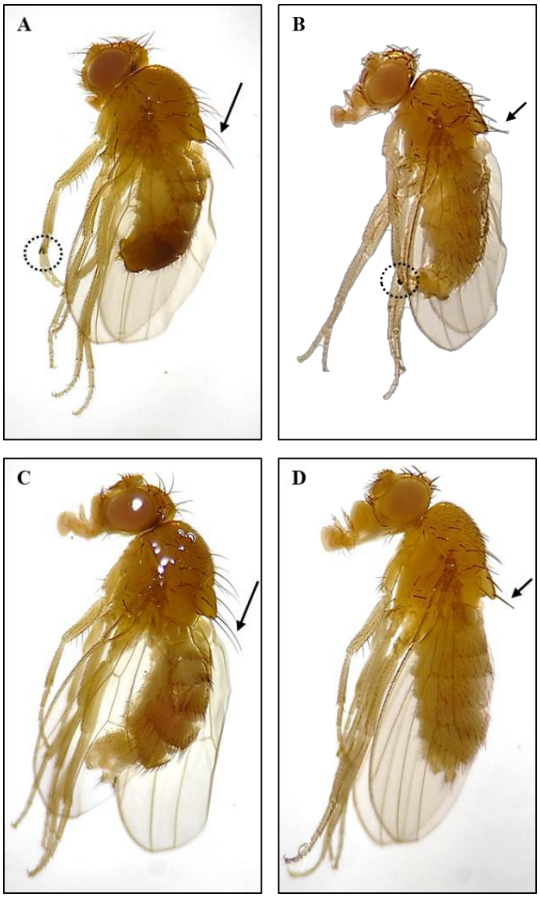


Fig. 1. Photomicrographs of *D. melanogaster* obtained by light stereomicroscope at 25X magnification, showing long fine hairs on the chest (long arrows) and short thick hairs on the chest (short arrows), that differentiate, respectively, the marked trans-heterozygous (MH) individuals from the balanced heterozygous (BH) ones. (A) MH male; (B) BH male; (C) MH female; (D) BH female. Dashed circle indicates the sex comb, exclusive to males.

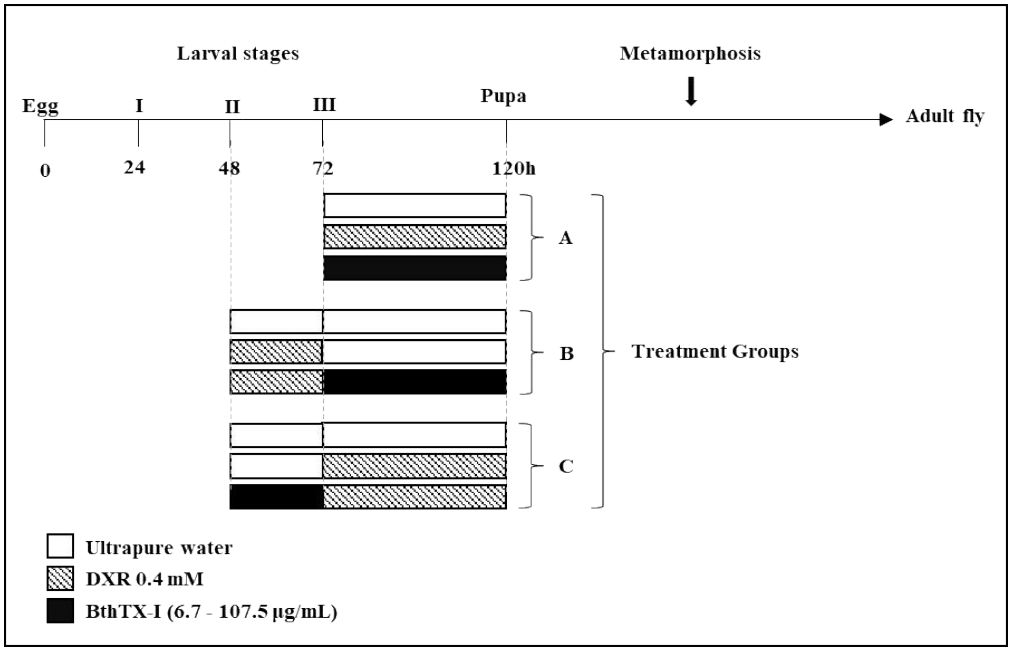


Fig. 2. Administration protocols for feeding larvae of the Epithelial Tumor Test (ETT) in *D. melanogaster*.

