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VINÍCIUS QUEIROZ OLIVEIRA

EFEITO ANTITUMORAL E ANTIANGIOGÊNICO DA BthMP, UMA METALOPROTEASE ISOLADA DA PEÇONHA DE *Bothrops moojeni*

Vitória da Conquista, BA

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Dissertação apresentada ao Programa de Pós-Graduação em Biociências, Universidade Federal da Bahia, como requisito para obtenção do título de Mestre em Biociências.

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Esta dissertação foi julgada adequada à obtenção do grau de Mestre em Biociências e aprovada em sua forma final pelo Programa de Pós-graduação em Biociências, Universidade Federal da Bahia. Vitória da Conquista – BA, 2022.

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(Instituto Butantan)

Para todos os curiosos que buscam na ciência uma forma de encontrar soluções para transformar o mundo.

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"Palavras são, na minha nada humilde
opinião, nossa inesgotável fonte de
magia."

(Alvo Dumbledore)

RESUMO

O câncer de pulmão é segundo tipo de câncer mais prevalente e o primeiro em número de óbitos em todo o mundo. Neste contexto, diversos estudos têm demonstrado que toxinas isoladas da peçonha de serpentes vêm sendo investigadas com o objetivo de desenvolver futuros fármacos para terapia contra o câncer. Assim, avaliamos os efeitos antitumorais e antiangiogênicos da metaloprotease da classe PI, BthMP, isolada do veneno de *Bothrops moojeni* em células de câncer de pulmão e em células endoteliais da veia umbilical humana (HUVEC). A BthMP foi citotóxica para células de câncer de pulmão (A549) apresentando inibição da formação de colônias pelo ensaio clonogênico e aumento dos níveis de LDH nas concentrações de 40 e 5μg/mL. Além disso, nessas mesmas concentrações, a BthMP inibiu a invasão, migração e adesão das células A549 além de aumentar os níveis de espécies reativas de oxigênio e óxido nítrico. Interessantemente a toxina não interferiu nos processos de citotoxicidade e adesão das células de pulmão não tumorigênica (BEAS-2B). A BthMP inibiu a adesão e migração de HUVECs e inibiu a angiogênese *in vitro* de forma dependente de VEGF, um fator pró-angiogênico essencial. Além disso, a toxina foi capaz de inibir o processo angiogênico *ex vivo* no modelo de anel aórtico. Por fim, foi possível observar, que a inativação da atividade catalítica da toxina aboliu a inibição da migração das células A549, demonstrando que a atividade antitumoral da metaloprotease está associada à sua atividade catalítica. Portanto, esses resultados demostram que a toxina BthMP tem um efeito antitumoral e antiangiogênico significativo no câncer de pulmão e em células endoteliais e representa uma importante ferramenta biotecnológica visando uma nova forma de terapia contra o câncer.

Palavras-chave: Peçonha de serpente. Metaloprotease. *Bothrops moojeni.* Câncer. Angiogênese.

ABSTRACT

Lung cancer is the second most prevalent type of cancer and the first in number of deaths worldwide. In this context, several studies have shown that toxins isolated from snake venom have been investigated with the aim of developing future drugs for cancer therapy. Thus, we evaluated the antitumor and antiangiogenic effects of BthMP, a PI metalloprotease isolated from *Bothrops moojeni* venom on lung cancer cells and Human Umbilical Vein Endothelial Cells (HUVEC). BthMP was cytotoxic to lung cancer cells (A549) showing inhibition of colony formation by the clonogenic assay and increased levels of LDH at concentrations of 40 and 5µg/mL. Furthermore, at these same concentrations, BthMP inhibited the invasion, migration and adhesion of A549 cells in addition to increasing the levels of reactive oxygen and nitric oxide species. Interestingly, the toxin did not interfere in the cytotoxicity and adhesion processes of non-tumorigenic lung cells (Beas) BthMP inhibited the adhesion, migration of HUVECs and angiogenesis *in vitro* dependent on VEGF, an essential pro-angiogenic factor. In addition, the toxin was able to inhibit the angiogenic process through an ex vivo assay of the aortic ring. Finally, it was possible to observe that the inactivation of the catalytic activity of the toxin abolished the inhibition of the migration of A549 cells induced by the metalloprotease, demonstrating that the antitumor activity of the metalloprotease is associated with its catalytic activity. Therefore, these results demonstrate that BthMP toxin has a significant antitumor and antiangiogenic effect on lung cancer and endothelial cells and represents an important biotechnological tool targeting a new form of cancer therapy.

Key words: Snake venom. Cancer. Angiogenesis. Metalloproteinase. *Bothrops Moojeni.*

Sumário

LISTA DE SIGLAS

Globocan - Global Cancer Statistics
INCA - Instituto Nacional do Câncer
NSCLC - Non-small-cell lung cancer
SCLC - small-cell lubg carcinoma
TEP - Transição epitélio mesenquimal
FAC - Fibroblastos associados ao câncer
VEGF - Fator de crescimento endotelial vascular
FGF - Fator de crescimento de fibroblasto
PIGF - Fator de crescimento placentário
EGF - Fator de crescimento epidérmico
TGF-β - Fator transformador de crescimento-β
NRP-1 - Neuropilin-1
MEPS - Metaloendopeptidases
MMP - Metaloproteases de matriz
ADAMs - A desintegrin and metalloproteinase
ADAMTSs - A desintegrin and metalloproteinase with trombospodin-like
SVMP - Snake venom metalloproteinase
LDH - Lactato desidrogenase
ANGPT1 - Angiopoetina 1
SFLT-1 - Soluble fms-like tyrosine kinase-1
MEC - Matriz extracelular
SFB - Soro fetal bovino
bFGF - Fator de crescimento fibroblástico básico
RPMI - Roswell park memorial institute
DNA - Ácido desoxirribonucleico
cDNA - Ácido desoxirribonucleico recombinante
GAPDH - Gliceraldeído 3-fosfato desidrogenase
NO - Óxido nítrico
ROS - Espécies reativas de oxigênio
DCF - Diclorofluoresceína
EDTA - Ácido etilenodiamino tetra-acético
ATCC - American type culture collection
HUVEC - Célula endotelial de veia umbilical humana
SDS - Dodecil sulfato de sódio
RNA - Ácido ribonucleico
RAEC - Linhagem celular de anel ártico de coelho
VEGFR - Receptor do fator de crescimento vascular endotelial
NO-cGMP - Óxido nítrico guanosina 3', 5' monofosfato cíclico
LDL - Lipoproteina de baixa densidade

LISTA DE FIGURAS

1. INTRODUÇÃO

De acordo com o GLOBOCAN, em 2020, o câncer de pulmão foi o segundo tipo de câncer com mais casos e o primeiro em número de mortes no mundo, representando 11,4% dos casos e 18% das mortes por câncer (SUNG, *et al.*, 2021). Para o Brasil, a estimativa do Instituto Nacional de Câncer José Alencar Gomes da Silva (INCA) para o triênio 2020-2022, é de 30 mil casos no país, correspondendo a 4,8% do número total de casos previstos.

O câncer é caracterizado pela divisão descontrolada de células, que fogem dos mecanismos de controle do ciclo celular e da apoptose, invadindo outros tecidos, promovendo angiogênese e metástases (HANNAHAN & WEINBERG, 2011). A metástase é um processo responsável por mais de 90% da mortalidade por câncer e a inibição dos processos angiogênicos e metastáticos é um dos alvos das atuais terapias anticâncer. Por exemplo, o Bevacizumab, usado na quimioterapia do câncer de pulmão, é um anticorpo monoclonal que bloqueia a ação do VEGF (fator de crescimento endotelial vascular), impedindo o crescimento de vasos sanguíneos no microambiente tumoral (PRESTA, 1997; WAN *et al.*, 2019; RECK *et al.*, 2020).

Nos últimos anos, diversos estudos demonstram o uso de toxinas isoladas da peçonha de serpente como ferramentas para descoberta de novas drogas para o tratamento do câncer. Neste contexto, as metaloproteases isoladas da peçonha de serpentes (SVMPs), responsáveis por causar danos locais e sistêmicos após o envenenamento, são moléculas com alto valor biológico e seu efeito antitumoral e antiangiogênico vem sendo estudado ao longo dos anos (GABRIEL *et al.*, 2012; GUIMARÃES *et al.*, 2017). Contudo, o efeito antimetástatico e antiangiogênico dessa classe de toxinas sobre câncer de pulmão ainda precisa ser melhor investigado.

Assim, o presente estudo visa, a partir do uso de diferentes técnicas experimentais *in vitro* e *ex vivo*, avaliar as ações antitumorais e antiangiogênicas da metaloprotease de classe P-I BthMP,isolada da peçonha de *Bothrops moojeni*, em células de câncer de pulmão e células endoteliais.

2. REVISÃO DE LITERATURA

2.1 Câncer

2.11 Câncer de pulmão: aspectos epidemiológicos e características gerais

Segundo o *Global Cancer Statistics* (GLOBOCAN), órgão da Agência Internacional de Pesquisa ao Câncer, em 2019, o câncer era a principal causa de morte do mundo. De acordo com o estudo, os casos de câncer ocuparam o primeiro ou segundo lugar em pessoas com menos de 70 anos em 112 dos 183 países do mundo, além de ocupar terceiro ou quarto lugar em mais 23 países nessa mesma faixa de idade (SUNG *et al.*, 2021).

De acordo com o GLOBOCAN 2020, foram diagnosticados 19,3 milhões de novos caso de câncer mundialmente, sendo o principal deles o câncer de mama (2,2 milhões de casos – 11,7%), seguido de pulmão (2,20 milhões – 11,4%), próstata, (1,41 milhão – 7,3%) e pele não melanoma (1,19 milhão – 6,2%) (SUNG *et al.*, 2021). No que se refere à taxa de mortalidade, o câncer de pulmão é o que mais leva a óbito no mundo (1,79 milhão de mortes – 18%), seguido pelo câncer de fígado (830 mil – 8,3%), estômago (768 mil – 7,7%), mama (684 mil – 6,9%) e cólon (576 mil – 5,8%) (SUNG *et al.*, 2021).

No Brasil, a estimativa do Instituto Nacional de Câncer José Alencar Gomes da Silva (INCA) para o triênio 2020-2022, uma incidência total de 625 mil casos no país, sendo o câncer de pele não melanoma o mais comum (177 mil casos), seguido pelo câncer de mama e próstata (66 mil casos), cólon e reto (41 mil casos) e pulmão (30 mil casos). Em termos de mortalidade, o câncer de pulmão apresenta taxa crescente. Em 2015, a doença foi responsável por 26 mil mortes e em 2020 gerou mais de 28 mil mortes, frente a uma estimativa de 30 mil novos casos (INCA, 2020).

Esse aumento na incidência e na mortalidade de cânceres, em geral, se associa com o envelhecimento e o crescimento populacional, bem como com a distribuição e prevalência dos principais fatores de risco relacionados com a doença (SUNG *et al.*, 2021). O desenvolvimento de cânceres pode depender da exposição do indivíduo a uma série de fatores intrínsecos e/ou extrínsecos que podem levar a uma tendência de aparecimento da doença. Os agentes promotores extrínsecos, são associados a mais de 80% dos cânceres existentes, por meio de exposição à fumaça de cigarro, consumo excessivo de bebidas

alcoólicas, exposição frequente ao sol, radiação e inatividade física e os agentes intrínsecos correspondem a disfunções hormonais, condições imunológicas ou mutações. (WHITEMAN & WILSON, 2016).

Em relação ao câncer de pulmão, o tabagismo é o principal fator de risco associado, além do fumo passivo que afeta de 33 a 35% de pessoas não-fumantes, aumentando de 20 a 30% o risco de desenvolvimento de câncer de pulmão. Mais recentemente, o aumento no uso de cigarros eletrônicos e "vapes" e com estudos demonstrando que a fumaça dos aparelhos induz padrões semelhante ao do cigarro, demonstram também uma condição de aumento no risco de desenvolver esse tipo de doença (PARK *et al.*, 2014; BARTA *et al.*, 2019). Além do tabagismo, há ainda a exposição à poluição do ar, doenças pulmonares, alimentação e os fatores genéticos (BARTA *et al.*, 2019).

O câncer de pulmão pode ser classificado em dois tipos diferentes, de acordo com a histologia do tecido: 1) o câncer de pulmão de células não pequenas (*Non-small-cell lung cancer* – NSCLC) e 2) o câncer de pulmão de pequenas células (*smal-cell lung carcinoma* – SCLC) (BRENNAN *et al.*, 2011).

Os NSCLC podem ser divididos em três grupos: 1) carcinoma de células escamosas, localizado centralmente e derivados de células epiteliais dos brônquios, está também associado ao histórico de tabagismo e à expressão de queratina em alto peso molecular; 2) adenocarcinoma, que são tumores epiteliais malignos derivados de células epiteliais alveolares, brônquicas ou bronquiolares os quais metastizam principalmente por mecanismos linfáticos, podendo normalmente alcançar cérebro, ossos, glândulas adrenais e fígado; 3) carcinoma de células grandes, que constituem 5% dos casos de cânceres de pulmão, não mostrando evidência de diferenciação glandular (LANGER *et al.*, 2010; BRENNAN *et al.*, 2011).

O SCLC compreende a forma mais agressiva de câncer de pulmão, correspondendo a 14% dos casos nos EUA, com aproximadamente 31 mil casos anuais. O desenvolvimento do SCLC está relacionado à exposição ao tabaco na maioria dos casos, combinado com mutação no gene TP53, promovendo a progressão de uma doença agressiva e molecularmente complexa (BYERS & RUDIN, 2015).

O tratamento para o câncer de pulmão geralmente envolve a administração simultânea de mais de uma abordagem, como radioterapia e

quimioterapia, através da radioquimioterapia, podendo também ocorrer pré-cirurgia (terapia neoadujvante) ou pós-cirurgia (terapia adjuvante) (HAMMERSCHMIDT & WIRTZ, 2009).

2.2 Tumorigênese

A maioria das células segue um ciclo tradicional de vida, crescendo, se proliferando e morrendo de maneira ordenada, porém essa sequência pode não ocorrer com células cancerosas. As células cancerosas, ou neoplásicas, ao invés de morrerem, perdem a capacidade de controlar a sua proliferação, produzindo novas células de maneira rápida, agressiva e descontrolada, podendo se espalhar para outras áreas do corpo. Essas células podem gerar um tumor benigno, formado por células semelhantes às do tecido, de crescimento progressivo e que não gera metástase ou tumor maligno, formado por células diferentes das do tecido normal, de crescimento rápido e que gera metástase mais frequentemente (HANAHAN & WEINBERG, 2011; FOUAD & AANEI, 2017).

O processo de formação do câncer é denominado carcinogênese ou oncogênese, é lento e muitas vezes ocorre por efeito cumulativo de diferentes agentes cancerígenos. O processo oncogênico ocorre em três etapas, o estágio de iniciação, onde ocorre a ação dos agentes cancerígenos nos genes, o estágio de promoção, no qual os agentes oncopromotores irão atuar na célula já alterada e o estágio de progressão, que é o momento em que ocorre a multiplicação descontrolada das células (HANAHAN & WEINBERG, 2011; FOUAD & AANEI, 2017). As principais características das células tumorais (Figura 1), são as de resistir à morte celular, ser capaz de evitar os supressores de crescimento, sustentar a sinalização proliferativa, permitir a replicação imortal, ativar mecanismos de invasão, metástase e angiogênese (HANAHAN & WEINBERG, 2011).
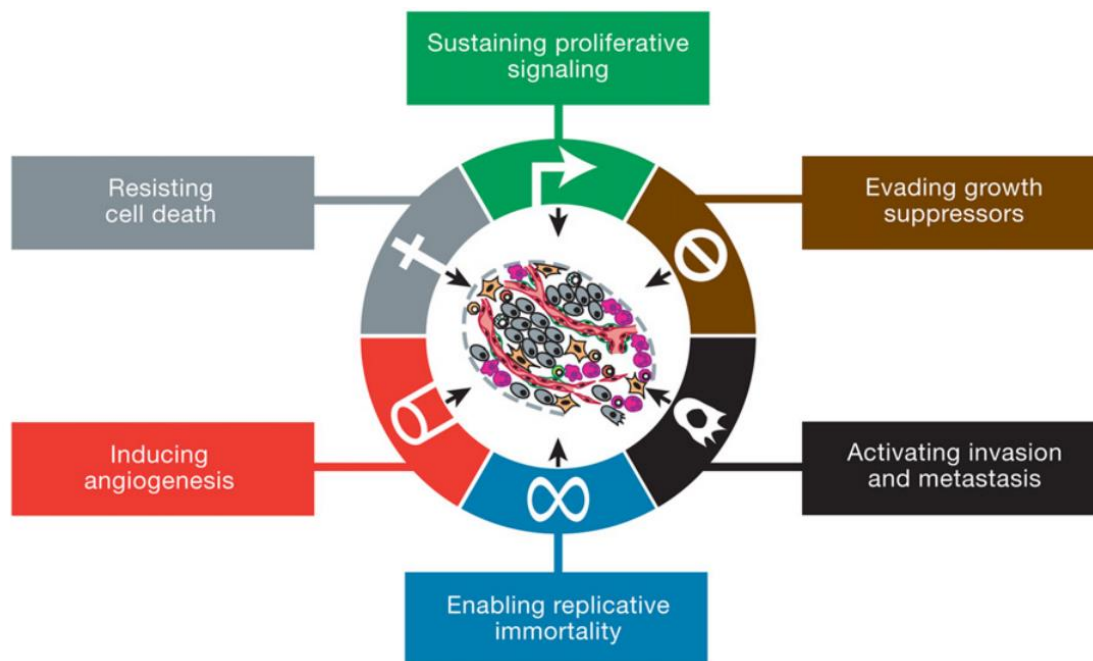
Figura 1: Principais características das células tumorais. Abrange os seis principais recursos característicos propostos para as células com características tumorais no início dos anos 2000 (HANNAHAN & WEINBERG, 2011).

2.3 Metástase

A sequência de eventos que leva ao processo de metástase, possui várias etapas interligadas, levando a uma cascata de processos concorrentes e/ou sobrepostos. A primeira etapa, inclui a ruptura da barreira da membrana basal e transição epitélio mesenquimal (TEP), ocorrendo a transição do tumor local e realizando o processo invasivo para órgão adjacente, com as células sendo liberadas e rompendo a barreira da membrana basal, para em seguida adquirirem atributos que se assemelham ao epitélio mesenquimal e induzir invasão (THIERY, J.P. 2002; GEIGER & PEEPER, 2009).

A segunda etapa é a dissociação das células tumorais do tumor, essa liberação leva a terceira etapa que é a invasão do tecido vizinho, onde as células desenvolvem a capacidade de migrar para invadir, realizando esse processo tanto em células únicas quanto em aglomerados (GEIGER & PEEPER, 2009).

A quarta etapa do processo de metástase se refere ao intravasamento em vasos sanguíneos e linfáticos que existentes ou que sejam pré-formados. Nesse caso somente o processo invasivo não é capaz de levar as células cancerígenas para difusão no organismo, bem como tumores avasculares, não conseguem crescer a tamanho maiores que 1mm de diâmetro, dessa forma, a vascularização

tumoral depende do processo angiogênico, que permite às células criarem vasos a partir de vasos pré-existentes, conseguindo então se disseminar pelo corpo e obter nutrientes (GIMBRONE, *et al.*, 1972; GEIGER & PEEPER, 2009).

A quinta etapa corresponde ao transporte através dos vasos, a sexta o extravasamento a partir dos vasos e a sétima a instalação no sítio secundário. A oitava e última etapa do processo metastático se refere ao crescimento de micro e macro metástases (GEIGER & PEEPER, 2009).

As células nos tecidos, possuem uma estrutura e conformação específicas, que geram para elas limites comportamentais, suprimento vascular com acesso a nutrientes e oxigênio. Células que consigam contornar essa organização, podem ficar expostas a uma série de pressões ambientais, como falta de oxigênio, carência de nutrientes, oscilações de pH, espécies reativas de oxigênio e mediadores inflamatórios. Esses estresses ambientais, podem fazer com que as células tumorais sejam selecionadas e possam desenvolver características agressivas (CHIANG & MASSAGUÉ, 2008).

No câncer de pulmão de células não-pequenas, é comum a presença de desmoplasias, que é caracterizado pela presença de Fibroblastos Associados ao Câncer (FAC). Nos tumores, os FACs mediam invasão, metástase, proliferação, angiogênese e resistência a drogas. Em FACs isolados de câncer de pulmão foi possível observar também um aumento na secreção de Interleucina-6, que estimula o transdutor de sinalização Janus-quinase-2 e ativa o STAT-3, essa cascata sinalizatória estimula a metástase *in vivo*, por exemplo, um aumento na expressão da integrina α11β1, gerando rigidez da matriz extracelular, que contribui para crescimento e metástase de xenoenxertos do paciente (ZHU, *et al.*, 2007; BREMNES *et al.*, 2011; WANG, *et al.*, 2017; ALTORKI, *et al.*, 2019).

Um componente importante para a disseminação dos tumores, é a presença de vasos sanguíneos, que além de permitirem a expansão do tumor, fornecem uma eficiente via de saída para as células tumorais saírem do tumor primário e entrarem na corrente sanguínea, permitindo atingir outros locais no corpo. O número de metástases formadas, geralmente é proporcional ao número de células eliminadas do tumor primário para a corrente sanguínea (ZETTER, 1998). Essa relação de metástase e angiogênese pode ser comprovada em experimentos onde animais com tumores primários estabelecidos, recebem tratamento que inibem o processo angiogênico, como sulfato de protamina

(TAYLOR & FOLKMAN, 1982), talidomida (D'AMATO *et al.*, 1994), angiostatina (O'REILLY *et al.*, 1994) e endostatina (O'REILLY *et al.*, 1997), com a diminuição da vascularização, sendo proporcional à diminuição das colônias metastáticas (ZETTER, 1998).

2.4 Angiogênese tumoral

As células de organismos mamíferos possuem uma necessidade de oxigênio e nutrientes necessários para a sobrevivência, sendo assim, essas células estão localizadas próximas aos vasos sanguíneos. Para os organismos multicelulares crescerem, é necessário recrutar novos vasos através do processo de vasculogênese e angiogênese e esse processo ocorre através de uma relação entre genes pró e antiangiogênicos. Essa relação se torna inviável ou desbalanceada em doenças como câncer, onde o crescimento tumoral e a metástase dependem do processo angiogênico (CARMELIET & JAIN, 2000; NISHIDA *et al.*, 2006). Uma vez que não há formação de vasos sanguíneos para fornecer os nutrientes ao tumor, este não irá conseguir crescer para além do tamanho crítico ou metastatizar para outro órgão (CARMELIET & JAIN, 2000).

O processo de angiogênese tumoral segue uma sequência de eventos que culminam na formação de vasos sanguíneos, responsáveis por nutrir o tumor: 1 – as células tumorais secretam fatores de crescimento como VEGF (Fator de Crescimento Endotelial Vascular) e FGF (Fator de Crescimento de Fibroblasto), afim de promover atração das células endoteliais na direção do tumor; 2 – as células endoteliais secretam enzimas para promover degradação de proteínas presentes na membrana basal de vênulas capilares ou pós-capilares; 3 – células endoteliais nas extremidades do tumor iniciam processo de migração ou brotamento, seguindo em direção ao gradiente de estímulo; 4 – as células endoteliais seguem a migração, com as células atrás da ponta principal, denominadas células estaminais, se proliferando e alinhando; 5 – ocorre transformação e criação de lúmen no centro do vaso formado; 6 – ocorre perfusão do sangue através do lúmen do novo vaso formado (BIELENBERG & ZETTER, 2015).

Uma série de moléculas são responsáveis pela angiogênese, com a família VEGF sendo uma das mais importantes. Os fatores de crescimento vascular VEGF-A, VEGF-B, VEGF-C, VEGF-D e o PlGF (Fator de Crescimento

Placentário) são os que mais se destacam (CARMELIET & JAIN, 2000). Sinais de isquemia e hipóxia iniciam muitos processos relacionados com a angiogênese e o crescimento tumoral somente se torna possível, devido ao microambiente hipóxico criado que ativa a rede angiogênica e os sinais angiogênicos (HOFF & MACHADO, 2012). A hipóxia regula os níveis de VEGF, através de estabilização do RNA mensageiro e citocinas como EGF (Fator de Crescimento Epidérmico) e TGF-β (Fator Transformador de Crescimento-β), também podem aumentar a expressão de VEGF por mecanismos variados (HOFF & MACHADO, 2012).

O VEGF-A é o mais bem estudado fator de crescimento da família VEGF e é observado em tumores como os de pulmão, mama, rim e bexiga. Suas principais ações, incluem forte ação mitogênica nas células endoteliais vasculares, promoção de ativação de enzimas que realizam a degradação da matriz extracelular, ação pró-sobrevivência via inibição da apoptose, mobilização de precursores endoteliais da medula óssea e aumento da permeabilidade vascular (HOFF & MACHADO, 2012). O VEGF-B possui uma função de fator de sobrevivência maior que pró-angiogênico, regulando positivamente genes pró-sobrevivência como NRP-1, no entanto possui ainda uma ação menor no processo angiogênico (LEE *et al.*, 2009). O VEGF-C e D possuem atividade menos conhecida, mas estão associados com aumento do risco metastático, promoção de linfangiogênese no tumor e potencial disseminação adicional de células tumorais que já colonizaram (HOFF & MACHADO, 2012).

Nos últimos anos muitos alvos antiangiogênicos foram descobertos, atuando em fatores de crescimento, metaloproteases de matriz, células estromais associadas ao tumor (LIN *et al.*, 2016). Muitas toxinas isoladas de toxinas de serpente já se mostraram capazes de atuar inibindo processos angiogênicos, sendo então um importante alvo terapêutico e dentre essas moléculas destacam-se as fosfolipases A2, lectinas tipo-c, l-aminoácidos oxidases e metaloproteases (GUIMARÃES *et al.*, 2017; POLLONI *et al.*, 2021; VAN PETTEN *et al.*, 2022).

2.5 Metaloprotease de Peçonha de Serpente

As enzimas proteolíticas são parte importante do organismo ao longo da sua vida, sendo consideradas todas aquelas enzimas que tenham a capacidade de hidrolisar ligações peptídicas seja em proteínas, seja em fragmentos de

proteínas. Após realizar essa hidrólise, essas enzimas destroem ou modificam de maneira irreversível o substrato ao qual reagiu, possuindo então, alta importância biológica (BARRETT, 2001). As peptidases correspondem a 2% de todos os produtos gênicos no organismo, nas vias metabólicas e de sinalização celular. Além disso, microrganismos são capazes de secretar peptidases para o meio externo, com o intuito de realizar a degradação de proteínas ou componentes cujos produtos de hidrólise são fontes de carbono e nitrogênio para o seu crescimento (BARRETT, 2001).

Atualmente a classificação das enzimas proteolíticas envolve três critérios: a similaridade das propriedades da enzima, que mostrem a mesma especificidade de atividade catalítica, pH e sensibilidade aos inibidores, possuir semelhança na sequência de aminoácidos e a árvore evolucionária das unidades das peptidases cujas sequências possam ou não divergir ao mesmo tempo, nos organismos em que aparecem (BARRETT, 1999). Dessa forma, as enzimas proteolíticas são divididas em dez grupos, aspartilproteases, cisteinoproteases, metaloproteases, serinoproteases, treoninoproteases, glutamilproteases, inibidores que são proteínas, peptidases de tipos catalíticos mistos, asparagina liases e as que os resíduos envolvidos no processo de catálise são desconhecidos (BARRETT, 1999; RAWLINGS, 2008; RAWLINGS, 2018).

Metaloproteases ou metaloendopeptidases (MEPS; EC subclasse 3.4.24) são enzimas hidrolíticas que dependem da ligação a um metal (zinco, cobalto, níquel ou manganês). Essas enzimas possuem ligações peptídicas como alvos em proteínas ou oligopeptídeos e são capazes de realizar a digestão ou degradação destas e realizar em tecidos o desenvolvimento, manutenção e remodelamento. Estão envolvidas em processos proteolíticos e participam de mecanismos regulatórios, estes processos são necessários para o controle da pressão sanguínea, a homeostase dos hormônios, regulação da transdução de sinais, modulação de interações proteína-proteína e célula-célula (RUGGIERO, *et al.* 2011; WANG, *et al.* 2012; CHANG, *et al.* 2016; GRZECHOCIŃSKA, *et al.* 2019).

As metaloproteases são produzidas e secretadas na forma de proenzimas inativas, com o seu sítio ativo sendo caracterizado pela presença de uma fenda que é projetada para acomodar o substrato que será hidrolisado. A ativação dessa enzima ocorre após liberação do sítio de zinco, que na sua forma inativa

possui um grupo tiol de uma cisteína presente, que se liga a esse zinco (RAMOS & SELISTRE-DE-ARAÚJO, 2006).

As metaloproteases são encontradas em organismos de todos os grupos de seres vivos e a sua classificação pode ser dividida em quatro níveis hierárquicos: Classe, Tribo, Clã e Família (Figura 2). Essa divisão permite separar as metaloprotease zinco-dependentes, por meio das suas características estruturais, estrutura de ligação, a forma com que se liga ao zinco e suas similaridades. Sendo assim, existem três divisões das metaloproteases dependentes de zinco: Zincinas, Inverzincinas e αβα-Exopeptidases (GOMIS-RÜTH, 2003; GOMIS-RÜTH *et al.*, 2012).
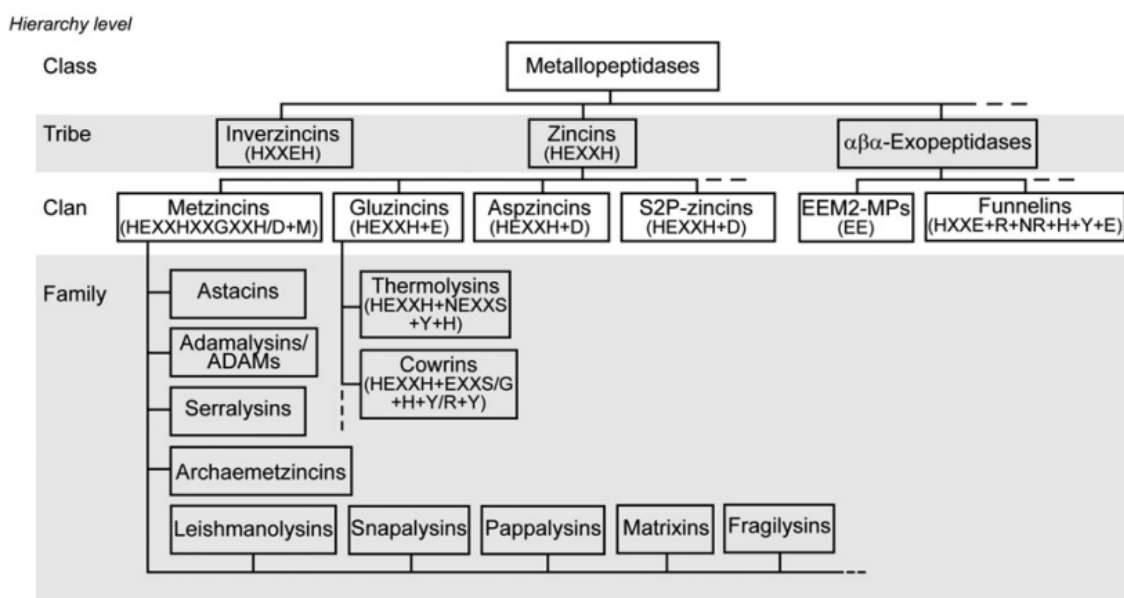
Figura 2: Classificação das metaloproteases de acordo com a ligação do zinco (Gomis-Rüth *et al.*, 2012).

O maior grupo compreende as Zincinas e se divide em quatro clãs: as Gluzincinas, Aspzincinas, S2P-zincinas e as Metzincinas, essa divisão depende da natureza do terceiro resíduo de ligação ao zinco e de demais características estruturais (GOMIS-RÜTH *et al.*, 2003; GOMIS-RÜTH *et al.*, 2012). Dentro do grupo das Metzincinas existem nove famílias, dentre elas algumas possuem importância biológica. As metaloproteases de matriz ou MMPs (Matrixinas), são proteases descobertas pela primeira vez em 1962, sendo capazes de realizar a remodelação da matriz extracelular. Entretanto, têm sido cada vez mais observado que a quebra dos componentes da matriz extracelular ou moléculas da superfície, promove alteração na relação célula-célula ou célula-matriz,

liberando componentes que podem ser substrato para as MMPs. Entre as atividades biológicas desempenhadas pelas MMPs, temos processos inflamatórios (HARO *et al.*, 2000), migração (KAJITA *et al.*, 2001), agregação plaquetária (SAWICKI *et al.*, 1997), apoptose (LIANG *et al.*, 2019), neovascularização (ZHANG *et al.*, 2020), crescimento celular, vasoconstrictor (NUGEN *et al.*, 2016), entre outras funções.

A família das Adamalisinas é composta pelas proteínas ADAMs (a desintegrin and metalloproteinase) e ADAMTSs (a desintegrin and metalloproteinase with trombospodin-like) que são expressas em mamíferos e as SVMPs (snake venom metalloproteinase) de peçonha de serpente (Figura 3).
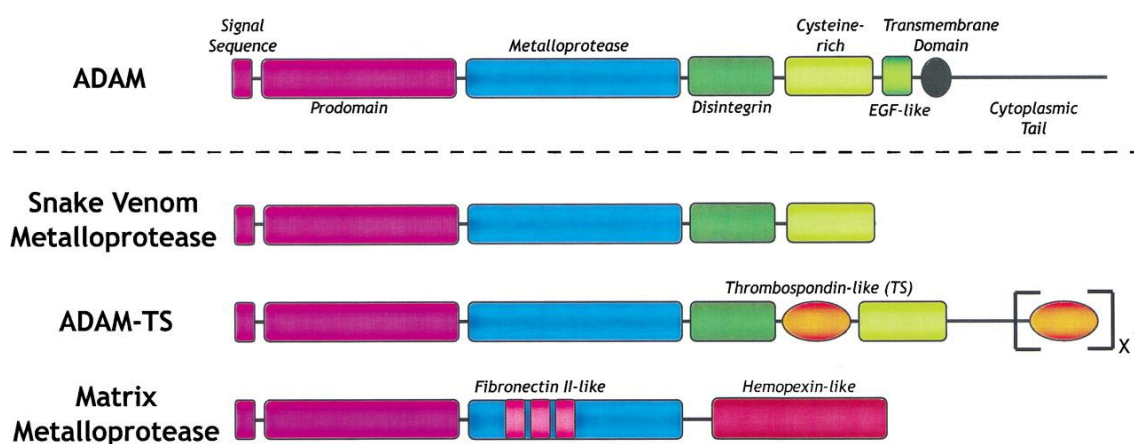
Figura 3: Principais domínios estruturais das ADAMs, ADAMTSs, SVMP e MMP. Topografia das ADAMs e metaloproteases relacionadas, sendo divididas em quatro grupos distintos. A composição dos grupos varia de acordo com seus domínios estruturais, apresentando peptídeo sinal, pró-domínio, domínio metaloprotease, desintegrina, rico em cisteína, tipo-fibronectina-II, tipo-trombospodina, tipo-EGF, tipo-hemopexina ou domínio transmembrana (SEALS e COURTNEIDGE, 2003).

As ADAMs são proteínas transmembranas que possuem uma metaloprotease e atividade catalítica de ligação ao receptor de integrina, além de um domínio citoplasmático. Essas moléculas possuem capacidades biológicas atribuídas ao controle de fusão de membrana, liberação de citocinas e fatores de crescimento, migração celular e determinação de destino celular (SEALS & COURTNEIDGE, 2003).

As ADAMTSs são proteases encontradas em mamíferos e invertebrados, sua estrutura é composta por um domínio metaloprotease, um domínio desintegrina e um domínio trombospondina, são enzimas extracelulares cujas funções estão o processamento de colágeno, clivagem dos proteoglicanos da

matriz, inibição da angiogênese e homeostase da coagulação sanguínea (PORTER *et al.*, 2005).

As SVMPs são proteínas dependentes de zinco que apresentam massas moleculares variando de 20 a 110 kDa podem possuir tamanho entre 20 e 110kDa, além de compor pelo menos 43% da composição da peçonha de serpentes da família Viperidae (CALVETE, *et al.* 2007; FOX E SERRANO, 2008; GEORGIEVA, *et al.*, 2008). Segundo os dados de proteoma do estudo de Georgieva *et al.*, 2008, as SVMPs são o grupo de toxinas mais abundantes entre os viperídeos, seguido pelas fosfolipases A$_2$ e as serinoproteases. Essa alta concentração de metaloproteases e serinoproteases na peçonha, explica os efeitos de coagulopatia presentes após a picada de indivíduos dessa família.

As metaloproteases de peçonha de serpente são sintetizadas na forma de proenzimas inativas e apresentam na sua estrutura domínios que auxiliam na realização das suas funções, além de participarem no direcionamento, toxicidade e inativação (RAMOS E SELISTRE-DE-ARAÚJO, 2006; FOX E SERRANO, 2008). A estrutura das SVMPs é composta pelo peptídeo sinal, pró-domínio, metaloprotease, interdomínio e ainda possui regiões que variam de acordo com a classe, podendo apresentar domínios desintegrina, tipo desintegrina, rico em cisteína e domínio lectina tipo-C (FOX E SERRANO, 2008). Essa estrutura inicial se reduz após a toxina atingir sua forma madura, perdendo então o peptídeo sinal e o pró-domínio.

As metaloproteases são classificadas em três classes e onze subclasses de acordo com os seus domínios estruturais (Figura 4). As de classe P-I compreendem as proteínas somente com o domínio catalítico (metaloprotease) na forma madura, podem apresentar massa entre 20 e 30kDa, além de possuir pouca ou nenhuma atividade hemorrágica. As SVMPs da classe P-II possuem massa molecular de 30 a 60kDa, possuindo um domínio desintegrina na sua estrutura na forma ativa. As metaloproteases de classe P-III possuem massa acima de 60kDa e possuem uma alta atividade hemorrágica, se diferenciando das demais por possuírem na sua estrutura domínios desintegrina-*like* e domínios ricos em cisteína (FOX E SERRANO, 2008; MARKLAND, 2013; OLAOBA, *et al.* 2020).
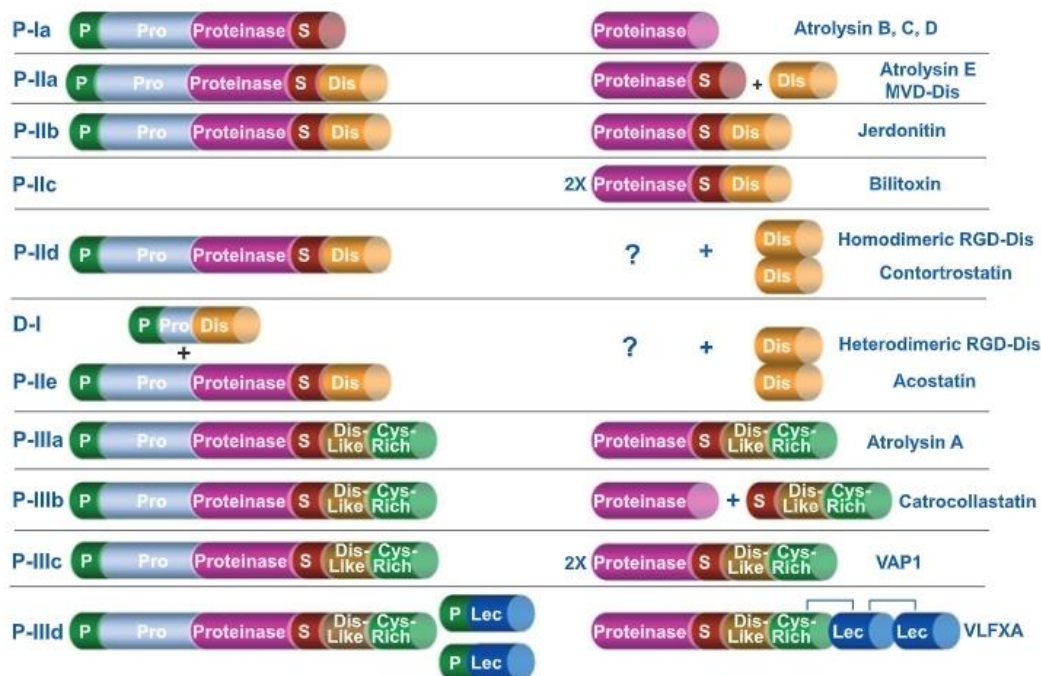
Figura 4: Esquema das classes de SVMPs. Trechos marcados com (?) indicam que o produto processado ainda não foi identificado na peçonha. Na figura, P significa peptídeo sinal, Pro significa pró-domínio, S significa espaçador, Dis corresponde ao domínio desintegrina ou Dis-like, que é o domínio tipo-desintegrina, Cys-Rich representa o domínio rico em cisteína e Lec o domínio lectina (FOX E SERRANO, 2008).

As SVMP são responsáveis por causar danos locais nos tecidos e sistêmicos após o envenenamento. A lesão local tem como característica a presença de inflamação, dor, hemorragia, edema e mionecrose, o dano sistêmico apresenta coagulopatia, choque hipovolêmico e traumas renais (ANAI *et al*, 2002; ESCALANTE, *et al*. 2011). Esses efeitos são produzidos devido à capacidade das SVMPs de interagir diretamente sobre proteínas da matriz extracelular endotelial e subendotelial dos vasos sanguíneos, juntamente com plaquetas, colágeno, laminina e fibronectina, fatores de coagulação, receptores de superfície e sistemas celulares (MOURA-DA-SILVA *et al*, 2007; MOURA-DA-SILVA & BALDO, 2012; MARKLAND E SWANSON, 2013). Apesar das SVMPs poderem causar efeitos danosos durante o envenenamento, estas podem atuar na alteração da hemostasia tendo atividade fibrinogenolíticas (OYAMA *et al*., 2015), fibrinolítica e antiplaquetárias (SANCHEZ, *et al*, 2016), ação hemorrágica (CAMACHO *et al*., 2019) ou não hemorrágica (SONG *et al*., 2013), tornando-as moléculas com alto valor biológico.