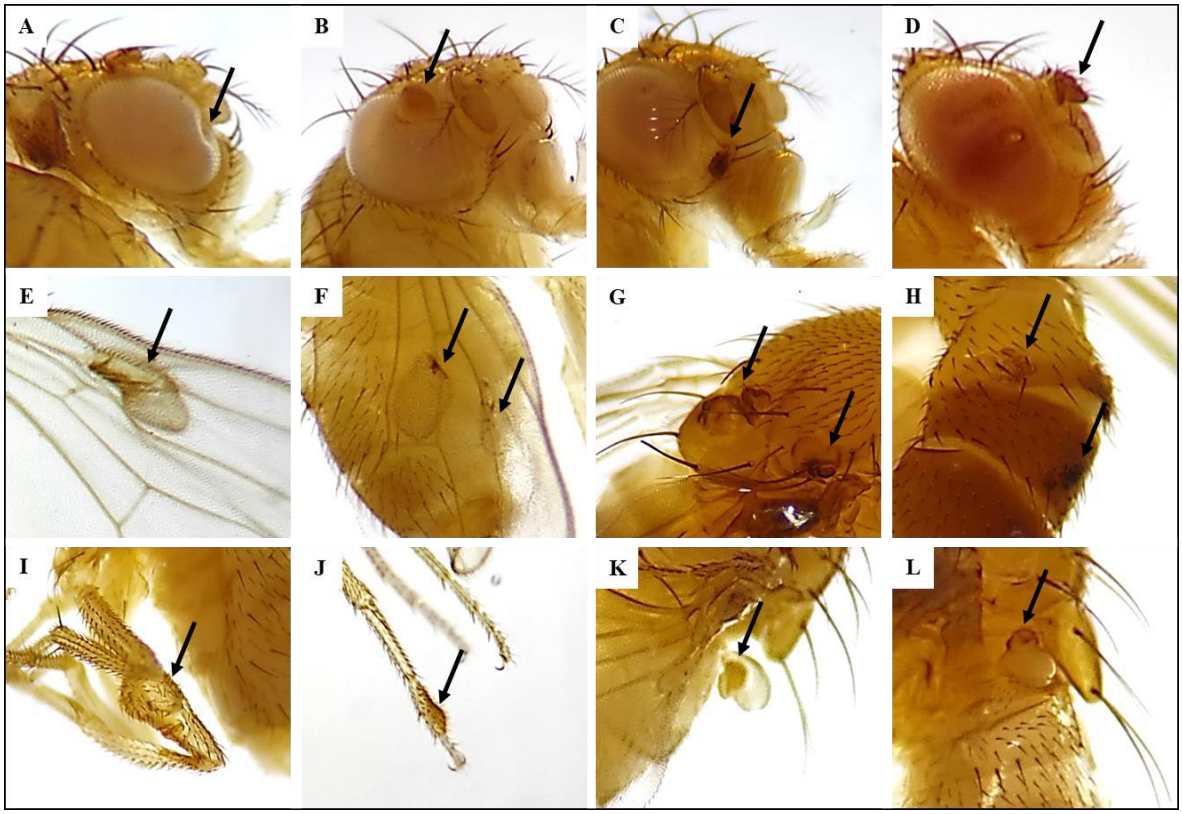
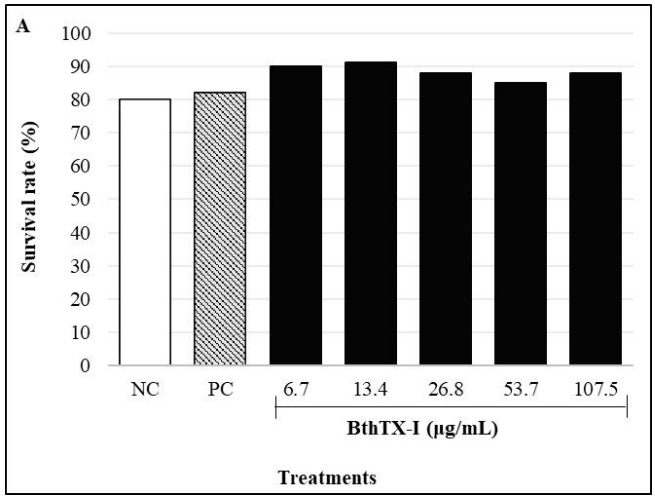
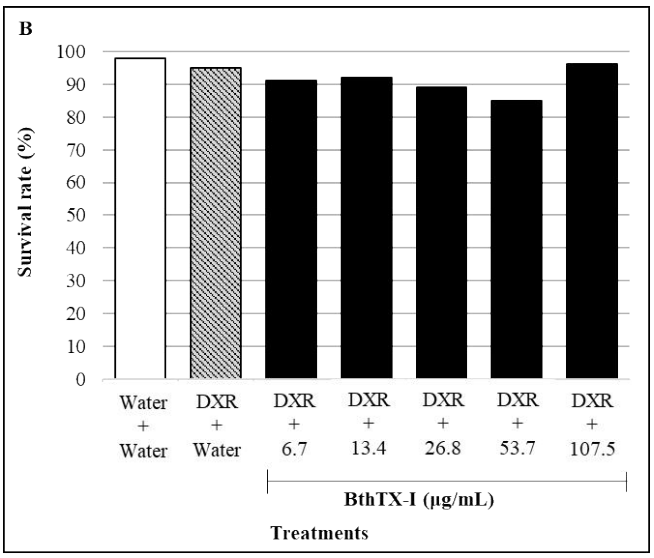