Várias SVMPs já foram estudadas e seus efeitos antitumorais e antiangiogênicos já foram comprovados. A Jararagina, metaloprotease de classe

P-III isolada de peçonha de *Bothrops jararaca*, reduz a viabilidade nas células Skmel-28, de melanoma humano, promovendo também redução nos processos metastáticos de adesão, migração e invasão, além disso, a toxina é capaz de promover alterações morfológicas nas células de melanoma testadas, promovendo aumento no número de células arredondadas e sem o formato espraiado comum das células e aglomerações dessas (CORRÊA, *et al.*, 2002).

Bothropoidina, uma P-III isolada da peçonha de *Bothrops pauloensis*, possui efeito significativo na citotoxicidade celular de linhagem tumoral de mama, MDA-MB-231 com 30% de morte das células na concentração de 100µg/mL. Além disso, a toxina promoveu aumento de apoptose, redução dos processos metastáticos de adesão e migração na linhagem tumoral de mama e induziu redução na formação *in vitro* de vasos em linhagem endotelial murina, tEnd (GUIMARÃES, *et al.*, 2017).

Apesar de muitos estudos abordarem os efeitos antitumorais e antiangiogênicos com metaloproteases de classe P-III, poucos estudos focam nas toxinas da classe P-I. Resultados sobre inibição da angiogênese também foram obtidos com BpMP-II, uma metaloprotease de classe P-I isolada da peçonha de *Bothropoides pauloensis*, onde a toxina foi capaz de inibir a formação de vasos em linhagem endotelial murina (ACHÊ, *et al.*, 2015). Assim nesse estudo, tivemos como objetivo avaliar os efeitos da metaloprotease de classe P-I.

A BthMP é uma metaloprotease da classe P-I, inicialmente isolada por GOMES *et al.*, 2009, é uma toxina da peçonha de *Bothrops moojeni*, com caráter fracamente hemorrágico, possuindo uma estrutura polipeptídica com 23,5kDa, pI=7.1 e cadeia N-terminal bloqueada. Ainda segundo GOMES *et al.*, 2009, a toxina é composta por alta atividade proteolítica sobre caseína, fibrina e fibrinogênio bovino, não possui atividade coagulante, esterase ou fosfolipase A_2, sendo também inibida por EDTA, EGTA e 1,10 fenantrolina e mantém sua atividade em pH de 7,0 a 9,0 e temperatura de 5-40ºC. Ensaios com íons metálicos mostram que Ca^{2+} é um ativador, enquanto Zn^{2+} e Hg^{2+} inibem cerca de 50 e 80% da sua atividade, respectivamente. E por fim é capaz de produzir alterações histológicas no músculo gastrocnêmio de camundongos, provocando a indução de hemorragia, necrose e infiltrado leucocitário.

Segundo SANTOS, 2016, a BthMP possui acentuada atividade fibrinogenolítica e alto consumo de fibrinogênio *in vivo*, que reforçam seu caráter anticoagulante, além de interferir na cascata de coagulação.

3. OBJETIVOS

3.1 Objetivo geral

Avaliar os efeitos antimetastáticos e antiangiogênicos da metaloprotease BthMP, isolada da peçonha da serpente *Bothrops moojeni* em modelos *in vitro* e *ex vivo*.

3.2 Objetivos específicos

3.2.1 Efeito antimetastático

- Avaliar a viabilidade e proliferação celular utilizando as células de câncer de pulmão (A549) e células de pulmão não tumorigênicas (BEAS-2B) tratadas com a BthMP;
- Investigar o efeito da BthMP nos processos de adesão, migração e invasão celular das células A549 e BEAS-2B;
- Avaliar os níveis de LDH e óxido nítrico e a formação de espécies reativas de oxigênio, utilizando as células de câncer de pulmão (A549) e células de pulmão não tumorigênicas (BEAS-2B) tratadas com a BthMP.

3.2.2 Efeito antiangiogênico

- Avaliar a viabilidade e proliferação de células endoteliais do cordão umbilical humano (HUVECs) tratadas com a metaloprotease BthMP;
- Avaliar os níveis de LDH e óxido nítrico e a formação de espécies reativas de oxigênio utilizando a linhagem HUVEC;
- Investigar o efeito da toxina na adesão e migração de HUVECs;
- Avaliar a ação antiangiogênica da BthMP em modelo *in vitro* em Matrigel utilizando HUVEC;

- Investigar a influência da toxina nos níveis de VEGF liberados pelas HUVECs e a modulação da expressão gênica de VEGF-A, ANGPT1 (angiopoetina 1) e SFLT-1 (Soluble fms-like tyrosine kinase-1);
- Avaliar a ação antigangiogênica da BthMP em modelo *ex vivo* por meio do ensaio de anel de aorta.

4. MATERIAIS E MÉTODOS

4.1 Purificação BthMP

A BthMP foi purificada da peçonha da serpente *Bothrops moojeni,* por meio de combinação de cromatografia de troca iônica (DEAE-Sephacel) e exclusão molecular com Sephadex G-75, como previamente descrito por (GOMES *et al.*, 2009)

4.2 Animais

Todas as experiências foram realizadas de acordo com as diretrizes e regulamentos do Comitê para o Uso Ético dos Animais. Foram utilizados 6 camundongos da linhagem Balb-c (machos, 6 semanas e aproximadamente 25g) mantidos sob condições padrão (ciclo claro/escuro de 12h, temperatura 22±1°C, umidade relativa 60±5%), com dieta e água *ad libitum*. Os procedimentos realizados com animais foram enviados e aprovados pelo Comitê de Ética na Utilização de Animais da Universidade Federal da Bahia Protocolo nº082/2020.

4.3 Cultura de Células

Foram utilizadas para este estudo a linhagem de células endoteliais humanas (HUVEC), linhagem tumoral de pulmão (A549) e linhagem de pulmão não tumorigênica (BEAS-2B), obtidas da American Type Culture Collection (ATCC). As células foram mantidas a 37°C em incubadora umidificada contendo 5% de CO_2, em meio de cultura celular RPMI suplementado com Soro Fetal Bovino 10%, antibiótico 1%, aminoácidos essenciais e piruvato de sódio.

4.4 Avaliação da Citotoxicidade pelo Ensaio de MTT

As células, foram semeadas a $3x10^4$ células por poço em microplacas de 96 poços. Após 24hrs, um novo meio contendo BthMP (60; 30; 15; 7,5; 1,875; 0,9375; 0,46875 μg/mL) ou meio (controle) foi adicionado e incubado a 37°C e 5% de CO_2 por 24h. Após o tratamento, as células foram incubadas com MTT (5mg/mL, 10μL/poço (MTT: 3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazólio brometo) por 3hrs a 37°C. Em seguida, foi adicionado 100μL/poço de PBS contendo 10% de SDS e 0,01M de HCl (18h, 37°C e 5% de CO_2). A absorbância foi lida em um espectrofotômetro de varredura de múltiplos poços a 550nm.

Com base nos resultados deste ensaio, foram escolhidas as concentrações a serem utilizadas nos experimentos posteriores.

4.5 Ensaio de proliferação celular ou formação de colônias

O ensaio de formação de colônias foi realizado conforme descrito por FRANKEN *et al.*, 2006 com modificações. Células ($3×10^4$ células/poço) foram plaqueadas em microplacas de 96 poços e incubadas por 24h a 37ºC em 5% de CO^2. Em seguida ocorreu o tratamento com a BthMP nas concentrações de (40 e 5μg/mL) ou meio de cultura (grupo controle) por 24h. Após o tratamento, as células foram tripsinizadas e transferidas (100 células/poço) para placas de 6 poços, sendo mantidas em meio de cultura por duas semanas a 37ºC em 5% de CO_2, momento em que ocorreu a remoção do meio, lavagem das células com PBS, fixação das mesmas com metanol gelado por 10 min, seguida da coloração com Panotic Kit (Laborclin, Brasil). As colônias formadas por mais de 50 células foram quantificadas e fotografadas.

4.6 Ensaio de Liberação de LDH

O ensaio de liberação de LDH foi realizado conforme LEGRAND *et al.*, 1992 com modificações. As células foram semeadas a $3x10^4$ células por poço em microplacas de 96 poços e após 24hrs, um novo meio contendo BthMP (40 e 5μg/mL) ou meio (controle) foi adicionado e incubado a 37°C e 5% de CO_2 por 24h e o sobrenadante foi coletado após esse período. Seguindo as intruções do kit Desidrogenase Lática LDH UV (Bioclin) foi produzida uma solução de trabalho com quatro partes do reagente 1 e uma parte do reagente 2 e foi adicionado

20µL da solução em cada sobrenadante correspondente aos tratamentos e controles. Em microplaca de 96 poços a mistura foi lida em leitora de placa a 370nm, previamente aquecida a 37ºC e os valores verificados com intervalo de 1 minuto. Os valores obtidos foram colocados na equação presente no kit após adaptação para a leitura em microplaca e em seguida analisados.

4.7 Inibição da Adesão de HUVEC em Proteínas da Matriz Extracelular

Para avaliar a interação entre BthMP e componentes da matriz extracelular (MEC), colágeno IV (10µg/mL em 0,1M de ácido acético), fibronectina (5µg/mL em PBS) ou matrigel (1mg/mL em PBS) foram revestidos em placa de 96 poços durante a noite a 4ºC e bloqueados com 1% de BSA. Posteriormente, a linhagem (3×10^4 células/poço) foi pré-incubada com diferentes concentrações de BthMP (40 e 5µg/mL) ou meio (grupo controle) por 30min a 37ºC, as células foram semeadas e incubadas durante 2h a 37ºC em 5% de CO_2. As células não ligadas foram removidas por lavagem com PBS e células aderidas quantificadas pelo ensaio MTT conforme descrito anteriormente. Contagens com relação à morfologia das células foram realizadas observando o formato da célula após o tratamento, sendo divididas em arredondadas e espraiadas.

4.8 Ensaio de Wound Healing

A inibição da migração da linhagem endotelial foi medida por um ensaio de cicatrização de feridas conforme descrito por JUNG, 2013 com modificações de GIMENES *et al.*, 2017. As células foram plaqueadas em placas de 12 poços com 4×10^6 células/poço e após 24h o meio foi descartado e foi produzida uma ferida na monocamada com uma ponta de ponteira de 200µL para produzir uma região sem células. Na sequência houve o tratamento das células com a toxina BthMP (40 e 5µg/mL) ou com meio controle por 24h e 48h e o resultado foi observado em microscópio óptico invertido.

4.9 Migração e Invasão Celular por Transwell

Para avaliar a inibição da migração e invasão celular, foi utilizada a câmara Transwell com membrana de 8µm de acordo com GIMENES *et al.*, 2017. As células foram pré-incubadas com a BthMP (40 e 5µg/mL) por 30 min a 37°C e 5% CO_2 em meio de cultura sem SFB. Após a incubação, 7×10^4 célula/poço

foram semeadas na parte superior da câmara dos insertos e no compartimento inferior foi adicionado meio com 10% de SFB como quimioatrativo para as células. Nos grupos controle negativo, a camada inferior foi preenchida com meio de cultura sem soro fetal bovino. No ensaio de invasão celular, os insertos de cultura celular foram previamente revestidos com Matrigel (Corning® Matrigel® Matrix, USA) diluído a uma proporção de 1:10 (PBS: Matrigel, v/v) com meio sem SFB (overnight), antes das células serem semeadas. Em ambos os ensaios o excesso de células na parte superior do transwell são retiradas com auxílio de um cotonete, as células migradas ou invadidas serão coradas com Panotic Kit (Laborclin, Brasil), as células serão fotografadas e contadas. O controle positivo é representado por células com meio e 10% de SFB e o controle negativo é representado por células com meio sem SFB.

4.10   Angiogênese *in vitro* (Ensaio de formação de tubos em HUVEC)

Para avaliar a angiogênese *in vitro* foi realizado o ensaio de verificação da formação de vasos em Matrigel segundo GIMENES *et al.*, 2017. As células HUVEC, $5x10^5$ células/poços, foram pré-incubadas com 40µg/mL, e 5µg/mL da BthMP ou meio (grupo controle) suplementado com bFGF (10ng/mL) durante 30 mim a 37°C. Posteriormente, as células foram semeadas em placas de cultura de 24 poços revestidas com 50µL de Matrigel (Corning Matrigel Matrix, EUA), sendo mantidas a 37°C em incubadora umidificada com 5% de CO^2. Após 18h, os resultados foram fotografados em microscópio óptico invertido e feita a quantificação dos vasos. O sobrenadante foi coletado para dosagem de VEGF em ensaio seguinte.

4.11   Dosagem de VEGF no sobrenadante de células HUVEC

Para quantificar os níveis de VEGF foi utilizado o sobrenadante das células HUVEC obtidos após o ensaio da formação de vasos *in vitro*, utilizando a BthMP (40 e 5µg/mL). A dosagem foi realizada utilizando um Kit CBA para proteínas celulares (BD–EUA), seguindo as recomendações do fabricante. Resumidamente, uma curva padrão foi construída com diferentes concentrações de VEGF conhecidas (2500, 1250, 625, 312,5, 156, 80, 40, 20 e 10 pg/mL), posteriormente adicionou-se 25µL do Mix de Beads (Diluent Beads e Capture

Beads). Em seguida, as amostras foram incubadas por 1h em temperatura ambiente e em seguida adicionados 25µL do Mix PE Detection Reagent, sendo então incubado por mais 2h em temperatura ambiente. Após esta incubação as amostras foram lavadas com 500µL de Wash Buffer e centrifugadas a 200g por 5 minutos. O sobrenadante foi então retirado e o pellet formado ressuspendido em 300µL de Wash Buffer. As amostras foram submetidas ao citômetro de fluxo Cytoflex (Beckman Coulter, Estados Unidos), e os dados gerados foram analisados pelo software FlowJo (versão 7.6.3). A partir dos dados gerados pela curva de concentrações conhecidas do VEGF, foi calculada uma curva padrão, a qual permitiu a determinação da concentração de VEGF, expressa em pg/mL, presente no sobrenadante oriundo das diferentes concentrações experimentais.

4.12   Ensaio de Angiogênese em linhagem HUVEC *ex vivo*

A angiogênese *ex vivo* foi avaliada pelo método do anel aórtico. O ensaio foi realizado conforme BAKER *et al*, 2011 com modificações. O fragmento da aorta teve tamanho entre 1mm e 1,5mm sendo retirado de camundongos Balb-c com seis semanas de idade. Os fragmentos removidos passaram por lavagem com solução PBS gelada e suplementada com 1% de penicilina-estreptomicina e colocados em placas de 48 poços contendo 50µL Matrigel (Corning® Matrigel® Matrix, USA), sendo incubados em meio RPMI suplementado com bFGF (10ng/ml) por 24h. Na sequência, ocorreu o tratamento com a BthMP (40 e 5µg/mL) durante 14 dias em intervalos de dois dias. Ao final do experimento, os anéis aórticos foram fotografados em microscópio ótico invertido e a presença de vasos contabilizada.

4.13   Ensaio de expressão gênica por PCR em tempo real (RT-PCR)

Células da linhagem HUVEC foram plaqueadas a $3,5 \times 10^5$ células/poço em microplacas de 24 poços, tratadas com a BthMP (40 e 5µg/mL), meio de cultura com VEGF (controle positivo) ou meio de cultura sem VEGF (controle negativo) por 24h, sendo mantidas em incubadora a 37ºC com 5% de CO_2. Após o tratamento, o RNA total das culturas foi extraído por meio do kit RNeasy Microarray (Qiagen, EUA), de acordo com as instruções do fabricante. Ao final do processo de extração, o RNA foi tratado com RNase-Free DNase (Qiagen, EUA) para digestão de possíveis fragmentos contaminantes de ácido

desoxirribonucleico (DNA). A concentração (ng/µL) e pureza (A260:A230 e A260:A280) do RNA total foram avaliadas por espectrofotometria (NanoDropTM 2000, Thermo Fisher Scientific, EUA). O cDNA foi sintetizado com 2µg do RNA total por meio do kit de transcrição reversa High-Capacity RNA-to-cDNATM (Applied Biosystem, EUA), seguindo as instruções do fabricante. Os níveis de expressão dos genes VEGFA, Angiopoietina 1 e sFlt-1 foram quantificados por PCR em tempo real utilizando o sistema Power UpTM SYBRTM Green Master Mix (Thermo Fisher Scientific, EUA) a partir das amostras de cDNA na concentração de 100ng/µL. Os dados foram normalizados utilizando GAPDH (Gliceraldeído 3-fosfato desidrogenase) como controle endógeno da reação e todos os procedimentos foram realizados em triplicata e sistema StepOne Plus (AppliedBiosystem, EUA) foi utilizado para termociclagem, conforme descrito: um ciclo de 50°C por 2 min, um ciclo de 95 °C por 10 min, 40 ciclos de 95 ºC por 15 s, 40 ciclos de 60ºC por 1 min e a curva de melting (15 a 95ºC, 1 min a 60ºC, 15 s a 95ºC). As sequências específicas dos primers para os genes na análise do PCR em tempo real foram obtidas na literatura e os anelamentos testados pela ferramenta BLAST do NCBI (Tabela 1). A partir da obtenção dos resultados de Ct (cycle treshold) fornecidos pelo termociclador, calculou-se a expressão relativa (foldchange) por meio do método comparativo ($2^{-\Delta\Delta Ct}$) em função do grupo tratado apenas com o meio de cultura (LIVAK; SCHMITTGEN, 2001).

Tabela 1: Sequência dos primers utilizados na qPCR.

Gene	Sequência 5' - 3'	Referência
VEGFA	*Forward*: AGGGCAGAATCATCACGAAGT	(XU *et al.*, 2019)
	Reverse: AGGGTCTCGATTGGATGGCA	
ANGIOPOIETINA-1	*Forward*: GGCAGTACAATGACAGTTTC	(KIM *et al.*, 2000)
	Reverse: CTTTGTTGCTTTCATAATCGC	
SFLT-1	*Forward*: AGGGGAAGAAATCCTCCAGA	(ABOU-FAYCAL *et al.*, 2019)
	Reverse: CAACAAACACAGAGAAGG	
GAPDH	*Forward*: CGAGATCCCTCCAAAATCAA	(ABOU-FAYCAL *et al.*, 2019)
	Reverse: ATCCACAGTCTTCTGGGTGG	

4.14   Produção de óxido nítrico (NO)

As linhagens foram plaqueadas a $3x10^4$ células por poço em microplacas de 96 poços e após 24hrs, um novo meio contendo BthMP (40 e 5µg/mL) ou meio (controle) foi adicionado e incubado a 37°C e 5% de CO_2 por 24h e o sobrenadante coletado após esse período. Os níveis de óxido nítrico (NO) foram quantificados a partir da concentração final do metabólito final, nitrito, utilizando o ensaio de Griess conforme o trabalho de GREEN, *et al.*, 1982. Alíquotas de 50µL da solução de teste (padrão, controle, amostra experimental) foram adicionadas a 0,1% de solução de reagente de Griess (50 µL de 3,9 mM N-(1-naftil)etilenodiamina em 5% (v/v) de ácido fosfórico) e incubados no escuro em temperatura ambiente durante 10 minutos. A solução de sulfanilamida (1% em ácido fosfórico) foi adicionada à mistura e a absorbância do produto foi medida a 540nm usando leitor de microplacas. Soluções padrão de nitrato de sódio (0,1 mM) foram utilizadas para construir uma curva padrão como forma de determinar as concentrações reais das amostras.

4.15   Produção de Espécies Reativas de Oxigênio (ROS)

A produção de espécies reativas de oxigênio (ROS) em células foi realizada segundo oxidação dependente de peróxido intracelular de diacetato de 2',7'-diclorodihidrofluoresceína (H2DCF-DA) (Invitrogen, número de catálogo: D399) para formar o composto fluorescente 2',7'-diclorofluoresceína (DCF), conforme descrito anteriormente (TEIXEIRA *et al.*, 2020). As células A549 e BEAS-2B foram plaqueadas a $3x10^4$ células/poço em microplacas escuras de 96 poços. Após 24h, com a adesão, um novo meio contendo BthMP (40 e 5µg/mL) ou meio (controle) foi adicionado e incubado a 37°C e 5% de CO_2 por 24h. Após o tratamento, as células foram lavadas com 1x PBS e incubadas com 150µL de H2DCF-DA (10µM; diluído em 1x PBS contendo 10% de Soro Fetal Bovino) por 45 minutos a 37°C e 5% de CO_2 no escuro. A intensidade de fluorescência DCF foi detectada imediatamente usando um espectrofotômetro de varredura multipoços GloMax Explorer (Promega, Madison, WI, EUA). Os resultados foram apresentados como intensidade de fluorescência. O peróxido de hidrogênio

(H_2O_2) foi utilizado como controle positivo da produção de ROS e o controle negativo corresponde a células tratadas apenas com meio de cultura.

4.16   Migração por Transwell com Adição de EDTA

Para avaliar o efeito do EDTA na BthMP, escolhemos o ensaio de inibição da migração utilizando a câmara Transwell com membrana de 8µm de acordo com GIMENES *et al.*, 2017. Foram divididos dois grupos, um somente toxina e outro da toxina com adição de solução 1mM de EDTA, com o grupo EDTA, passando por pré-incubação de 1h a 37ºC. As células A549 e BEAS-2B foram pré-incubadas com a BthMP (40 e 5µg/mL) dos dois grupos, por 30 min a 37°C e 5% CO_2 em meio de cultura sem SFB. Após a incubação, 7×10^4 célula/poço foram semeadas na parte superior da câmara dos insertos e no compartimento inferior foi adicionado meio com 10% de SFB como quimioatrativo para as células. Nos grupos controle negativo, a camada inferior foi preenchida com meio de cultura sem soro fetal bovino e no controle positivo a camada inferior possuía meio de cultura com soro fetal bovino.

4.17   Análises Estatísticas

Os dados deste trabalho foram apresentados por uma média de 3 experimentos e desvio padrão. Primeiramente, todos os dados foram verificados para distribuição normal. Para os dados paramétricos, as diferenças estatísticas foram determinadas pelos testes T de Student ou ANOVA one-way, seguido do pós-teste de Tukey. Para os dados não paramétricos, foram utilizados os testes de Mann-Whitney e Kruskal-Wallis, seguido do pós-teste de Dunn, de acordo com o projeto experimental (GraphPad Prism Software version 7.0). Os dados foram considerados estatisticamente significativos quando $p < 0,05$; $p < 0,01$; $p < 0,0001$.

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CAPÍTULO 1

Antiangiogenic effects of BthMP, a P-I metalloproteinase from *Bothrops moojeni* snake venom

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Abstract

Angiogenesis is the process of forming blood vessels from pre-existing ones. The tumor angiogenesis plays a critical role in the nutrition and oxygen supply during tumorigenic process, and a considerable number of cancers have been reported to be dependent on angiogenesis and responds to antiangiogenic therapies. In this sense, we evaluated both *in vitro* and *ex vivo* antiangiogenic effects induced by BthMP a P-I metalloproteinase from *Bothrops moojeni* snake venom, using the methodologies of *in vitro* angiogenesis and *ex vivo* with aortic mouse ring. BthMP did not show an expressive decrease in cell viability on HUVECs by MTT assay and wasn't able to significantly reduce the capacity of HUVECs to undergo division and produce colonies. Interestingly, BthMP inhibited the adhesion and migration of HUVECs and blocked *in vitro* angiogenesis in a VEGF-dependent manner, an essential proangiogenic factor. In addition, BthMP was capable of inhibiting the sprouting angiogenic process through an ex vivo aortic ring assay. Together these results indicate that BthMP has a potent in vitro and *ex vivo* antiangiogenic effect and represents a biotechnological tool to understand the antiangiogenic response of metalloproteinases in cancer cells.

Keywords: adhesion, antiangiogenic, cancer, metalloproteinase, snake venom.

1. Introduction

Metastasis is responsible for more than 90% of cancer-associated mortality, an event that requires several interconnected molecular mechanisms, including cell adhesion, migration and invasion [1]. Also, tumor angiogenesis, defined as new blood vessels form from pre-existing vessels, plays a critical role in the nutrition and oxygen supply during tumorigenic processes [2]. Thus, inhibiting tumor angiogenesis is a promising approach for treatment of cancers and has been successfully applied in recent years [3] In this scenario, due their therapeutic potential, snake venom-derived toxins have been widely explored for the discovery of new bioactive compounds and stand out as an alternative source for therapeutics for a variety of diseases, including cancer.

Snake venom metalloproteinases (SVMPs) are the majoritaries components of bothropic snake venoms [4]. These toxins can interact with the components of the extracellular matrix, being responsible for local tissue damage, hemorrhage and coagulopathy during an envenomation [5]. According to their domain composition, SVMPs are classified into P-I, P-II and P-III classes. P-I, has only a metalloproteinase domain; P-II, contains a metalloproteinase domain followed by a disintegrin domain; while P-III, comprise catalytic Zn-dependent metalloproteinase, disintegrin-like and cysteine-rich domains [6]. Interestingly, despite being responsible for various toxic effects, the antitumor and antiangiogenic effect of this class of proteins has already been characterized. It was reported that SVMPs are capable of promoting impairment in cell adhesion of melanoma cells [7], cytotoxic effects in melanomas, glioblastomas, and carcinomas [8], inhibition of cell proliferation and induction of changes in cellular morphology [9].

Bothropoidin, a P-III hemorrhagic metalloprotease isolated from *Bothrops pauloensis* snake venom, inhibited apoptosis and migration of breast cancer cells MDA-MB-231, as well as impaired the angiogenesis in an *in vitro* endothelial cell model [10]. BthMP is a weakly P-I hemorrhagic metalloprotease with high proteolytic activity isolated from *B. moojeni* snake venom. This protein has a molecular weight of 23.5kDa, an isoelectric point of 7.1, and represents 2.3% of the crude venom of *B. mooje*ni [11]. Although some studies have addressed the antitumor activity of SVMPs, the role of these proteins on angiogenesis is still unclear. In this context, the present study showed the antiangiogenic properties of BthMP using *in vitro* and *ex vivo* models of angiogenesis,

highlighting their possible use as a promising tool for the development of new therapeutic models to inhibit angiogenesis in different pathological contexts.

2. Materials and Methods

2.1 BthMP Purification

BthMP was purified from *Bothrops moojeni* snake venom by a combination of ion exchange chromatography (DEAE-Sephacel) and molecular exclusion of Sephadex G-75, as previously described by (GOMES *et al.,* 2009) [11].

2.2 Animals

All experiments involving the use of animals were carried out in accordance with the guidelines and regulations of the Committee for the Ethical Use of Animals. BALB/c mice (males, 6 weeks and approximately 25g) kept under standard conditions (light/dark cycle of 12h, temperature 22±1°C, relative humidity 60±5%), with diet and *water ad libitum* were used. The procedures performed with animals were approved by the Ethics Committee on the Use of Animals of the Federal University of Bahia Protocol No.083/2020.

2.3 Cell Culture

The human umbilical vein endothelial cell (HUVEC) was obtained from the American Type Culture Collection (ATCC). The cells were maintained in RPMI cell culture medium supplemented with 10% inactivated fetal bovine serum (FBS), 2mM l-glutamine, 2 mM sodium pyruvate, 1mM non-essential amino acids, 100 U/mL penicillin and 100 g/mL streptomycin at 37°C in a humidified incubator containing 5% CO_2.

2.4 Evaluation of cytotoxicity by MTT assay

HUVECs (3×10^4 cells/well) were seeded into a 96-well plate for adhesion. After 24 h, a new medium containing a two-fold serial dilution of BthMP (ranging from 60 to 0.46875 μg/mL) or culture medium (control) was added and incubated at 37°C and 5% CO_2 for 24 h. After treatment, the cells were incubated with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (5mg/mL, 10μL/well) for 3 h at 37°C. Then, 100μL/well of phosphate buffered saline (PBS) containing 10% of sodium

dodecyl sulfate (SDS) and 0.01 M of HCl (18 h, 37°C and 5% of CO_2) were added. Absorbance was read in a multi-well scanning spectrophotometer at 570 nm.

2.5 Colony formation assay

The colony formation assay was performed as described by FRANKEN *et al.,* 2006 [12] with modifications. HUVECs (3×10^4 cells/200 μL/well) were plated into a 96-well plate and incubated for 24 h at 37ºC in 5% CO_2. Then, cells were incubated with BthMP (40 and 5μg/mL) or culture medium (control group) for 24 h. After treatment, the cells were harvested and seeded (100 cells/2000 μL/well) into a 6-well plate, being kept in culture medium for two weeks at 37ºC at 5% CO_2. After, the culture medium was removed, and then the cells were carefully rinsed with PBS, and fixed with 100% cold methanol for 10 min, followed by staining with Panotic Kit (Laborclin, Brazil). The colonies formed by more than 50 cells were quantified and photographed.

2.6 LDH Assay

The LDH assay was performed according to LEGRAND *et al.,* 1992 [13] with modifications. HUVECs (3×10^4 cells/well) were seeded into 96-well plate microplates. After 24 h, a new medium containing BthMP (40 and 5μg/mL) or culture medium (control) was added and incubated at 37°C and 5% of CO_2 for 24 h. Next, the culture supernatant was collected and submitted to the LDH measurement. Following the instructions of the LDH UV Lactic Dehydrogenase kit (Bioclin), a working solution with four parts of Reagent 1 and one part of Reagent 2 was produced and 20μL of the solution was added to each supernatant corresponding to the treatments and controls. In a microplate of 96 wells, the mixture was read in a plate reader previously heated to 37ºC and the values were verified with an interval of 1 minute. The values obtained were placed in the equation present in the kit after adaptation to the reading in microplate and then analyzed.

2.7 Cell adhesion inhibition assay

In order to investigate the possible interaction between BthMP and components of the extracellular matrix (ECM), a cell adhesion assay were performed using MTT reagent as in the study by GIMENES *et al.,* 2017 [14]. Briefly, 96-well plates were coated with 50 μL of collagen IV (10μg/mL in 0.1M of acetic acid), fibronectin (5μg/mL in PBS)

or matrigel (1mg/mL in PBS) for 18 h at 4ºC. The wells were carefully washed twice with PBS, and blocked with bovine serum albumin (BSA) in PBS (0.1 %, 50 μL). A control without any extracellular matrix coat was performed. For the cell adhesion inhibition experiment, HUVECs (3×10^4 cells/well) were pre-incubated with BthMP (40 and 5μg/mL) or culture medium (control group) for 30min at 37ºC and 5% CO_2, and then seeded into a 96-well plate previously coated or not, and allowed to interact with ECM components for 2 h at 37ºC and 5% CO_2. Finally, the detached cells were removed by two careful washes with PBS, and the adhered cells were quantified by the MTT assay as described above.

2.8 Scratch wound-healing assay

The inhibition of horizontal cell migration was measured by a wound-healing assay as described by JUNG, 2013 [15], with minor modifications [14]. HUVECs (4×10^6 cells/well) were plated into 12-well plate for 24 h. Next, the culture medium was discarded, and culture cell monolayers were scratched with a plastic pipette tip of 200μL. Subsequently, the medium was then replaced either in the absence (control group) or presence of BthMP (40 and 5μg/mL) for 24 h and 48 h. Images were captured with an inverted phase-contract microscope (Nikon Eclipse TS100) immediately after scratching (t = 0h) and upon 24 h or 48 h of treatment (t = Δh). The cell migration toward the wounds was measured by Image J software, and expressed as percentage of wound closure: % wound closure = $[(A_{t=0h} - A_{t=\Delta h})/A_{t=0h}]$ x 100, where "$A_{t=0h}$" h is the area of wound measured after scratching and "$At_{=\Delta h}$" is the area of wound measured 24 h or 48 h after scratching.

2.9 Migration and cell invasion assay

To evaluate the inhibition of vertical cell migration and invasion, a transwell chamber with 8μm membrane was used according to GIMENES *et al.,* 2017 [14]. For cell migration experiment, HUVECs (7×10^4 cell/well) were pre-incubated or not with BthMP (40 and 5μg/mL) for 30 min at 37°C and 5% CO_2 in culture medium without FBS. As control, cells were pre-incubated with culture medium only. After, cells were seeded at the top of the insert chamber, and in the lower compartment was added culture medium with 10% FBS as chemoattractive factor to the cells. As negative control of cell migration, the lower layer was filled with serum-free medium. For the cell invasion assay, the upper

chamber of transwell plates were previously coated with Matrigel (Corning® Matrigel® Matrix, USA) diluted at a ratio of 1:10 (PBS: Matrigel, v/v) with serum-free medium for 1 h, and the cells were added to upper chamber of transwell plates. After 24 h, the migrated or invaded cells were stained with Panotic Kit (Laborclin, Brazil), and the images were captured by using a brightfield microscope (Nikon Eclipse TS100). Cellular migration and invasion rates were determined by counting of the cells in a total of 6 fields examined randomly.

2.10 Matrigel tube formation assay

The influence of BthMP on *in vitro* angiogenesis was evaluated by use of the Matrigel tube formation assay, according to GIMENES *et al.,* 2017 [14]. HUVECs ($5x10^5$ cells/wells) were pre-incubated with BthMP (40 and 5μg/mL) or culture medium (control) supplemented with basic fibroblast growth factor (bFGF) (10ng/mL) for 30min at 37ºC and 5% CO_2. After, the cells were seeded in a 24-well plate previously coated with Matrigel (5.25mg/mL, 50μl) (Corning Matrigel Matrix, USA), being kept at 37ºC in a humidified incubator with 5% CO_2. After 18h, images were acquired at 20 x magnification by using an inverted optical microscope, and the tubes were quantified. Also, culture supernatant was collected and stored at -80 °C for further measurement of VEGF. The morphometric analysis of endothelial tube formation assay was performed using Angiogenesis Analyzer, a toolset of Image J software, which allows the analysis of cellular networks [18,19]

2.11 Nitric oxide (NO) production

The HUVEC cells were plated to $3x10^4$ cells per well in microplates of 96 wells, and after 24 hrs, a new medium containing BthMP (40 and 5μg/mL) or medium (control) was added and incubated at 37°C and 5% CO_2 for 24 h and the supernatant collected after this period. Nitric oxide (NO) levels were quantified from the final concentration of the final metabolite, nitrite, using the Griess assay according to the work of GREEN, *et al.,* 1982 [16]. The 50μL aliquots of the test solution (standard, control, experimental sample) were added to 0.1% of Griess reagent solution (50μL of 3.9 mM N-(1-naphthyl)ethylenediamine in 5% (v/v) of phosphoric acid) and incubated in the dark at room temperature for 10minutes. Sulfanilamide solution (1% in phosphoric acid) was added to the mixture, and the absorbance was measured at 540nm using a microplate

reader. Standard sodium nitrate (0.1 mM) solution was used to construct a standard curve to determine the actual concentrations of the samples.

2.12 Vascular endothelial growth factor (VEGF) quantification

The levels of VEGF were quantified in the HUVEC supernatants that were obtained from the *in vitro* angiogenesis assay by using a CBA Kit for cellular proteins (BD-USA), following the manufacturer's recommendations [14]. Briefly, a standard curve was constructed with different known VEGF concentrations (2500, 1250, 625, 312.5, 156, 80, 40, 20, and 10pg/mL), then 25μL of the Beads Mix (Diluent Beads and Capture Beads) was added. Then, the samples were incubated for 1 h at room temperature and then added 25μL of the MIX PE Detection Reagent, and then incubated for another 2 h at room temperature. After, the samples were rinsed with 500μL of Wash Buffer and centrifuged at 200g for 5 min. The supernatant was then removed and the pellet formed resuspended in 300μL of Wash Buffer. The samples were submitted to the Cytoflex flow cytometer (Beckman Coulter, USA), and the data generated were analyzed by FlowJo software (version 7.6.3). From the data generated by the curve of known concentrations of VEGF, a standard curve was calculated, which allowed the determination of VEGF concentration, expressed in pg/mL from different experimental conditions.

2.14 Total RNA isolation and real-time polymerase chain reaction (RT-PCR)

HUVECs (3.5×10^5 cells/well) were plated in a 24-well plate for 24 h, treated with BthMP (40 and 5μg/mL), culture medium with VEGF (positive control) or culture medium without VEGF (negative control) for 24 h, being kept in incubator at 37ºC and 5% CO_2. After, the total RNA was extracted from cells with RNeasy Microarray kit (Qiagen, USA), according to the manufacturer's instructions. At the end of the extraction process, RNA was treated with RNase-Free DNase (Qiagen, USA) for digestion of possible contaminant fragments of deoxyribonucleic acid (DNA). The concentration (ng/μL) and purity (A260:A230 and A260:A280) of the total RNA were evaluated by spectrophotometry (NanoDropTM 2000, Thermo Fisher Scientific, USA). cDNA was synthesized with 2μg of total RNA using the High-Capacity RNA-to-cDNA^{TM} reverse transcription kit (Applied Biosystem, USA), following the manufacturer's instructions. The gene expression of VEGFA, Angiopoietin-1 and sFlt-1 were quantified by real-time PCR using the Power UpTM SYBRTM Green Master Mix (Thermo Fisher Scientific).

StepOne Plus system (AppliedBiosystem, USA) was used for thermocycling, as described: a cycle of 50°C for 2 min, a cycle of 95 °C for 10 min, 40 cycles of 95 ºC for 15 s, 40 cycles of 60ºC for 1 min and the melting curve (15 to 95ºC, 1 min at 60ºC, 15 s at 95ºC). The specific sequences of primers for genes in real-time PCR analysis were obtained in the literature and the ringing tested by the NCBI BLAST tool (Table 1). The data were normalized by using GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) as a housekeeping gene. The data were obtained from the comparative threshold cycle (Ct) provided by the thermocycler, where the relative expression (foldchange) was calculated using the comparative method ($2^{-\Delta\Delta Ct}$) in relation to the group treated with culture medium only [20].

Table 1: Sequence of primers used in RT-PCR.

Gene	Sequence 5' - 3'	Reference
VEGFA	F: AGGGCAGAATCATCACGAAGT R: AGGGTCTCGATTGGATGGCA	(XU *et al.,* 2019) [21]
ANGIOPOIETIN-1	F: GGCAGTACAATGACAGTTTC R: CTTTGTTGCTTTCATAATCGC	(KIM *et al.,* 2000) [22]
SFLT-1	F: AGGGGAAGAAATCCTCCAGA R: CAACAAACACAGAGAAGG	(ABOU-FAYCAL *et al.,* 2019) [23]
GAPDH	F: CGAGATCCCTCCAAAATCAA R: ATCCACAGTCTTCTGGGTGG	(ABOU-FAYCAL *et al.,* 2019) [23]

F: Forward; R: Reverse

2.14 Mouse aortic ring assay

Ex vivo angiogenesis was evaluated by the mouse aortic ring method. The experiment was carried out according to BAKER *et al*, 2011 [17], with minor modifications. The fragments of the aorta (1mm-1.5mm) were surgically removed from BALB/c mice at six weeks of age. The fragments were washed with ice-cold PBS solution supplemented with 1% penicillin-streptomycin, and then placed on top of matrigel (5.25 mg/mL, 50μL) coated in a 24-well plate, and posteriorly another 50 μL of matrigel was added to cover the fragments. The aortic rings were incubated in RPMI medium supplemented with bFGF (10ng/ml) and 10% FBS for 48 h at 37ºC and 5% CO_2. Following, the aortic rings were treated or not with BthMP (40 and 5μg/mL) for 14 days at two-day intervals. At the end of the experiment, the aortic rings were photographed

under an inverted optical microscope and the all area (expressed in pixel) covered by the sprouts in each image was measured using the Image J software [18, 19].

2.15 Statistical Analysis

The experiments were performed in triplicate and the data were expressed as means ± standard deviation (SD). First, all data were checked for normal distribution. For the parametric data, the statistical differences were determined by the Student's *t*-test or one-way ANOVA, followed by the Tukey post-test. For the non-parametric data, the Mann-Whitney and Kruskal-Wallis tests were used, followed by Dunn's post-test, according to the experimental design (GraphPad Prism Software version 7.0). The data were considered statistically significant when $p < 0.05$.

3. Results and Discussion

3.1 Effect of P-I BthMP at HUVEC cells

Studies addressing the potential use of snake venom-derived toxins for the design of new drugs has been increasingly frequent [24, 25], however, the use of SVMPs as antiangiogenic and/or antitumor agents remains little explored [10,25]. In the present work, we evaluated the antiangiogenic effects of BthMP, a P-I SVMP obtained from *B. moojeni*, through *in vitro* and *ex vivo* experimental models.