Fig. 3. Photomicrographs of *D. melanogaster* obtained by light stereomicroscope at 25x magnification, showing epithelial tumors (arrows) in different parts of the fly: (A-B) eyes; (C-D) head; (E-F) wings; (G-H) body; (I-J) legs; (K-L) halters.


A
Survival rate (%)
100
90
80
70
60
50
40
30
20
10
0
NC
PC
6.7
13.4
26.8
53.7
107.5
BthTX-I (µg/mL)
Treatments


B
Survival rate (%)
100
90
80
70
60
50
40
30
20
10
0
Water + Water
DXR + Water
DXR + 6.7
DXR + 13.4
DXR + 26.8
DXR + 53.7
DXR + 107.5
BthTX-I (µg/mL)
Treatments

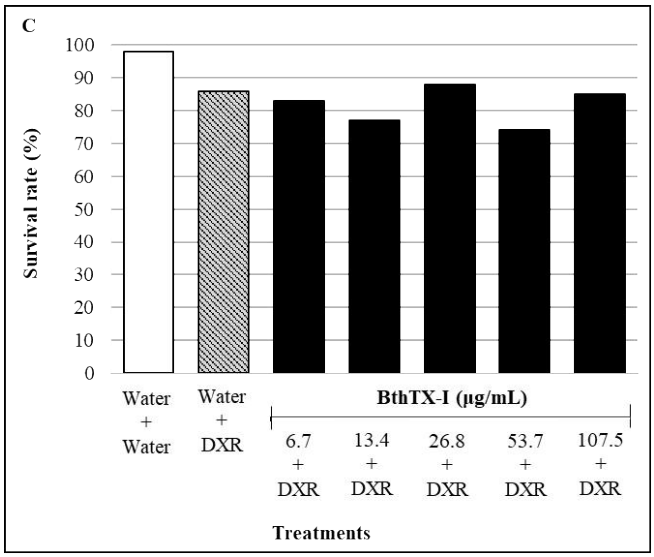

C
Survival rate (%)
100
90
80
70
60
50
40
30
20
10
0
Water + Water
Water + DXR
BthTX-I (µg/mL)
6.7 + DXR
13.4 + DXR
26.8 + DXR
53.7 + DXR
107.5 + DXR
Treatments