Before evaluating the direct impact of BthMP in the angiogenesis process, we assessed the viability of HUVECs exposed to BthMP treatment for 24 h by MTT, colony formation, and LDH assays. Interestingly, BthMP did not promote loss of viability of HUVECs at none of the concentrations tested in relation to the control group (untreated cells) (Figure 1A, B, and C). Based on the cytotoxicity assays and previous studies from our research group [10], we chose the dosage of 5 and 40μg/mL, as nontoxic and safe concentrations to investigate the antiangiogenic effects of BthMP using HUVECs, a normal endothelial cell line.
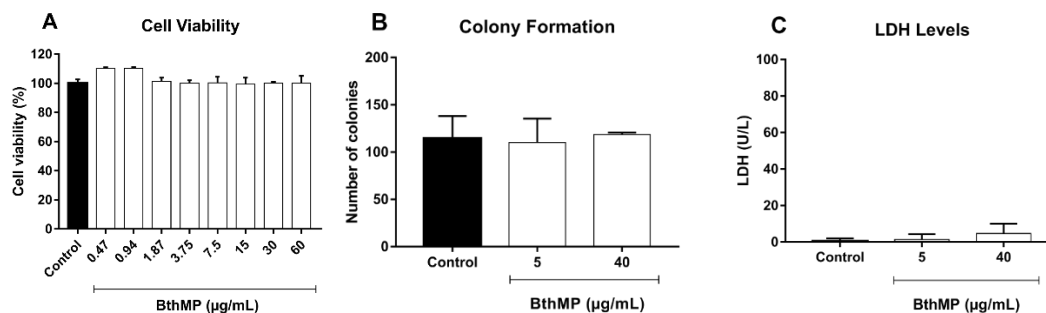
Figure 1. **Viability of HUVECs exposed to the BthMP treatment for 24 h**. **A**) Cell viability assay evaluated by MTT assay. HUVECs were treated with BthMP at different concentrations (0.46, 0.94, 1.87, 3.75, 7.5, 15, 30 and 60μg/mL) for 24 h. Negative control: culture medium. **B**) Cell proliferation assay. The cells were treated or not with BthMP (5 or 40μg/mL). **C**) LDH assay. HUVECs were treated or not with BthMP (5 or 40μg/mL) for 24 h.

3.2 Effect of BthMP at *in vitro* and *ex vivo* angiogenesis

To assess the antiangiogenic effect of BthMP, we first evaluated the *in vitro* vessel formation by HUVECs. BthMP (5 and 40μg/mL) significantly inhibits the bFGF-induced vessel formation compared to the control group (Figure 2A). Also, we observed a reduction of 9,6 and 17,32 in the average number of nodes in the cells treated with the toxin at 5 and 40μg/mL, respectively, compared to the control, having 24,2 (Figure 2B). For the tubules, we also observed reduction of 15,9 at 5μg/mL and 21,6 at 40μg/mL in comparison with control, 32,8 (Figure 2C). In agreement with our data, it was reported that SVMPs from other species of the genus *Bothrops* spp. have also demonstrated ability to inhibit *in vitro* angiogenesis, such as the P-III SVMP Bothropoidin, and the P-I SVMP BpMP-II, both toxins isolated from *B. pauloensis* snake venom [10, 26].
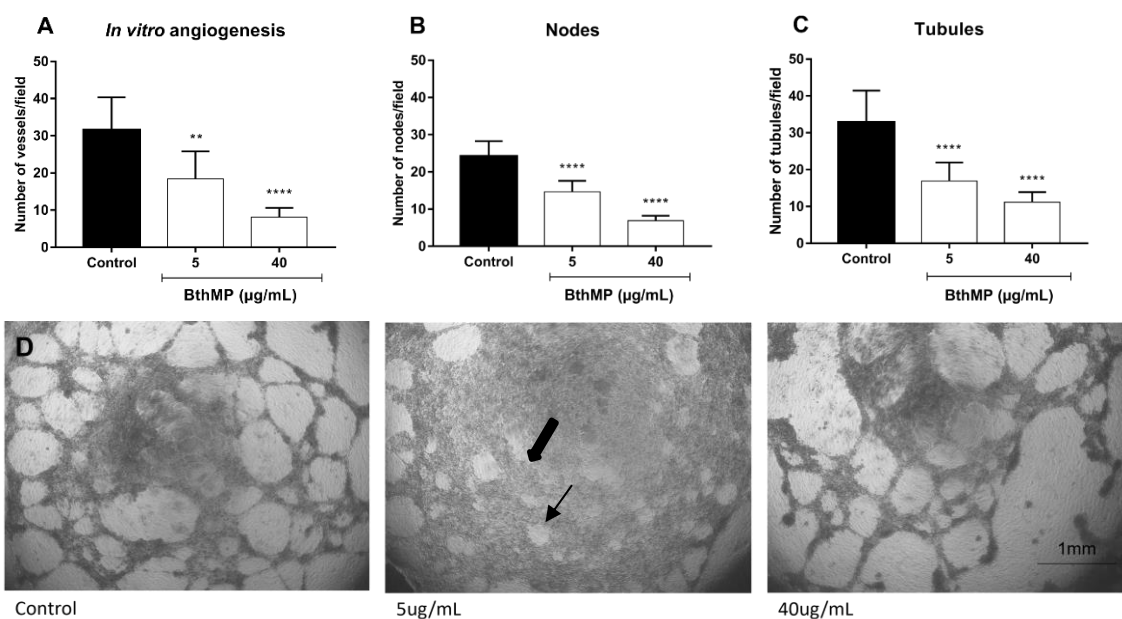

Figure 2. *In vitro* **angiogenesis inhibition assay**. HUVECs were pre-treated with BthMP (5 and 40μg/mL) or medium (negative control) for 30 min and allowed to interact with Matrigel for 18 h at 37ºC and 5%

CO_2. **A**) Number of vessels formed by field. **B**) Number of nodes formed by field. **C**) Number of tubules formed by field. **D**) Representative photomicrography of the formation of vessels in Matrigel with different concentrations of toxin or culture medium (control) [18, 19]. Scale bar: 1mm. Thick arrow: tubules; thin arrow: nodes; Symbol (*) indicates a significant difference when compared to the control group. **p<0.01: ****p<0.0001.

In order to extrapolate our *in vitro* angiogenesis findings, we performed an *ex vivo* aortic angiogenic assay. This assay is an alternative method that allows cultivating native endothelial cells that have quiescent cells, promoting migration, proliferation, formation, and remodeling of vessels [28]. Our data revealed that the treatment of the aortic rings with BthMP for 14 days was able to decrease the area covered by the new vessels emerged from the aortic rings compared to the control, by reducing of 52% at the 5μg/mL, and 66% at 40μg/mL (Figure 3A). Illustrative images highlight the antiangiogenic effect of BthMP (Figure 3B). Similarly, other SVMPs have been used for *ex vivo* angiogenesis assay, such as ADAMTS-15, a disintegrin and metalloproteinase with thrombospondin motifs-15, which were capable of decreasing significantly the total area of formed tubes [29].
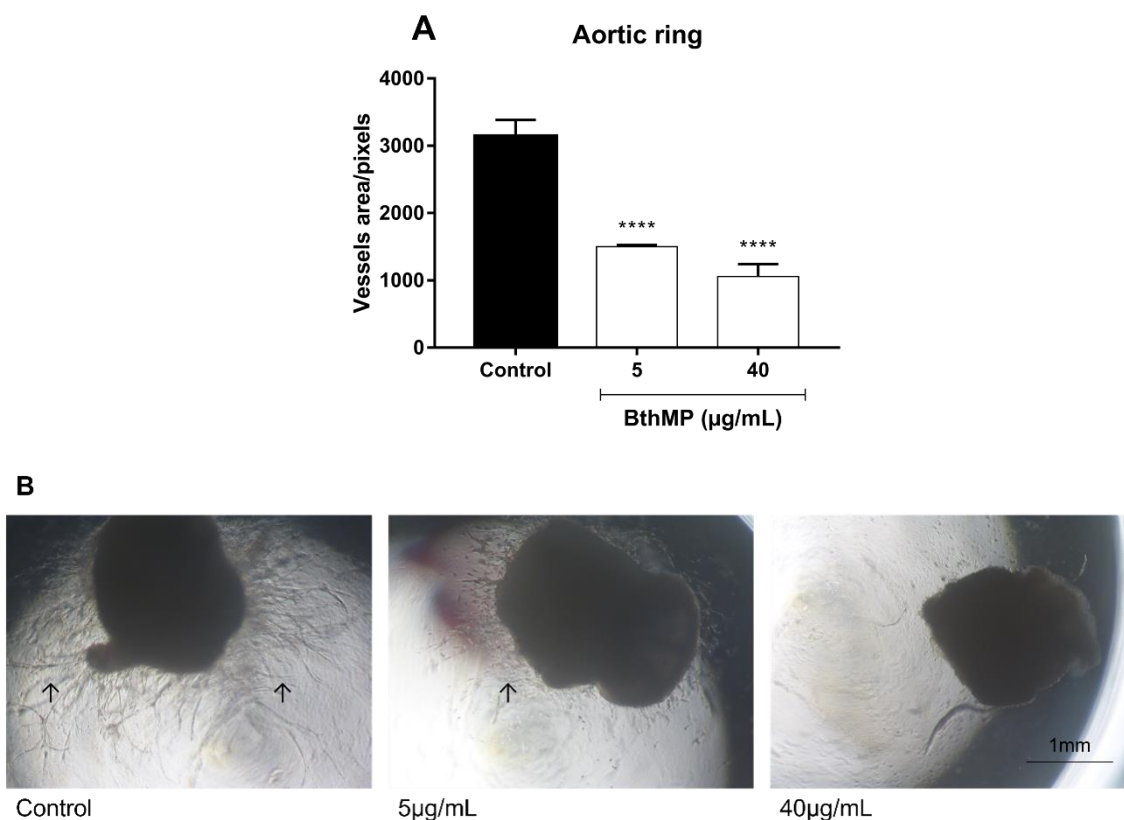

Figure 3. *Ex vivo* **angiogenesis assay**. Aortic rings were treated with BthMP (5 and 40μg/mL) or medium with bFGF (10ng/mL) (negative control) for 14 days at two-day intervals. **A**) Area of the formed vessels. **B**) Representative images of the formation of vessels from aortic rings in Matrigel in the presence of 5 and 40μg/mL of BthMP or culture medium (control). Cellular sprouting (1mm) is indicated by black arrows.

Scale bar: 1mm Symbol (*) indicates a significant difference when compared to the control group. ****p<0.0001.

3.3 Effects of BthMP on cell adhesion and migration

Antiangiogenic therapies can target distinct steps of sprouting angiogenesis, including cell-cell and cell-matrix interaction, proliferation, migration and invasion of endothelial cells [30, 31]. Our findings have revealed that BthMP is able to efficiently inhibit angiogenesis in different contexts. Thus, an interesting question was raised: what would be the direct impact of BthMP treatment on essential steps of the angiogenesis? We set out to assess the impact of BthMP in cell adhesion and migration of endothelial cells.

Firstly, we investigated the possible action of BthMP on cell adhesion inhibition upon different ECM components (*i.e.*, collagen IV, fibronectin, and matrigel). BthMP (40μg/mL) inhibited 33% and 28.5% of HUVEC adhesion in collagen IV and fibronectin, respectively, when compared to the control group (untreated cells) (Figure 4A). Similarly, in the absence of ECM components (no coating group), 40μg/mL of the toxin also reduced cell adhesion about 45.5% in relation to untreated cells (Figure 4A). Curiously, we did not observe any statistical difference when Matrigel was used to coat the plates (Figure 4A).
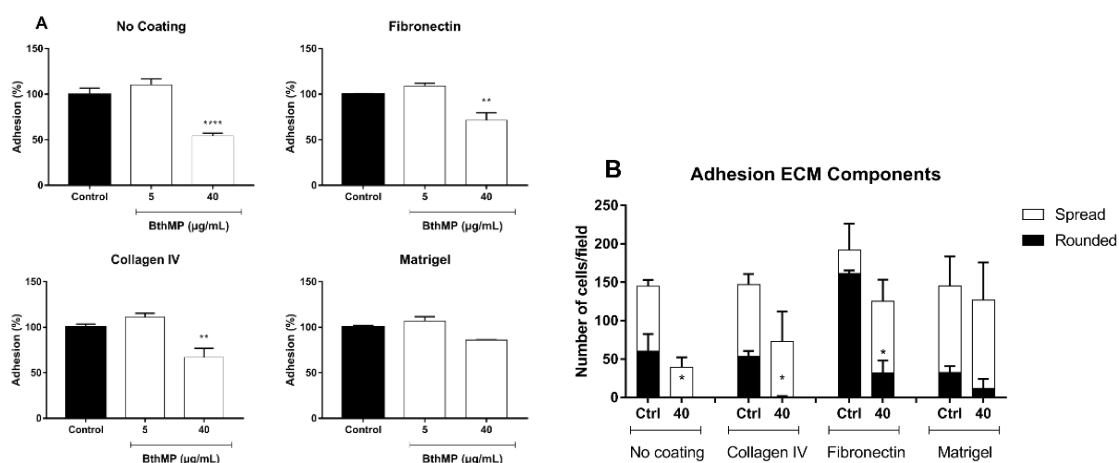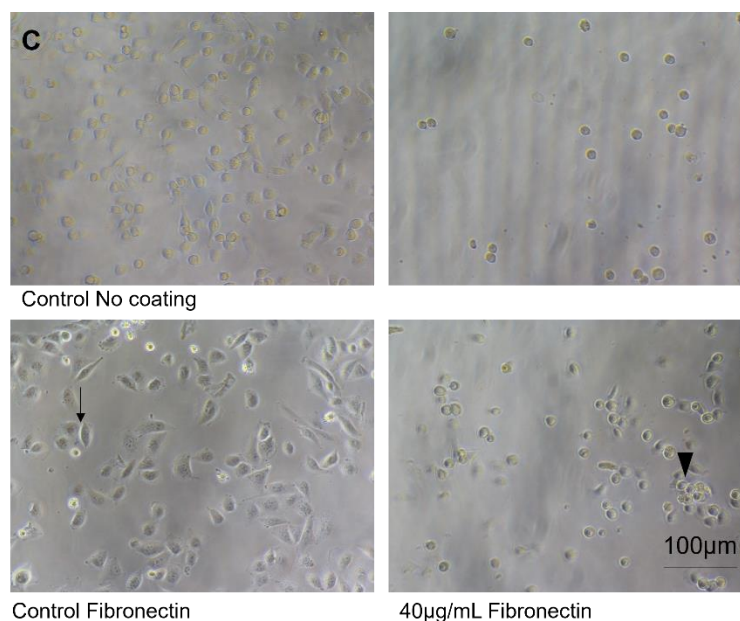
Figure 4. Cell adhesion inhibition assay on different ECM substrates. HUVECs were pre-treated with different concentrations of BthMP (5µg/mL and 40µg/mL) or culture medium (negative control) for 30 min, and plated into a 96-well plate for 2 h at 37°C and 5% CO_2. **A**) Percentage of cell adhesion (%) of HUVECs onto a plate previously coated or not with ECM components. **B**) Amount of spread and rounded HUVEC in three extracellular matrix components with BthMP treatment (40µg/mL). **C**): Representative images of the difference between the two types of cell morphology. Arrow indicates spread cells and arrowhead indicates rounded cells. Scale bar: 100µm Symbol (*) indicates a significant difference when compared to the control group. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

Previous studies have shown the ability of SVMPs to inhibit endothelial cell adhesion. GREMSKI *et al.,*2007 [32] demonstrated that the pre-treatment of rabbit aortic ring cell line (RAEC) with different concentrations of Leucurolysin, a P-I metalloproteinase isolated from *B. leucurus* snake venom, reduced the rate of endothelial cell adhesion to fibronectin. Similarly, it was also reported that other two SVMPs, P-I BpMP-II and P-III Bothropoidin, were able to impair the adhesion of murine endothelial cells upon different ECM components [26, 9].

In our experiments, we observed that the HUVECs presented different structural shapes, with a smaller number of cells spread and more rounded cells, with a structure that was not with the polygonal format and not fully adhered after the treatment (Figure 4B). We observed that without coating, 100% of the cells after treatment with 40µg/mL were only viable, without the presence of cells with a polygonal shape and adherent structure. With the collagen IV substrate, this reduction of adhered cells was 98%, compared to the control, and finally, with fibronectin, after treatment, there was a reduction of 80% in the number of adhered cells compared to adhered cells and with the normal structure for the control group (Figure 4B). Representative imagens highlight the

impact of BthMP on cell morphology (Figure 4C). These differences in the cells' shape after treatment can be also observed in studies with the SVMPs BpMP-II and Bothropoidin [9,26].

Further, we evaluated the horizontal and vertical migration of HUVECs after treatment with BthMP. Regarding wound healing assay, our data demonstrated that BthMP (5 μg/mL) was not able to impair the horizontal migration after 24 and 48h of treatments compared to untreated cells; in contrast, 40 μg/mL of BthMP strongly inhibited the migration in both times analysed in comparison with control group (Figure 5A and B).

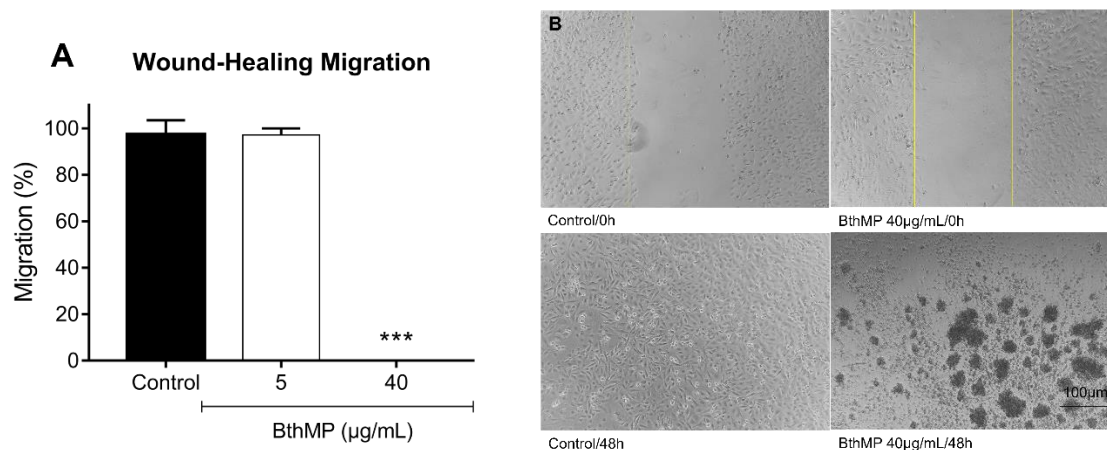**Figure 5. Cell migration by wound-healing assay**. HUVECs were treated with BthMP (5 and 40μg/mL) or culture medium (negative control). **A**) Graphical representation of the percentage of wound closure after 48 h of treatment with BthMP (5 and 40μg/mL). **B**) Wound closure after 48 h of HUVECs in treatment with BthMP 40μg/mL. Scale bar: 100μm. Symbol (*) indicates a significant difference when compared to the control group. ****p<0.0001.

Next, we performed the vertical migration test by transwell. Similarly, BthMP was also able to inhibits the vertical migration only at 40μg/mL in relation to control group after 24 h (Figure 6A, B). One difference between the result of both assays was that the 40μg/mL generated different results when compared to the two techniques however in the horizontal migration assay, the treatment with BthMP generated detachment of the cells from the plate.
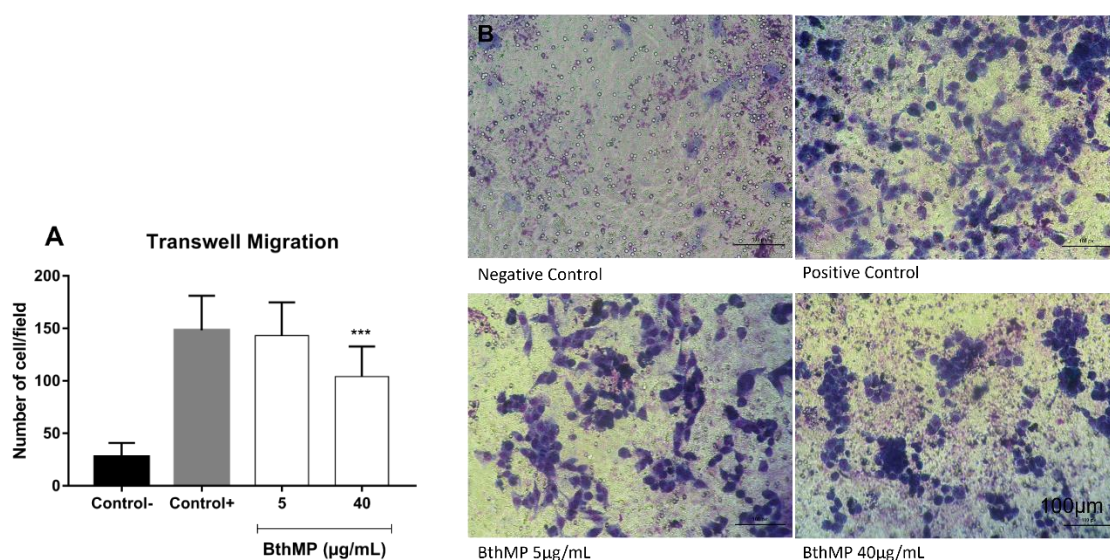
Figure 6. Cell migration by Transwell. The endothelial cells were pre-incubated with different concentrations of BthMP (5 and 40μg/mL) or culture medium. The lower part of the chamber was filled with a culture medium with 10% bovine fetal serum (positive control) and a culture medium without bovine fetal serum (negative control). **A**): Number of HUVEC migrated per field **B**): Photomicrography representative of transwell migration in HUVEC after toxin treatment. Scale bar: 100μm. Images were taken at 40x magnification. Symbol (*) indicates a significant difference when compared to the control group. ***p<0.001.

In agreement with the migration assays, BthMP-treated HUVECs demonstrated an impairment in their ability to invade matrigel only at 40 μg/mL, which was illustrated by a reduction of 43.5% in the number of invaded cells compared to the untreated cells (Figure 7A and B).
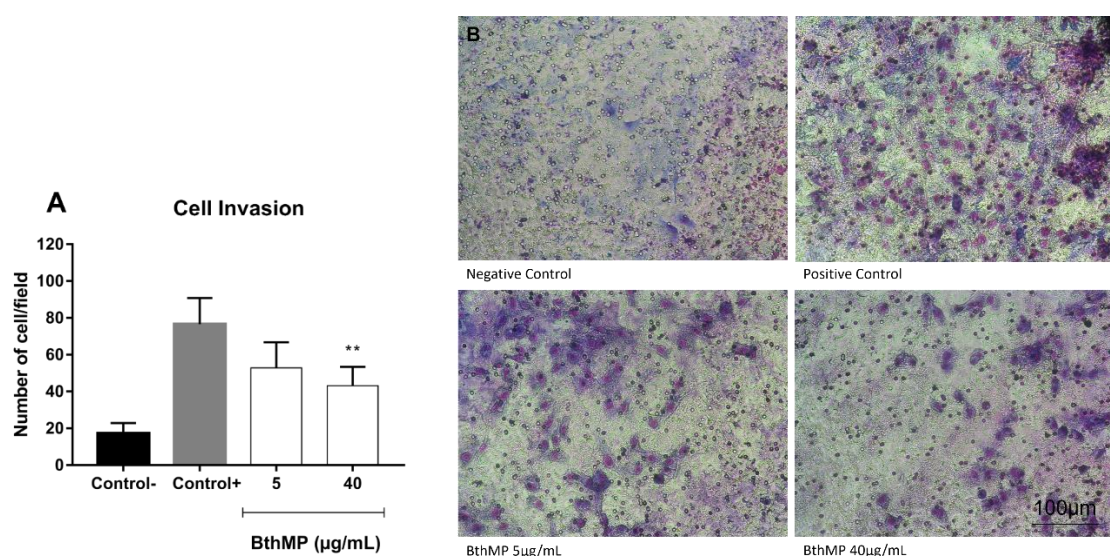

Figure 7. Inhibition of cell invasion by transwell assay. HUVECs were pre-incubated with BthMP (5 and 40μg/mL) or culture medium for 30 min, and added to the upper transwell chamber for 18h at 37°C and 5% CO_2. The lower part of the chamber was filled with a culture medium with 10% FBS (positive control) and a serum-free medium (negative control). **A)** Number of invaded HUVECs by field. **B)** Representative photomicrography of invasion in transwell after treatments with BthMP. Scale bar: 100μm. Symbol (*) indicates a significant difference when compared to the control group. **p<0.01.

The cell-cell and extracellular cell-matrix connection relationships are important for the maintenance of tissue integrity, and during angiogenesis and metastasis, these connections are remodeled, generating changes in shape and cell polarity. The alteration in this process contributes to the increase in invasiveness and metastatic potential [33].

Thus, BthMP was able to generate changes in adhesion, and invasion. For the angiogenic process, the criteria of cell adhesion and invasion are necessary for subsequent cell proliferation, being, in many characteristics, like the invasive behavior of metastatic tumors [33].

3.4 Antiangiogenic factors modulated by BthMP

Angiogenesis is known to be regulated by the balance between various pro- and antiangiogenic signals [34]. Based on our findings, we hypothesized that the interference mediated by BthMP on distinct steps of the angiogenesis process may be related to a direct modulation in the production of angiogenesis-associated molecules. Thus, we further assessed the gene expression of vascular endothelial growth factor A (VEGFA), angiopoeitin-1 (ANGPT-1), and soluble fms-like tyrosine kinase 1 (sFLT-1).

Our data demonstrated that BthMP (5 and 40 μg/mL) significantly downmodulated the expression of VEGFA and ANGPT-1, even in the presence of the recombinant pro-angiogenic VEGF, when compared to untreated cells (Figure 8A and B). As expected, the VEGF treatment regulated positively the expression of VEGFA and ANGPT-1 in relation to control group (Figure 8 A and B). Interestingly, both concentrations of BthMP upregulated the levels of SFLT-1 in VEGF-stimulated HUVECs in comparison with control group; in addition, the treatment with the exogenous VEGF reduced the SFLT-1 expression (Figure 8C). In order to corroborate with our data of gene expression, we also measured the protein levels of VEGFA and nitric oxide (NO). In agreement with the gene expression, we also observed that BthMP (40 μg/mL) reduced about 30% of the protein expression of VEGFA in the culture supernatant of HUVECs compared to untreated group (Figure 8D). Also, BthMP-treated HUVECs had a significant reduction in the NO levels in relation to untreated cells (control group) (Figure 8 E).

Taken together, our findings demonstrated that BthMP treatment downmodulated pro-angiogenic factors (*e.g.,* VEGFA, ANGPT-1 and NO), and upregulated the antiangiogenic factor SFLT-1. The current literature has reported that the NO can plays a

dual role during angiogenesis. The first is to generate an increase in the mitotic, migratory, and degradation of the ECM, favoring the dispersion of new vessels. The second is related to the release of VEGF, an essential growth factor for angiogenesis, which depends on a signaling pathway called NO-cGMP (nitric-guanosine oxide 3',5' cyclic monophosphate). Thus, the decrease in NO levels in the endothelium can compromises the response to VEGF and may result in resistance to neovascularization [35-37].

VEGF-A is one of the components of the VEGF family, which consists of five others that bind in an overlapping way to three receptor tyrosine kinase, VEGFR1, VEGFR2, and VEGFR3. The in vivo angiogenic response to VEGF-A depends mainly on the binding to VEGFR2, which initiates a signaling cascade including other mediators, and may have varied biological effects, including proliferation and survival, vascular permeability, cell migration, invasion of surrounding tissue, and endothelial inflammation [38,39]. BthMP increased the expression of SFLT-1, an endogenous potent VEGF inhibitor, promoting antiangiogenic activity and being produced mainly in endothelial cells [40, 41].

ANGPT-1 binds to the tyrosine kinase TIE-2 receptor and promotes vessel maturation and stabilization of newly formed vessels via the Akt/survivin pathway [2]. The role played by ANGPT-1 varies according to the tumor type evaluated and the conditions, but studies have already been able to use compounds to interfere with its binding to the TIE-2 receptor [42] and use vector for angpt-1 administration and promote increased blood vessels in lung tumor and normal cells, promoting greater dissemination of tumor cells [43]. ANGPT-1 is considered to affect the final stages of the metastasis, fixation, and extravasation process [44].

Nitric oxide has a role in stimulating angiogenesis since VEGF positively regulates NO release [45]. In 3D fibrin gel, human umbilical cord endothelial cells can stimulate NO and form capillary-like structures [46]. Angiogenesis is potentiated after the reduction of nitric oxide bioactivity, where in an ex vivo assay, with rabbit coronary aorta, capillary-like protuberances are inhibited by oxidized LDL cholesterol, an agent that reduces NO bioactivity [47]. Thus, our result, which demonstrates the decrease in the release of nitrates (Figure 8E), is consistent with the antiangiogenic character of BthMP, promoting a reduction in the expression of an important factor that promotes angiogenesis, maintaining significantly in both external visits, attended in 16μM in the concentration of 40μg/mL and 20μM in the 5μg/mL.
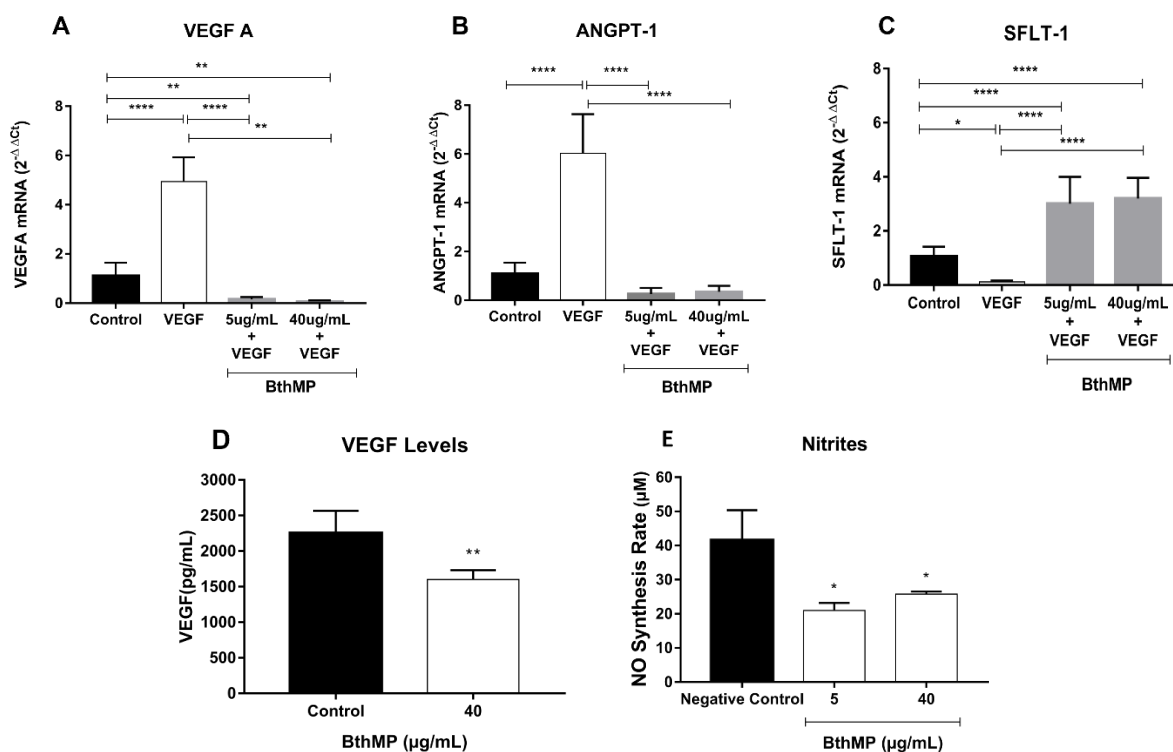
Figure 8. Real-time PCR (RT-PCR) gene expression assay and Nitrite levels in HUVECs supernatants. Expression of pro and antiangiogenic genes after treatment with BthMP (5 and 40μg/mL) or culture medium (Control). **A**) Expression of the VEGFA gene; **B**) Expression of the SFLT-1 gene; **C**) Expression of the ANGPT1 gene; **D**) VEGF dosage of HUVEC supernatant. Symbols (a, b, and c) indicate significant differences between groups, where each letter represents a statistical difference, to differentiate each group. **E**) Nitrite dosage. HUVECs were treated with BthMP (5 and 40μg/mL). Symbol (*) indicates a significant difference when compared to the control group. **p < 0.01.

4. Conclusion

The present study demonstrated the promising antiangiogenic activities of the P-I SVMP BthMP through *in vitro* and *ex vivo* experimental models. We suggested that the BthMP-mediated angiogenesis inhibition is related to an impairment in the processes of adhesion, migration, and invasion. We also observed that BthMP downmodulated and upregulated the expression of pro- and antiangiogenic factors, respectively, which could explain their ability to block angiogenesis. Therefore, BthMP can be used as interesting approach for the discovery of new possible antiangiogenic targets may leading to the development/production of biopharmaceuticals, with greater stability and mechanism of specific action, positively impacting public health worldwide.

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CAPÍTULO 2

Antitumoral effects of BthMP, a P-I metalloproteinase from *Bothrops moojeni*

snake venom, in lung cancer cells

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Abstract:

This work shows for the first time the antimetastatic effect of BthMP, a P-I metaloproteinase isolated from *Bothrops moojeni* venom on lung cancer cells. The results showed that BthMP reduced colony formation by clonogenic assay and increased LDH levels of A549 cells, showing a cytotoxic effect at concentrations of 40 and 5 μg/mL. BthMP specifically inhibited the invasion, migration, and adhesion, and increasing the levels of reactive oxygen and nitric oxide species in the tumor cell. Indeed, it was also possible to observe that BThMP pre-treated with EDTA was not able to reduce the A549 cell migration. In conclusion, these results showed that the BthMP has a significant antitumor effect in lung cancer and represents an important biotechnological tool to design new therapeutic strategies for treatment of lung cancer patients.

Key words: adhesion, antitumoral, cancer, metalloproteinase, snake venom.

1. INTRODUCTION

Lung cancer is the second most prevalent type of cancer and the first in death numbers, representing 11.4% of cases and 18% of cancer deaths [1]. In Brazil, for the triennium 2020–2022 are estimated 30,000 cases, corresponding to 4.8% of the total number of predicted cases [2]. According to the Global Cancer Statistics (GLOBOCAN), an organ of the International Agency for Research on Cancer, in 2019, cancer was the leading cause of death in the world, ranking first or second in people under 70 years of age in 112 of 183 countries in the world, in addition to occupying third or fourth place in another 23 countries in the same age group [1].

Lung cancer present huge genetic and phenotypic heterogeneity across the individuals being the one of the major obstacles to treat of cancer patients [1]. Therefore, many efforts are still mandatory to find new therapeutics molecules that can potently inhibit cancer cells.

Metastasis is a cancer-related process that is responsible for more than 90% of cancer mortality and is an event that requires distinct molecular mechanisms to occur, such as invasion, adhesion and migration by the

extracellular matrix [3,4]. In addition is necessary the angiogenic process, which is responsible for the subsistence of malignant cells, thus providing nutrients and facilitating their dissemination through metastasis [5]. Thus, preventing these processes can lead to the inhibition of metastasis and angiogenesis [6].

In this sense, the search for new terapeutic components in snake venoms, have been relevant. Snake venom metalloproteinases (SVMPs) are present in the most of snake venoms in the Viperidae and Crotalidae families and can trigger local hemorrhaging, inflammation and other local and systemic effects by causing changes in blood coagulation or interaction with the main components of the extracellular matrix such as collagen, laminin, and fibronectin [7-9]. SVMPs can be classified according to their domain organization in PI, P-II and P-III class. PI SVMPs exhibits only the metalloproteinase domains; P-II contains a metalloprotease domain followed by a disintegrin domain, and P-III has a metalloproteinase domain, a disintegrin type domain, and a cysteine-rich domain. Groups P-II and P-III still have subclasses, presenting variations in the products of enzyme processing [10].

Despite causing toxic effects, SVMPs have been studied, and their antitumor effects have been demonstrated. Jararagin, class P-III metalloprotease isolated from *Bothrops jararaca* venom, reduces the viability in of cell human melanoma Skmel-28, also promoting a inhibition of adhesion, migration and invasion [11]. Bothropoidin, a P-III, isolated from *Bothrops pauloensis* venom, has a significant effect on the cell cytotoxicity of the breast cancer cell, MDA-MB-231, and induced reduction in the *in vitro* formation of vessels in murine endothelial line, tEnd [12].

BthMP is a weakly hemorrhagic metalloprotease with high proteolytic activity isolated from the venom of *Bothrops moojeni*. It belongs to the P-I class, has a molecular weight of 23.5kDa, and represents 2.3% of *Bothrops mooje*ni crude venom [13].

The present study aims to characterize, through the use of different techniques of cell culture, the antitumor effect in lung cancer of the BthMP metalloprotease isolated from the venom of *Bothrops moojeni*. The results found here can provide important information for the development of new therapeutic strategies for treatment of lung cancer patients.

2. METHODOLOGY

Cell Culture

Human lung cells (A549) and bronchial epithelial cell line (BEAS-2B) were obtained from the American Type Culture Collection (ATCC) (ATCC® HTB-26) (Manassas, VA, USA). The cells were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 2mM sodium pyruvate, 1mM nonessential amino acids, 100U/mL penicillin and 100mg/mL streptomycin, and maintained at 37 °C in 5% CO_2.

Animals

Male BALB/c mice weighing 20–30g were maintained for two days in a 12:12 h light: dark cycle and received water and food ad libitum, before experiments. All experimental procedures were conducted according to the ethical parameters proposed by the International Society of Toxinology and the Brazilian College of Experimental Animals and were approved by the Ethical Committee for the Use of Federal University of Bahia, Instituto Multidisciplinar em Saúde, Campus Anísio Teixeira (Protocol 082/2020).

BthMP Purification

BthMP was purified from *Bothrops moojeni* snake venom by a combination of ion exchange chromatography (DEAE-Sephacel) and molecular exclusion of Sephadex G-75, as previously described by (GOMES et al., 2009) [13].

Evaluation of BthMP Cytotoxicity by MTT Assay

The viability of A549, BEAS-2B cells was evaluated by the MTT assay, according to GIMENES *et al.*, 2017 [14]. Briefly, ($3x10^4$ cell/well), were seeded in 96-well microplates for 24 hours at 37°C and 5% CO_2, and treated with BthMP (60; 30; 15; 7.5; 1.875; 0.9375; 0.46875μg/mL) or medium (control). The plates were maintained at 37°C for 24h. After, cells were incubated with 10μL/well of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (5mg/mL diluted in PBS) and maintained for 3 h at 37ºC. The formazan crystals

were dissolved in 100μL phosphate buffered saline (PBS) with 10% SDS and 0.01N HCL for 18h until complete dissolution of crystals; then the absorbance at 570nm was determined (Multiskan GO Thermo Scientific, USA). Cell growth inhibition (%) = [1 - (OD Treated/OD Control)] × 100.

Colony formation assay

The colony formation assay was performed according to the FRANKEN *et al.,* 2006 [15] with modifications. The cell lines A549 and BEAS-2B ($3{\times}10^4$ cells/well) were seeded in microplates of 96 wells and incubated for 24 h at 37ºC in 5% CO_2. Following the treatment with BthMP at concentrations of (40 and 5μg/mL) or culture medium (control group) for 24 h. After treatment, cells were trypsinized, and 100 cells per well were transferred to 6-well plates, being kept in a culture medium for two weeks at 37ºC at 5% CO_2. After that, the removal of the medium occurred, washing the cells with PBS, fixation of the same with cold methanol for 10 min, followed by staining with Panotic Kit (Laborclin, Brazil). The colonies formed by more than 50 cells were quantified and photographed.

LDH Release Assay

The LDH release assay was performed according to the work of LEGRAND *et al.,* 1992 [16], with modifications. A549 and BEAS-2B cells were seeded at $3{\times}10^4$ cells per microplate of 96 wells. After 24hours, BthMP (40 and 5μg/mL) or medium (control) were added and incubated at 37°C and 5% CO_2 for 24 hours. After, the supernatant was collected and the LDH release assay was performed according to LDH UV Lactic Dehydrogenase kit (Bioclin) instructions.

Inhibition of cellular interaction with extracellular matrix components

To determine interactions between BthMP and extracellular matrix components, collagen IV (10μg/mL in 0.1M of acetic acid), fibronectin (5μg/mL in PBS), or matrigel (1mg/mL in PBS) were coated on microtiter plate wells and incubated overnight at 4º C. After blocking with 1% of BSA, the cells A549 and BEAS-2B ($3{\times}10^4$ cells/well) were pre-incubated with BthMP (40 and 5μg/mL) or medium (control group) for 30min at 37ºC. After, cells were seeded and incubated for 2h to 37ºC in 5% CO_2 Two of them. The unbound cells were removed by

washing with PBS, and adhered cells were quantified by the MTT assay as described above.

Wound Healing Essay

The inhibition of cell migration was evaluated by the wound-healing assay according to JUNG, 2013 [17] with modifications of GIMENES *et al.,* 2017[14]. A549, BEAS-2B (4 x 10^6 cells/well) cells were seeded in 12-well plates and incubated overnight. After 24h, the medium was removed and a wounding was created in the cell monolayer with a 10μL pipette. Next, cells were incubated for 2h, and treated with BthMP toxin (40 and 5μg/mL) or with a medium (control) for 24h and the result was observed under an inverted optical microscope.

Migration and Cell Invasion by Transwell

To evaluate the inhibition of cell migration and invasion, the Transwell chamber with an 8μm membrane was used according to GIMENES *et al.*, 2017 [14] Cells A549 and BEAS-2B were pre-incubated with BthMP (40 and 5μg/mL) for 30 min at 37°C and 5% CO_2 in a culture medium without FBS. After incubation, 7×10^4 cells/well were seeded at the top of the insert chamber, and in the lower compartment was added medium with 10% FBS as chemoattractive to the cells. In negative control, the lower layer was filled with a culture medium without bovine fetal serum. In the cell invasion assay, using cells A549 and BEAS-2B, the cell culture inserts were previously coated with Matrigel (Corning® Matrigel® Matrix, USA) diluted at a ratio of 1:10 (PBS: Matrigel, v/v) with a medium without FBS (overnight), before the cells were seeded. The migrated or invaded cells were flushed with Panotic Kit (Laborclin, Brazil), and the cells were photographed and counted. The control and positive were represented by cells with medium and 10% FBS, and the negative control was represented by cells with medium without FBS.