Fig. 4. Survival rates (%) of individuals from Epithelial Tumor Test (ETT), (A) upon exposure to different concentrations of BthTX-I (6.7 - 107.5 µg/mL), ultrapure water as negative control (NC) and doxorubicin (DXR 0.4 mM) as positive control (PC); (B) upon exposure of larvae to pre-treatment (approximately 24 h) with ultrapure water or DXR 0.4 mM followed by treatment (approximately 48 h) with ultrapure water, DXR 0.4 mM or different concentrations of BthTX-I (6.7 - 107.5 µg/mL); (C) upon exposure of larvae to pre-treatment (approximately 24 h) with ultrapure water, DXR 0.4 mM or different concentrations of BthTX-I (6.7 - 107.5 µg/mL) followed by treatment (approximately 48 h) with ultrapure water or DXR 0.4 mM. Data are representative of survival tests performed only once, without replicate. Statistical comparisons were made by using Chisquare test for ratios for independent samples ($\alpha = 0.05$).

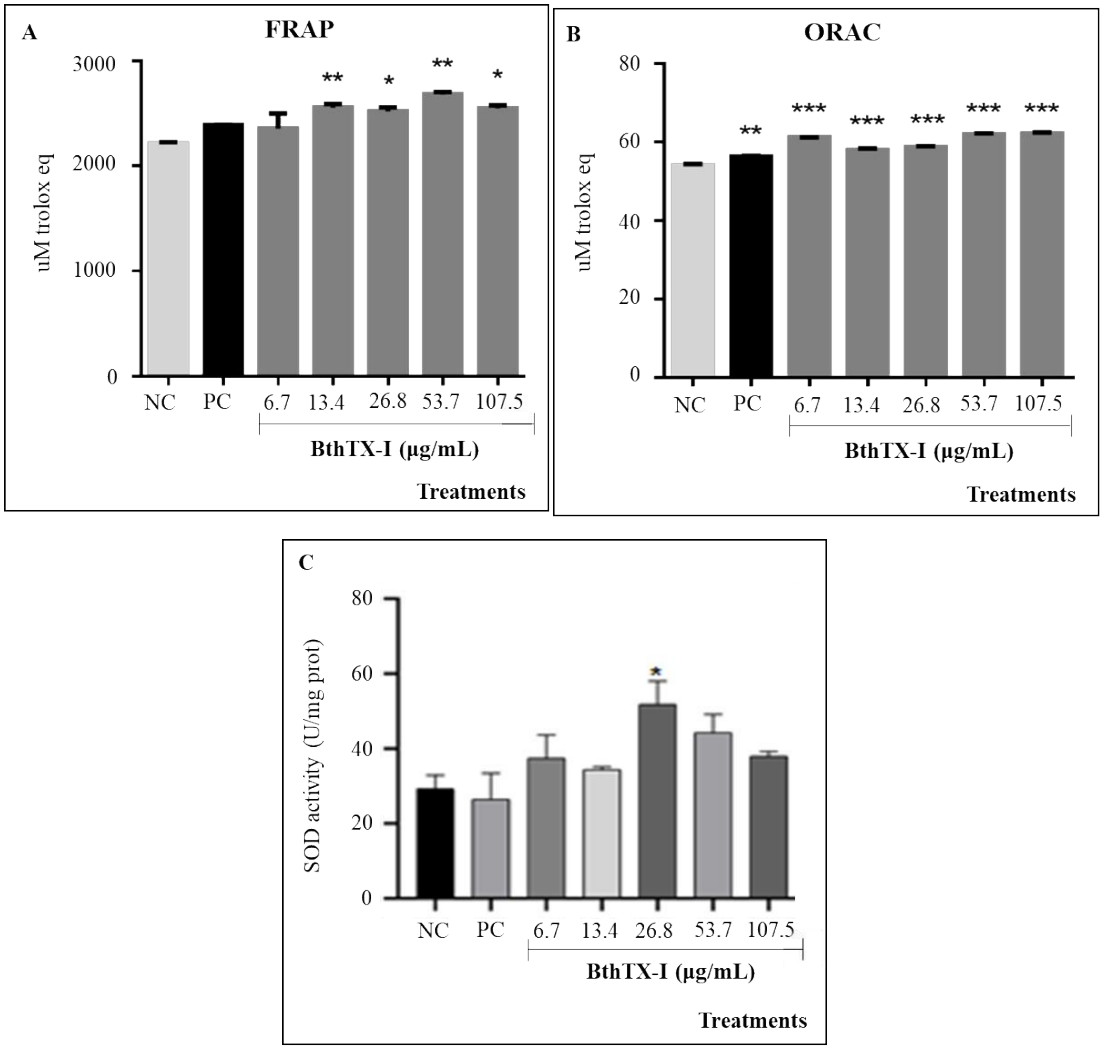


Fig. 5. Analysis of biomarker of oxidative stress and total antioxidant capacity in *D. melanogaster*: (A) FRAP assay; (B) ORAC assay; (C) Superoxide dismutase - SOD. Groups: ultrapure water as negative control (NC), doxorubicin (DXR 0.4 mM) as positive control (PC) and different concentrations of BthTX-I (6.7 - 107.5 µg/mL). *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (One-way ANOVA followed by Dunnett's of multiple comparisons to control).

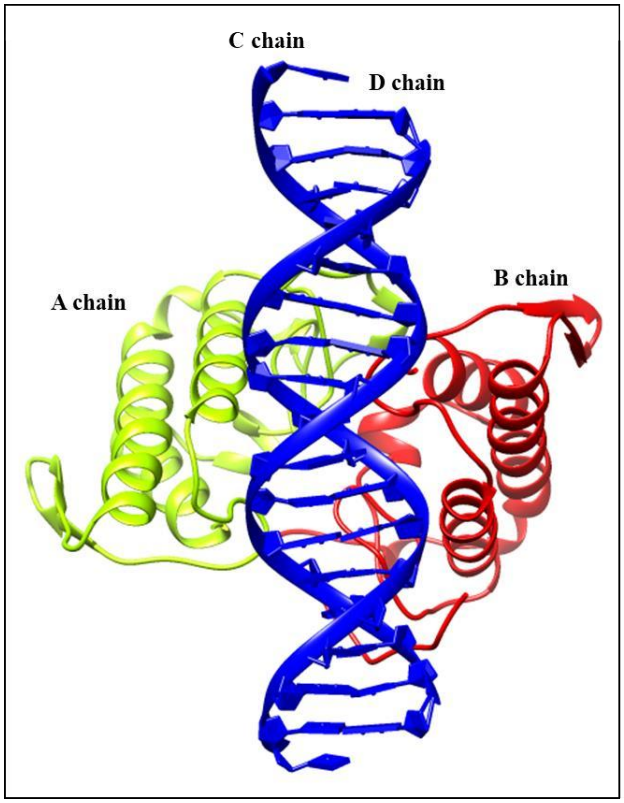


Fig. 6. Complex obtained during protein-DNA molecular docking presenting the BthTX-I of *B. jararacussu* composed of A chain (green), B chain (red) and DNA (C and D chains in blue).

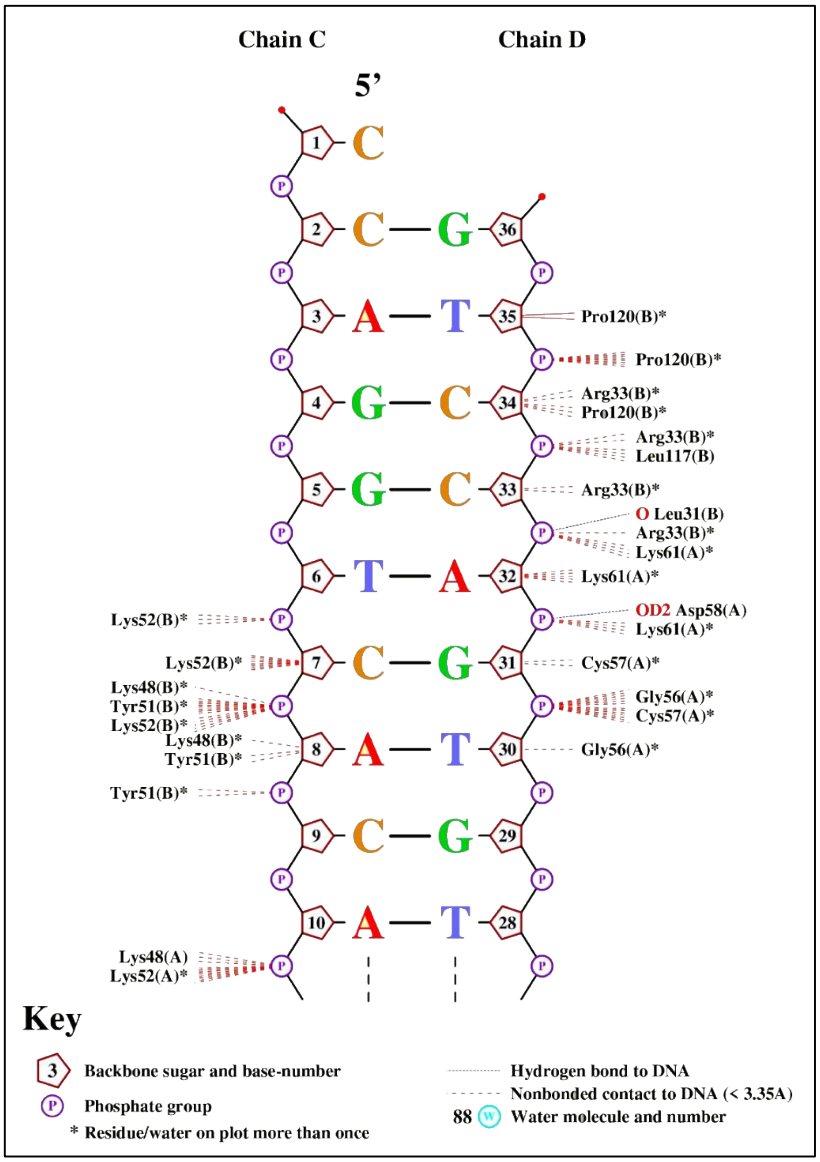


Fig. 7. 2D diagram of the interaction of BthTX-I with DNA.

Table 1. Summary of results obtained with the *D. melanogaster* Epithelial Tumor Test (ETT) after chronic treatment of larvae with ultrapure water as negative control, doxorubicin (DXR 0.4 mM) as positive control and different concentrations of BthTX-I (6.7 - 107.5 µg/mL)

Treatments	**Nº of flies**	**Frequency of tumors**						
		Eyes	**Head**	**Wings**	**Body**	**Legs**	**Halters**	**Total tumors**
Water	200	0.020 (04)	0.030 (06)	0.030 (06)	0.175 (35)	0.005 (01)	0.000 (00)	0.260 (52)
DXR	171	0.509 (87)*	0.152 (26)*	0.930 (159)*	0.830 (142)*	0.509 (87)*	0.140 (24)*	3.070 (525)*
BthTX-I								
6.7	200	0.075 (15)*	0.095 (19)*	0.060 (12)	0.345 (69)*	0.055 (11)*	0.010 (02)	0.640 (128)*
13.4	200	0.045 (09)	0.065 (13)	0.080 (16)*	0.290 (58)*	0.060 (12)*	0.005 (01)	0.545 (109)*
26.8	200	0.040 (08)	0.085 (17)*	0.065 (13)	0.320 (64)*	0.105 (21)*	0.010 (02)	0.620 (124)*
53.7	167	0.060 (10)*	0.060 (10)	0.054 (09)	0.263 (44)	0.036 (06)*	0.000 (00)	0.473 (079)*
107.5	200	0.070 (14)*	0.090 (18)*	0.050 (10)	0.295 (59)*	0.035 (07)*	0.005 (01)	0.545 (109)*