Nitric oxide (NO) production

The A549 and BEAS-2B (3×10^4 cells per well) were seeded in 96 microplates, and after 24 hours, BthMP (40 and 5μg/mL) or medium (control) was added to the cultures and incubated at 37°C and 5% CO_2. After 24 h, the

supernatant was collected to nitric oxide (NO) quantification. NO levels were quantified from the final concentration of the final metabolite, nitrite, using the Griess assay according to the work of GREEN, *et al.,* 1982 [18]. The 50µL aliquots of the test solution (standard, control, experimental sample) were added to 0.1% of Griess reagent solution (50µL of 3.9 mM N-(1-naphthyl)ethylenediamine in 5% (v/v) of phosphoric acid) and incubated in the dark at room temperature for 10minutes. Sulfanilamide solution (1% in phosphoric acid) was added to the mixture, and the absorbance was measured at 540nm using a microplate reader. Standard sodium nitrate (0.1 mM) solution was used to construct a standard curve to determine the actual concentrations of the samples.

Production of Reactive Oxygen Species (ROS)

The production of reactive oxygen species (ROS) in Cells A549 and BEAS-2B was performed according to intracellular peroxide-dependent oxidation of 2',7'-dichlorodihydrofluorescein (H2DCF-DA) (Invitrogen, catalog number: D399) to form the fluorescent compound 2',7'-dichlorofluorescein (DCF), as described above [19].The A549 and BEAS-2B cells were plated to $3x10^4$ cells/well in dark microplates of 96 wells. After 24h, with the support, a new medium containing BthMP (40 and 5µg/mL) or medium (control) was added and incubated at 37°C and 5% CO_2 for 24h. After treatment, the cells were washed with 1x PBS and incubated with 150µL of H2DCF-DA (10µM; diluted in 1x PBS containing 10% Bovine Fetal Serum) for 45 minutes at 37°C and 5% CO_2 in the dark. DCF fluorescence intensity was immediately detected using a GloMax Explorer multi-well scanning spectrophotometer (Promega, Madison, WI, USA). The results were presented as fluorescence intensity. Hydrogen peroxide (H_2O_2) was used as a positive control of ROS production and the negative control corresponds to cells treated only with a culture medium.

Statistical Analyses

The data of this work were presented by an average of 3 experiments and standard deviation. Data were checked for normal distribution. For parametric data, the statistical differences were determined by the Student's T-tests or one-

way ANOVA, followed by the Tukey post-test. For non-parametric data, the Mann-Whitney and Kruskal-Wallis tests were used, followed by Dunn's post-test, according to the experimental project (GraphPad Prism Software version 7.0). Data were considered statistically significant when $p<0.05$; $p<0.01$; $p<0.0001$.

3. RESULTS AND DISCUSSION

Lung cancer is one of the most aggressive malignances that lacks an efficient therapy being an important public health concerns. In this study, we demonstrate for the first time the antitumoral effects of a P-I SVMP BthMP isolated from *B. moojeni* venom on A549 lung cancer cells. The selective cytotoxicity of BthMP for cancer cells was investigated using no tumorigenic lung cell line, BEAS-2B.

The cytotoxic effect of BthMP on lung cancer cells, was first evaluated by MTT assay. The results showed that BthMP was not able to induce significant reduction in viability of A549 and BEAS-2B cells at none of the concentrations evaluated (Figure 1).
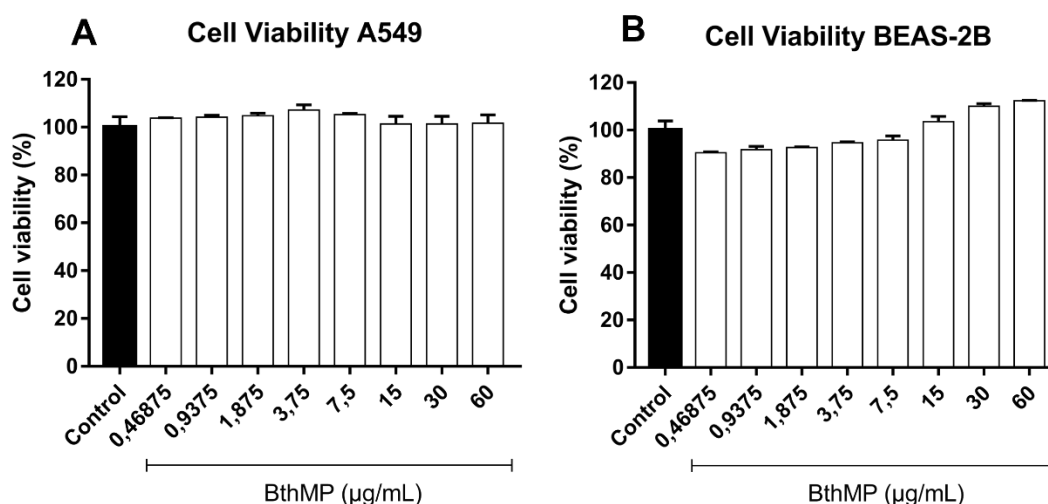

Figure 1. Cell Viability assay by MTT (A) Cell viability by MTT assay. A549 cells were treated with BthMP at different concentrations (0.46875 µg/mL, 0.9375µg/mL, 1.875µg/mL, 3.75µg/mL, 7.5µg/mL, 15µg/mL, 30µg/mL and 60µg/mL) for 24h. Negative control: culture medium. **(B)** Cell viability assay evaluated by the MTT assay. BEAS-2B cells were treated with BthMP at different concentrations (0.46875 µg/mL, 0.9375µg/mL, 1.875µg/mL, 3.75µg/mL, 7.5µg/mL, 15µg/mL, 30µg/mL and 60µg/mL) for 24h. Negative control: culture medium.

Similar results were described by an isolated metalloprotease of *Naja atra* venom, Atrase A. This toxin did not show cytotoxicity on A549 and K562 cells in MTT assay, but were able to detached adherent A549 cells from the substrate

[20]. Next we evaluated the influence of SVMP on the release of LDH by cells at 5 and 40µg/mL. These concentrations were defined according to previous studies that describe the cytotoxic effect of SVMPs [21]
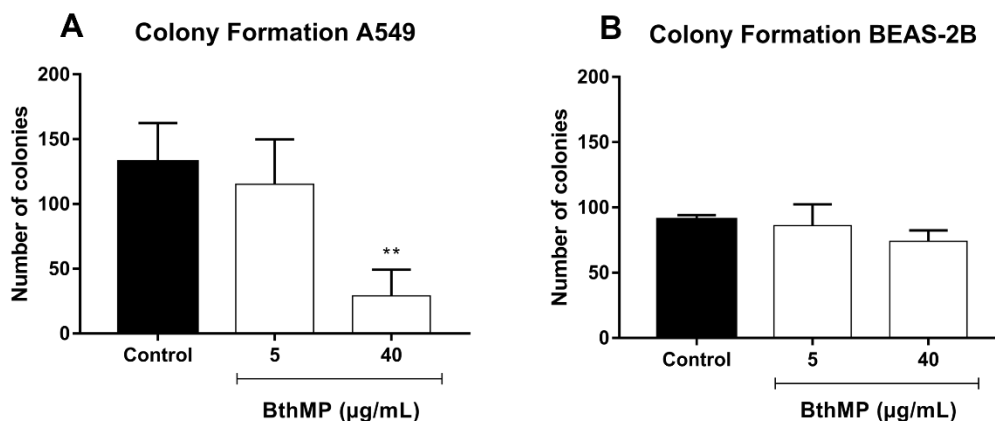

Figure 2. **Colony Formation assay**. A549 and BEAS-2B cells were treated with different concentrations of BthMP (5µg/mL and 40µg/mL) or culture medium. **(A)** Number of A549 colonies formed. Negative control: culture medium. **(B)** Number of BEAS-2B colonies formed. Negative control: culture medium. $**p < 0.01$.

BthMP induced a significative increase in LDH levels in both A549 and BEAS-2B cells at 40µg/mL (Figure 3). As show the figure 3, the level of LDH was 193,4 and 117,2 units in A549 and BEAS-2B cells respectively.

Subsequently, the cytotoxic effect of BthMP was also investigated by colony formation assay, at 40µg/mL and 5µg/mL Our results showed that BthMP induces significant reduction in viability of A549 cells at 40µg/mL(Figure 2A). Interestingly, in the same concentration, BthMP did not significantly affect the viability of non-tumorogenic BEAS-2B cells (Figure 2B). Thus, these 3 cytotoxic assay which might suggest that this toxin may preferentially target cancer cells
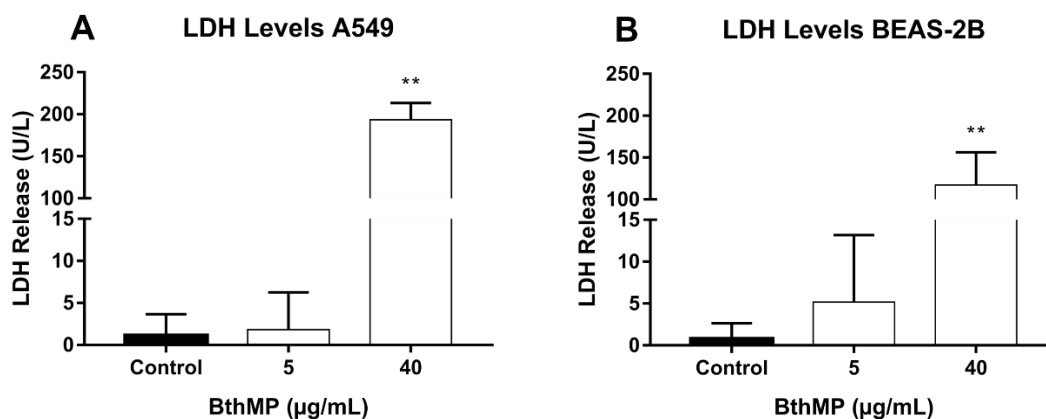

Figure 3. **LDH level.** Cells A549 and BEAS-2B were treated with different concentrations of BthMP (5µg/mL and 40 µg/mL) or culture medium. **(A)** LDH release in A549 cells in U/L **(B)** LDH release in BEAS-2B cells in U/L. $**p < 0.01$.

Although MTT cell survival assay is widely used for measuring cytotoxic potential of a compound, this method may overestimate the cytotoxicity by not considered the reversible damage or regrowth of cells resistant to the cytotoxic agent [22]. Like so, the association of a long-term assay, such as clonogenic assay and a biochemical assay as LDH release, decreasing the chance of underestimating the observed values [23]. The clonogenic assay is considered a gold standard test to detect the cytotoxic effect of anticancer agents [24]. Other studies have used the clonogenic assay to further confirm the cytotoxic effect of snake venom toxins, such as BnSP-7, a phospholipase A_2, isolated from *Bothrops pauloensis* venom, which was able to reduce the number of colonies formed in human endothelial cells (HUVEC) [24]. In addition to the crude venom of different snakes, tested in breast tumor cell line (MDA-MB-231), generating a reduction of over 85% [25].

Leucurolysin-B (leuc-B), a class PIII metalloproteinase isolated from *Bothrops leucurus*, also showed a notable cytotoxic effect against of many câncer cell lines, such as breast cancer, glioblastoma, Ehrlich ascites carcinonoma and melanoma [26].

Many mechanisms have been proposed to describe the anti-cancer activity of snake venom isolated components such as apoptosis, inhibition of signaling pathways, and angiogenesis reduction [27]. Thus, the ability of BthMP to inhibit the cell invasion of A549 and BEAS-2B were investigated using 40 μg/mL and 5 μg/mL. The results showed a significantly reduction of 83% and 57% in A549 cells invasion when 40μg/mL and 5μg/mL were used, respectively. In the non-tumor cells, there was a significant reduction (53%) only when the highest concentration was used. No significant effect on cell invasion was observed when treated with 5μg/mL (Figure 4).
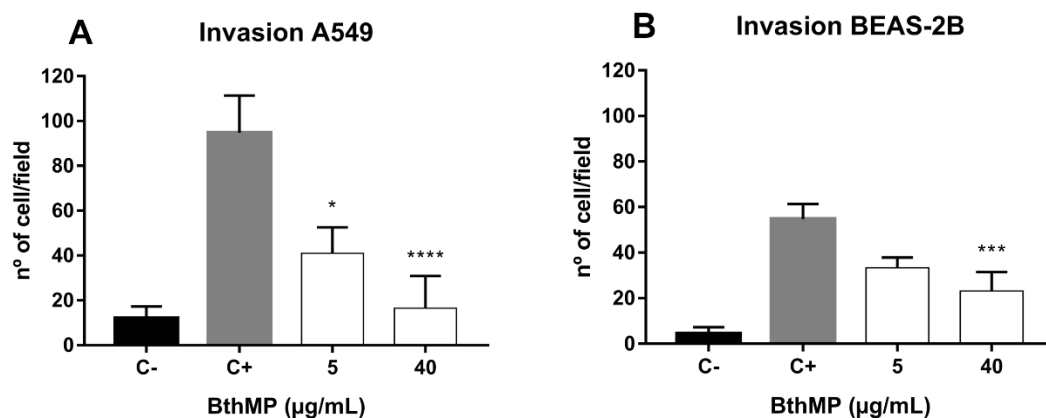
Figure 4. Inhibition of cell invasion by Transwell. Cells A549 and BEAS-2B were pre-incubated with different concentrations of BthMP (5μg/mL and 40μg/mL) or culture medium. The lower part of the chamber was filled with a culture medium with 10% bovine fetal serum (positive control) and a culture medium without bovine fetal serum (negative control). **A)** Number of A549 cells invaded per field. **(B)** Number of BEAS-2B cells invaded per field. *P< 0.05; ***p<0.001; p<0.0001.

Similar results were found by CORRÊA *et al.*, 2002 [11] that studied the antitumoral effect of a P-III SVMP jararhagin from the venom of *Bothrops jararaca*, on Skmel-28 lineage of human melanoma and observed that concentrations of 0.4μM and 0.8μM of jararhagin was able to inhibit the cell invasion d in 28.6% and 22.9% respectively. The metastatic process is crucial for tumor progression and are related to the invasion of cells from the primary tumor through adjacent tissues, basal membranes and/or blood circulation, migration, substrate adhering, and subsequent proliferation of cancer cells [28]

We proceeded evaluating the interference of BthMP on cell migration by two different methodologies. Horizontal migration was evaluated by wound healing method. The results showed that the lowest concentration of 5μg/mL of BthMP was able to promote a 57% of migration reduction in the A549 cells. In contrast, the toxin were able to affect only 19% in the non-tumor BEAS-2B cells after 48h of treatment (Figure 5).
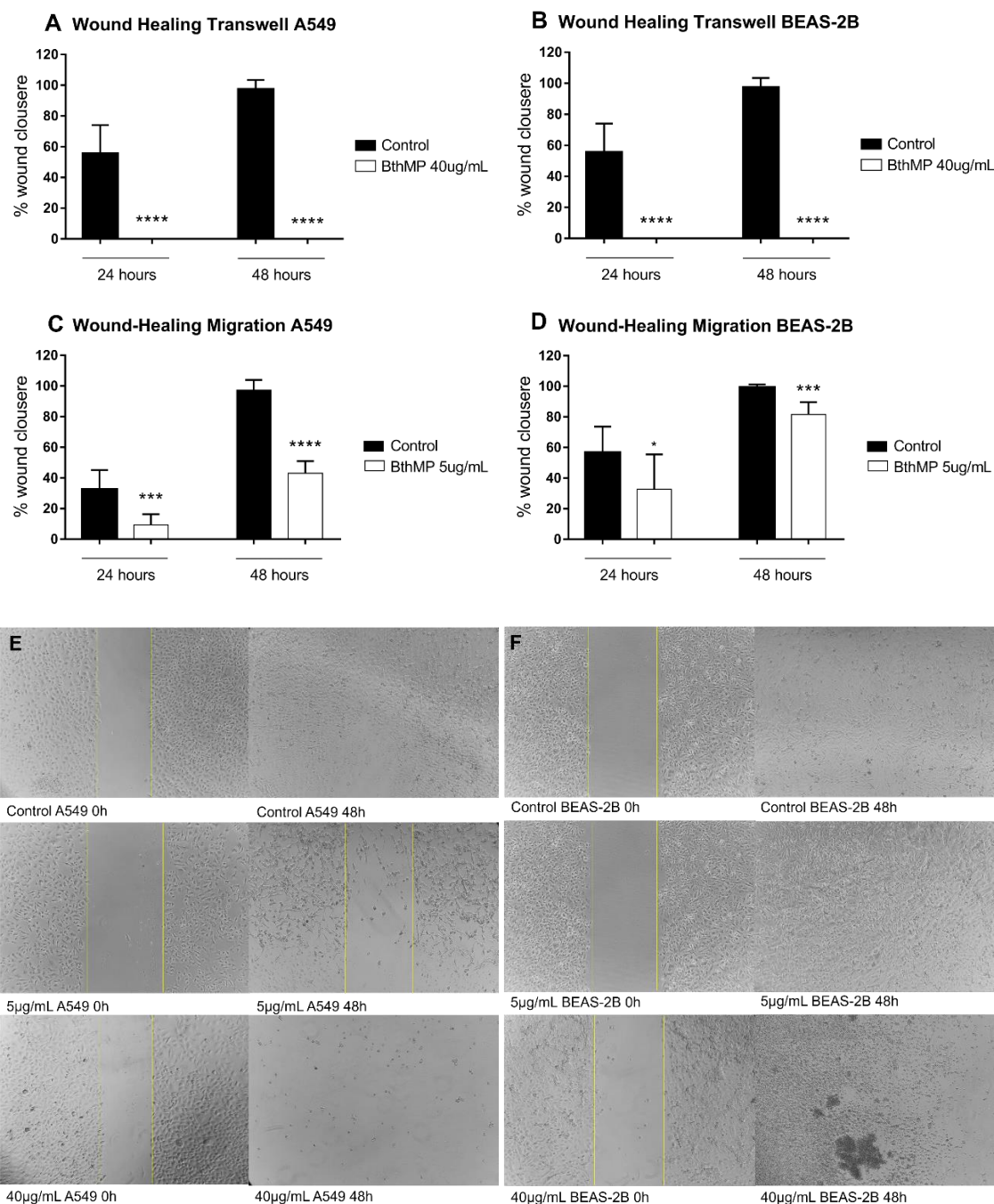
Figure 5. Cell migration test by Wound-Healing. The endothelial cell lines A549 and BEAS-2B were treated with different concentrations of BthMP (5μg/mL and 40 μg/mL) or medium (negative control). A) Percentage of wound closure in A549 cells after 24h in treatment with 40μg/mL of BthMP; B) Percentage of wound closure in BEAS-2B cells after 24h in treatment with 40μg/mL of BthMP; C) Percentage of wound closure in A549 cells after 24h in treatment with 5μg/mL of BthMP; D) Percentage of wound closure in BEAS-2B cells after 24h in treatment with 5μg/mL of BthMP; E) Wound closure after 48h of A549 and BEAS-2B in treatment with BthMP 5 and 40μg/mL. Symbol (*) indicates significant difference when compared to control group *p< 0.05; ***p<0.001; ****p < 0.0001.

The effect of other SVMPs in migration of cancer cells was already demonstrated in other study. In the work of GUIMARÃES *et al.*, 2017 [14] Botropoidin, a P-III SVMP isolated from the venom of *Bothrops pauloensis*, was

able to reduce by 45% of horizontal migration of MDA-MB-231 cells from breast cancer.

Regarding the morphological alteration resulting from treatment with BthMP, the ability of SVMP to alter cell morphology after treatment is previously described. The effect of TSV-DM, a P-III metalloprotease isolated from *Trimeresurus stejnegeri* on ECV304 cells, induced morphological changes in cells after treatment with the toxin at a concentration of 20µg/mL and after 24h, promoting the loss of polygonal format [29]. In other study, MASUDA, *et al.*, 2001 [30] observed a similar result with SVMP HV1 isolated from the venom of *Trimeresurus flavoviridis*, where the concentration of 1.5µg/mL has already been able to promote structural as well as biochemical changes.