Statistical diagnoses according to the Mann-Whitney test. Significance level ($p < 0.05$).

* Values considered different from the negative control (ultrapure water).

Table 2. Summary of results obtained with the *D. melanogaster* Epithelial Tumor Test (ETT) after pre-treatment of larvae with ultrapure water or doxorubicin (DXR 0.4 mM) and treatment with ultrapure water or different concentrations of BthTX-I (6.7 - 107.5 µg/mL)

Pre-treatments	Treatments	Nº of flies	Frequency of tumors						
			Eyes	Head	Wings	Body	Legs	Halters	Total tumors
Water	**Water**	200	0.065 (13)	0.050 (10)	0.040 (08)	0.295 (59)	0.035 (07)	0.005 (01)	0.490 (98)
DXR	**Water**	200	1.100 (220)[a]	0.845 (169)[a]	1.060 (212)[a]	1.210 (242)[a]	0.730 (146)[a]	0.260 (52)[a]	5.205 (1041)[a]
	BthTX-I								
DXR	6.7	200	1.105 (221)	0.850 (170)	1.025 (205)	1.135 (227)	0.530 (106)	0.130 (26)*	4.775 (955)
DXR	13.4	200	0.965 (193)	0.605 (121)*	0.925 (185)	0.895 (179)*	0.570 (114)	0.165 (33)	4.125 (825)*
DXR	26.8	200	1.130 (226)	0.900 (180)	0.985 (197)	1.055 (211)	0.660 (132)	0.205 (41)	4.938 (987)
DXR	53.7	200	0.705 (141)*	0.545 (109)*	0.760 (152)*	0.785 (157)*	0.390 (78)*	0.145 (29)	3.330 (666)*
DXR	107.5	200	0.860 (172)*	0.515 (103)*	0.775 (155)*	0.925 (185)*	0.515 (103)	0.150 (30)	3.740 (748)*

Statistical diagnoses according to the Mann-Whitney test. Significance level ($p < 0.05$).

[a] Values considered different from the Water + Water.

* Values considered different from the DXR 0.4 mM + Water.

Table 3. Summary of results obtained with the *D. melanogaster* Epithelial Tumor Test (ETT) after pre-treatment of larvae with ultrapure water or different concentrations of BthTX-I (6.7 - 107.5 µg/mL) and treatment with ultrapure water or doxorubicin (DXR 0.4 mM)

Pre-treatments	**Treatments**	**Nº of flies**	**Frequency of tumors**						
			Eyes	**Head**	**Wings**	**Body**	**Legs**	**Halters**	**Total tumors**
Water	**Water**	200	0.065 (13)	0.050 (10)	0.040 (08)	0.295 (59)	0.035 (07)	0.005 (01)	0.490 (98)
Water	**DXR**	200	0.130 (26)[a]	0.085 (17)	0.110 (22)[a]	0.305 (61)	0.100 (20)[a]	0.015 (03)	0.745 (149)[a]
BthTX-I									
6.7	**DXR**	200	0.175 (35)	0.080 (16)	0.100 (20)	0.350 (70)	0.075 (15)	0.045 (09)	0.825 (165)
13.4	**DXR**	200	0.240 (48)	0.085 (17)	0.170 (34)	0.330 (66)	0.055 (11)	0.010 (02)	0.890 (178)
26.8	**DXR**	200	0.180 (36)	0.080 (16)	0.165 (33)	0.360 (72)	0.080 (16)	0.030 (06)	0.895 (179)
53.7	**DXR**	200	0.205 (41)	0.080 (16)	0.185 (37)	0.325 (65)	0.095 (19)	0.025 (05)	0.915 (183)
107.5	**DXR**	200	0.105 (21)	0.100 (20)	0.115 (23)	0.360 (72)	0.120 (24)	0.010 (02)	0.810 (162)

Statistical diagnoses according to the Mann-Whitney test. Significance level ($p < 0.05$).