Vertical migration through the use of transwell were used in order to better understanding the effect of BthMP on cell migration. The results showed that, concentrations of 40µg/mL and 5µg/mL of toxin, were to reduce by 70% and 60% of BEAS-2B migration, respectively (Figure 6). In contrast, the treatment of BEAS-2B with BthMP induced a significant reduction on cell migration (60%) only when the highest dose was used. As observed by SELISTRE-DE-ARAÚJO [31], alternagin-C, a disintegrin-like from *Bothrops alternatus* was able to inhibit the vertical migration of breast cancer line MDA-MB-231 in concentrations ranging from 0.1-10nM.
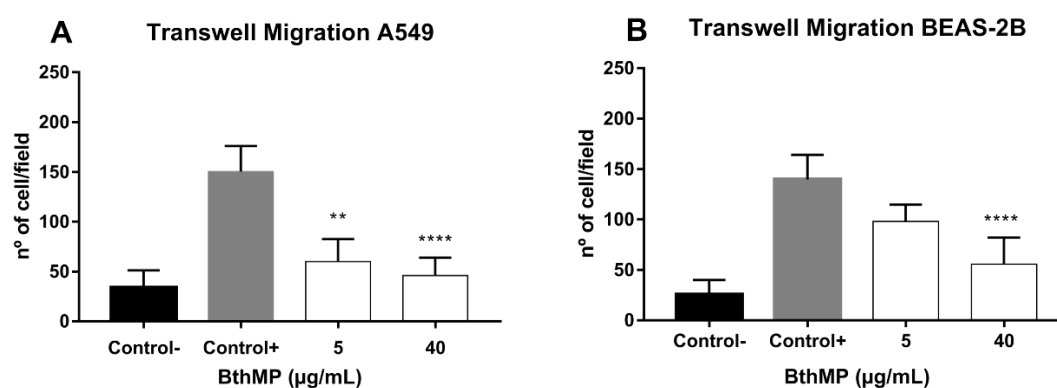

Figure 6. Cell migrationby Transwell. Cell lines were pre-incubated with different concentrations of BthMP (5µg/mL and 40 µg/mL) or culture medium. The lower part of the chamber was filled with a culture medium with 10% bovine fetal serum (positive control) and a culture medium without bovine fetal serum (negative control). (A): Number of A549 cells migrated per field (B): Number of BEAS-2B cells migrated per field. Symbol (*) indicates significant difference when compared to control group *p< 0.05; ****p < 0.0001.

BthMP presentes only catalytic domain and probably inhibits adhesion, migration and invasion by binding catalytic activity. In order to investigate the role of catalytic activity of BthMP effect, we evaluated the migration of A549 cells

treated with BthMP preincubated with EDTA. As show the Figure 7, that removal of the zinc from catalytic site by EDTA inhibit completely the effect of toxin on cell migration of A549 and Beas. BpMP-II, a PI SVMP isolated from *Bothrops pauloensis* snake venom, was also able to inhibitin vitro angiogenesis probably by a proteolytic mechanism [32]. However, Bothropoidin, from *Bothrops pauloensis* presents the disintegrin-like domain and probably inhibits adhesion and angiogenesis also by binding to integrins on the cell surface [14]. In addition, the SVMPs can interfere with the viability and adhesion of endothelial cells by degradation of extracelular matrix proteins [33, 34].
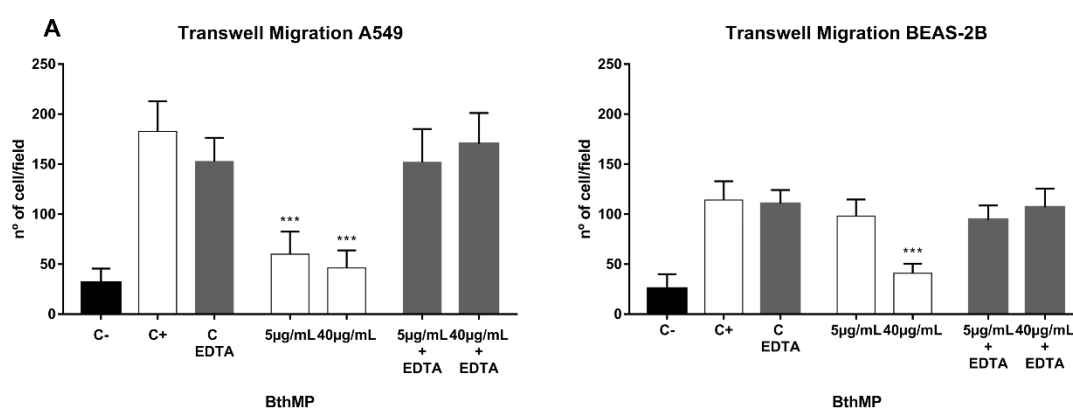

Figure 7. A) Transwell Migration assay. A549 and BEAS-2B cells were treated with different concentrations of BthMP (5µg/mL and 40µg/mL) or medium (negative control) or EDTA 1µg/mL + BthMP solution (5µg/mL or 40µg/mL). Symbol (*) indicates a significant difference when compared to the control group. ***p<0.001

The interaction of tumor cell to components of the extracellular matrix is one of the most important parameter for the metastatic process. Therefore, we investigated the capacity of BthMP in inhibit the adhesion of A549 and BEAS-2B cells to Fibronectin, Matrigel and Colagen IV. The results showed that BthMP promoted a significant alteration in A549 adhesion on fibronectin and also in uncoated plates. In the other hand, BthMP did not exhibit any significant reduction in fibronectin, collagen IV, and plaque without coating (Figure 8).
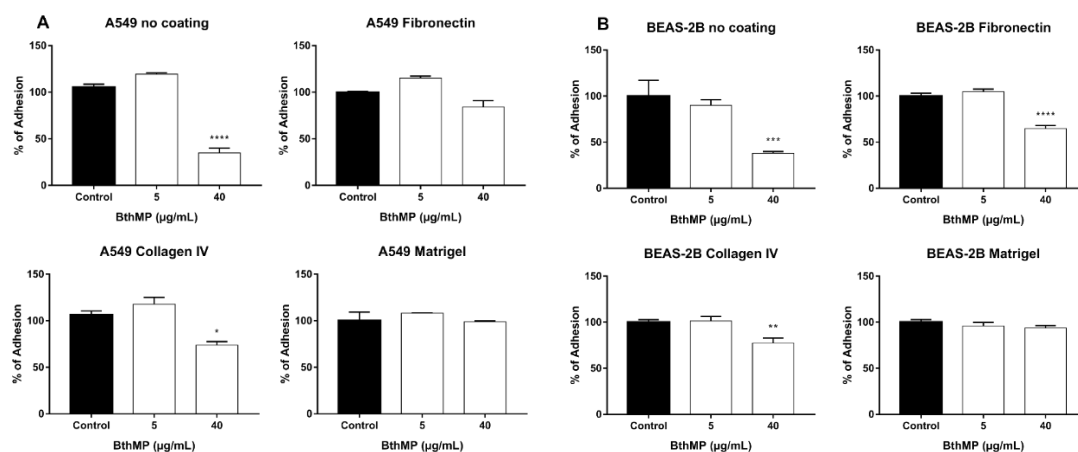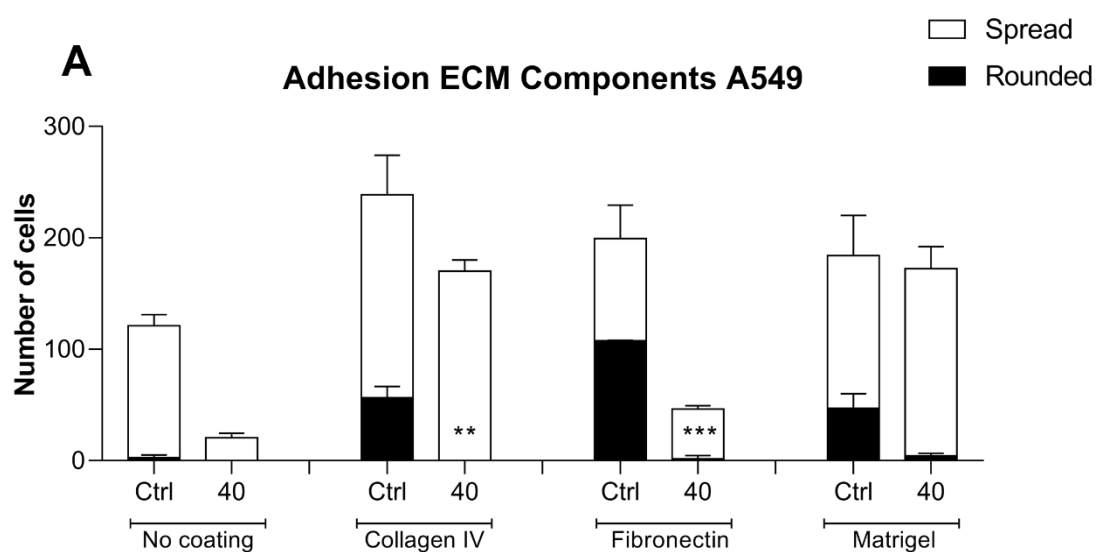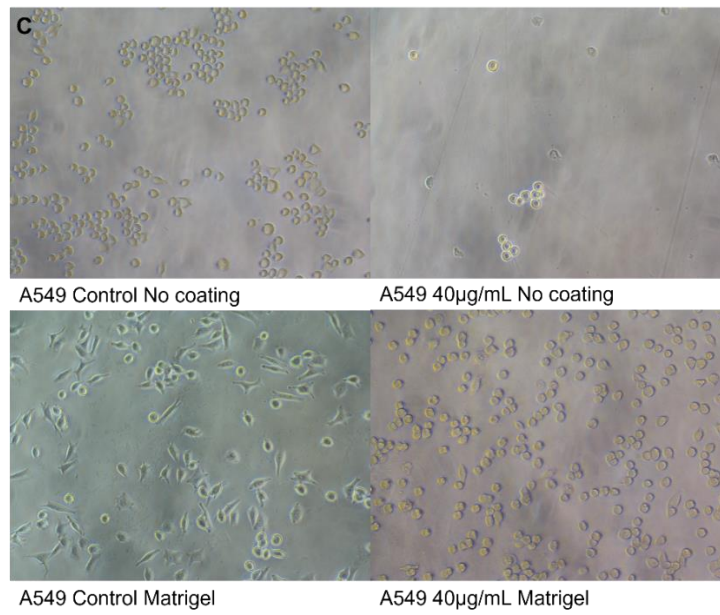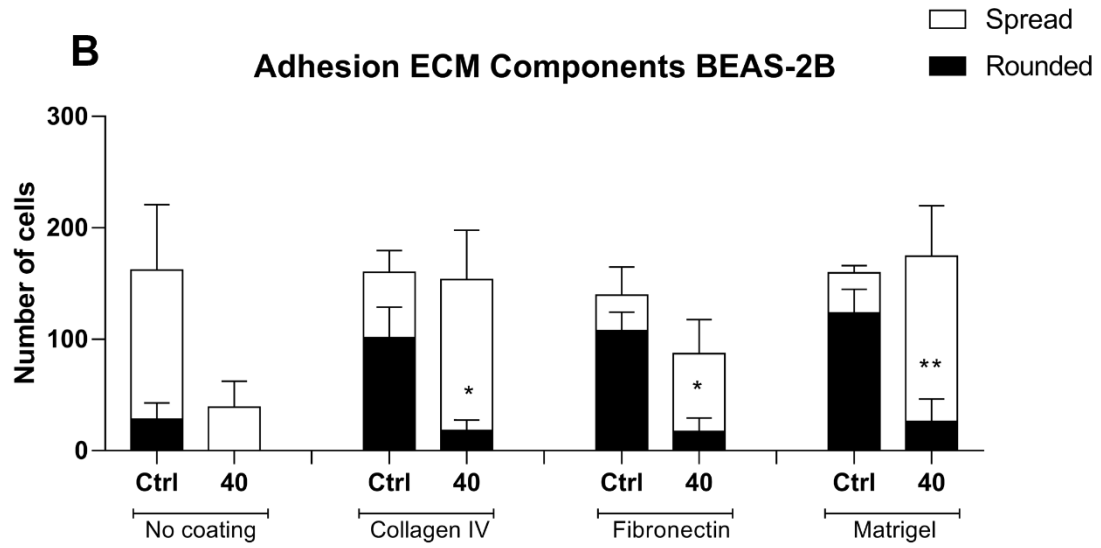
Figure 8. Adhesion inhibition assay on components of the extracellular matrix. Cells A549 and BEAS-2B were treated with different concentrations of BthMP (5µg/mL and 40µg/mL) or medium (negative control). **A**) Adhesion A549 cell inhibition of by BthMP a. **B)** Adhesion Beas cell inhibition of by BthMP. Symbol (*) indicates a significant difference when compared to the control group. *P< 0.05; ***p<0.01; p<0,001; p<0.0001.

However, it was possible to observe a difference between the cell lines with the treatment performed by BthMP generated different responses in the two lines tested compared to cell morphologies. The tumor cell was considerably more affected in morphology than the non-tumor cell. The A549 after treatment presented more rounded cells with agglomeration formation, more easily detachable, and fewer cells in polygonal format, while treatment in BEAS-2B generated a smaller number of rounded cells, fewer agglomerations, and an increased number of polygonal cells (Figure 9).

B

Adhesion ECM Components BEAS-2B

☐ Spread
■ Rounded

(Y-axis: Number of cells, 0 to 300)

No coating: Ctrl, 40
Collagen IV: Ctrl, 40 *
Fibronectin: Ctrl, 40 *
Matrigel: Ctrl, 40 **

C

A549 Control No coating

A549 40µg/mL No coating

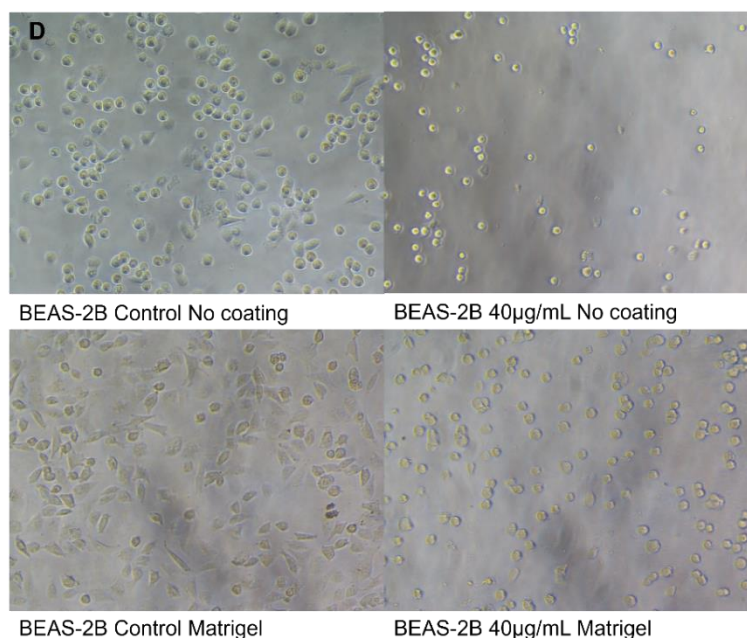A549 Control Matrigel

A549 40µg/mL Matrigel

Figure 9. Adhesion inhibition assay on different substrates. Cells A549 and BEAS-2B treated with different concentrations of BthMP (5µg/mL and 40µg/mL) or medium (negative control). A) Amount of polygonal and rounded cells of the A549 line in the four different substrates with BthMP treatment (40µg/mL); B) Amount of polygonal and rounded BEAS-2B cells in the four different substrates with BthMP treatment (40µg/mL); C) Images representing the difference in Cells A549; D) Images representing the difference in BEAS-2B cells. Symbol (*) indicates a significant difference when compared to the control group. *$p < 0.05$; **$p < 0.01$; $p < 0.001$.

Previous results showed that jararhagin was able to inhibit the adhesion of the murine intestinal epithelium IEC-6 cells to matrigel, in concentrations of 5, 10, and 15µg/mL resulting in an inhibition of 38, 67 and 73% respectively [35]. In other study it was also demostrated that BnP1 a P-I SVMP isolated from *Bothrops neuwiedi* was able to strong inhibit the adhesion in HUVECs [36]. After treatment Skmel-28 lineage of human melanoma with Jararhagin the authors also observed cell rounding, agglomeration formation, and cell lysis [20]. Likewise, Jararhagin also was able to promote formation of small cell clusters, plate detachment, apoptotic and necrotic bodies in B16F10 line of murine melanoma [37].

In this study, the levels of reactive oxygen and nitric oxide species in the A549 and BEAS-2B after treatment with BthMP was evaluated. Treatment of A549 with BthMP resulting in an increase of NO and ROS levels at 40µg/mL. BthMP was able to induced a reduction in NO levels and a mild ROS production for both 5µg/mL and 40µg/mL in Beas (Figure 10).
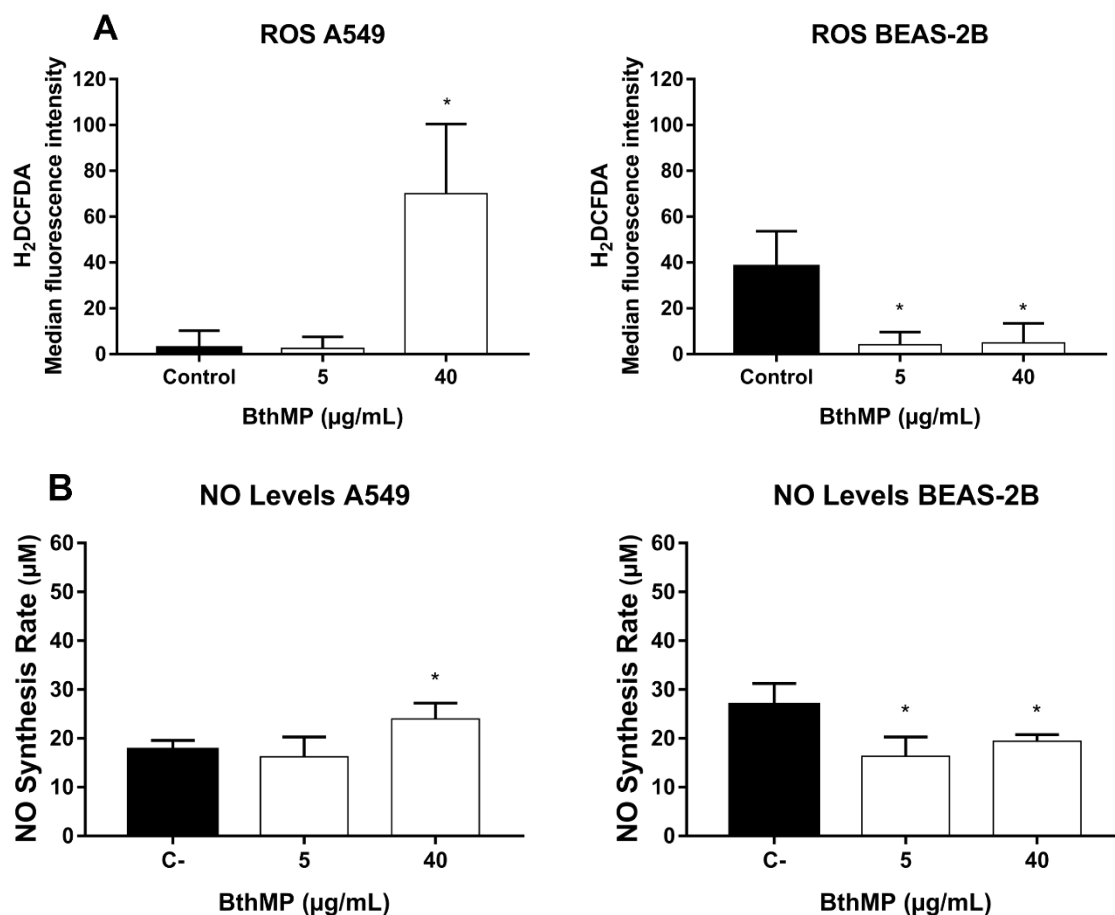
Figure 10. A) ROS levels. Cells A549 and BEAS-2B were treated with BthMP (5μg/mL and 40μg/mL); B) Nitrite dosage. Cells A549 and BEAS2-B were treated with BthMP (5μg/mL and 40μg/mL). Symbol (*) indicates a significant difference when compared to the control group. *p < 0.05

Thus, several studies have been showed that the excessive increase in NO levels increases the chances of cell apoptosis, while low levels of NO can increase vascularity and protect cells against apoptosis [43]. Patients with lung cancer have a significant increase in serum nitride protein levels, reinforcing the effects of oxidative stress [44]. In addition, at high concentrations, NO leads to the inactivation of the p53 protein, a tumor suppressor that regulates the cell cycle [45],

The increase in ROS levels is associated with, promoting tumor cell proliferation [46], cell invasion and migration [47, 48].

However, the role of snake toxins on oxidative stress parameters is not well studied. Previous studies showed that BpirMP, a SVMP isolated from *Bothrops pirajai* venom, promoted an increase in NO levels in the peritoneal fluid of mice [49]. The study of BROWN, 2019 [50], it was observed that *Crotalus atrox*

venom promotes an increase of reactive oxygen species levels, showing that this parameter is important for this venom to induce the apoptotic process.

4. CONCLUSION

Taken together, the results presented here demonstrate the ability of P-I SVMP BthMP in inhibiting the proliferation, invasion, adhesion and migration of lung cancer cells A549, important process involved in tumor growth and metastasis. However, further studies can contribute for better elucidation of the exact molecular mechanism involved in the action of metalloproteinase on lung cancer cell.

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