[a] Values considered different from the Water + Water.

* Values considered different from the Water + DXR 0.4 mM.

Conclusões Gerais

Conclusões gerais

Nas condições experimentais utilizadas no presente estudo, concluímos que a BthTX-I não alterou a taxa de sobrevivência da *D. melanogaster* em nenhum dos testes realizados.

A BthTX-I foi recombinogênica na maior concentração do cruzamento padrão e mutagênica/recombinogênica em concentrações intermediárias do cruzamento de alta ativação metabólica do SMART de asa de *D. melanogaster*.

A BthTX-I sozinha foi carcinogênica para *D. melanogaster* em todas as concentrações testadas.

Quando a BthTX-I foi administrada após o tratamento com DXR 0,4 mM, reduziu a frequência de tumores induzidos pela doxorrubicina.

BthTX-I administrada antes da DXR, não causou alteração na frequência de tumores nas moscas, não apresentando efeito na prevenção de tumores.

Essa toxina causou diminuição na viabilidade das células HUVEC e DU-145, além de ter sido genotóxica para essas células.

Os ensaios bioquímicos FRAP, ORAC e SOD, demonstraram que a BthTX-I aumentou a defesa antioxidante das moscas.

A análise *in silico* com docking molecular entre BthTX-I e DNA mostrou uma interação estável.

Assim, com base nos achados do nosso estudo e em dados da literatura, sugerimos que os efeitos modulatórios da BthTX-I podem estar relacionados ao reparo de quebra de fita dupla mediado por recombinação homóloga; sua capacidade de induzir apoptose, bem como seu efeito antioxidante, capaz de reduzir o estresse oxidativo induzido por DXR. Mais estudos devem ser realizados para esclarecer o possível mecanismo de ação antitumoral do BthTX-I.

Apêndice

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Mutagenic and genotoxic activities of Phospholipase A_2 Bothropstoxin-I from *Bothrops jararacussu* in *Drosophila melanogaster* and human cell lines

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ABSTRACT

Phospholipase A_2 Bothropstoxin-I (PLA_2 BthTX-I) is a myotoxic Lys49-PLA_2 from *Bothrops jararacussu* snake venom. In order to evaluate the DNA damage caused by BthTX-I, we used the Somatic Mutation and Recombination Test (SMART) in *Drosophila melanogaster* and Comet assay in HUVEC and DU-145 cells. For SMART, different concentrations of BthTX-I (6.72 to 430 μg/mL) were used and no significant changes in the survival rate were observed. Significant frequency of mutant spots was observed for the ST cross at the highest concentration of BthTX-I due to recombinogenic activity. In the HB cross, BthTX-I increased the number of mutant spots at intermediate concentrations, being 53.75 μg/mL highly mutagenic and 107.5 μg/mL predominantly recombinogenic. The highest concentrations were neither mutagenic nor recombinogenic, which could indicate cytotoxicity in the wing cells of *D. melanogaster*. *In vitro*, all BthTX-I concentrations (1 to 50 μg/mL) induced decrease in HUVEC cell viability, as well as in DU-145 cells at concentrations of 10, 25, and 50 μg/mL. The comet assay showed that in HUVEC and DU-145 cells, all BthTX-I concentrations promoted increase of DNA damage. Further studies should be performed to elucidate the mechanism of action of PLA_2 BthTX-I and its possible use in therapeutic strategies against cancer.

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