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Caroline de Freitas Zanon de Carvalho

Estudo dos mecanismos de ação do peptídeo Ac_{2-26} e da Piplartina nas células endoteliais de veias umbilicais humanas (HUVEC) ativadas pelo lipopolissacarídeo

São José do Rio Preto
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DEDICATÓRIA

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“A persistência é o caminho do êxito!”

Charles Chaplin

RESUMO

Os avanços recentes nos mecanismos de inflamação e a descoberta de vários mediadores endógenos anti-inflamatórios levaram a novas investigações sobre as possibilidades terapêuticas. A demonstração, em estudos científicos, da ação anti-inflamatória da proteína anexina A1 (ANXA1) e do seu peptídeo mimético Ac_{2-26}, têm sido fonte de sucesso para o desenvolvimento de novos fármacos. A descoberta da interação biofísica da piplartina (PL) com a região N-terminal da ANXA1 abriu um novo e instigante campo de investigação para o nosso grupo de pesquisa. Diante destas considerações, o objetivo do presente trabalho foi investigar a atuação da PL nas células endoteliais da veia umbilical humana (HUVEC), ativadas pelo lipopolissacarídeo (LPS). Ainda, se a interação Ac_{2-26}/PL influência nas ações anti-inflamatórias da PL, favorecendo ou atenuando os seus efeitos nos processos inflamatórios. Inicialmente foi utilizada a Espectroscopia de Absorbância UV-Vis e de Fluorescência para investigar as interações entre o ligante PL e Ac_{2-26}. *In vitro*, as células HUVEC foram ativadas pelo LPS (10 µg/mL), nos tempos de 8, 24, 48 e 72 horas, seguido pelos tratamentos com Ac_{2-26} (1µM) e/ou PL (10µM), demonstrando resultados expressivos em 24 e 72 horas. Os efeitos foram avaliados nos seguintes aspectos: proliferação celular, viabilidade celular, dosagens da quimiocina MCP-1 e das citocinas IL-8 e IL-1β e expressão da proteína α-tubulina. A presença de um anel trimetoxiaromático na estrutura da PL, o que pode favorecer uma interação com a tubulina, motivou as análises de *Western blotting*, as quais demonstraram que a PL inibiu a síntese da proteína endógena α-tubulina, em todas as condições experimentais. Nossos resultados mostraram efeitos pró-proliferativos do peptídeo Ac_{2-26} e, por outro lado, antiproliferativos e pró-apoptóticos da PL. Os níveis das citocinas pró-inflamatórias confirmaram o papel anti-inflamatório do Ac_{2-26} nas células ativadas pelo LPS, com redução dos níveis de MCP-1 e IL-8, em 24 horas. O tratamento PL reduziu os níveis de MCP-1 nas células ativadas pelo LPS, e aumentou IL-8, inclusive no tratamento associado Ac_{2-26}/PL. Em conjunto, os resultados com as células HUVEC indicam que a PL inibe a proliferação por meio da ativação da apoptose celular, regulada pela inibição da polimerização da α-tubulina e dos níveis elevados da citocina IL-8. A PL e o Ac_{2-26} mostraram ações anti-inflamatórias, e a interação Ac_{2-26}/PL parece ser neutra em relação às propriedades anti-inflamatórias da PL.

Palavras-chave: HUVEC; anexina A1; lipopolissacarídeo; inflamação; proliferação, apoptose, piplartina, piperlongumina.

ABSTRACT

Recent advances in inflammation mechanisms and the discovery of several endogenous anti-inflammatory mediators have led to further investigations into the therapeutic possibilities. The demonstration, in scientific studies, of the annexin A1 protein (ANXA1) anti-inflammatory action and its peptide mimetic Ac_{2-26}, have been a source of success for the development of new drugs. The discovery of the biophysical interaction of piplartin (PL) with the N-terminal region of ANXA1 opened a new and exciting field of investigation for our research group. In view of these considerations, the objective of the present study was to investigate the role of PL in lipopolysaccharide (LPS) activated human umbilical vein endothelial cells (HUVEC). Also, if the interaction Ac_{2-26}/PL influences the anti-inflammatory actions of PL, favoring or attenuating its effects on inflammatory processes. Initially, UV-Vis Absorbance Spectroscopy and Fluorescence were used to investigate the interactions between the PL ligand and Ac_{2-26}. In vitro, HUVEC cells were activated by LPS (10 μg/mL) at 8, 24, 48 and 72 hours, followed by treatments with Ac_{2-26} (1μM) and/or PL (10μM), demonstrating expressive results in 24 and 72 hours. The effects were evaluated in the following aspects: cell proliferation, cell viability, MCP-1 chemokine and IL-8 and IL-1β cytokines and α-tubulin protein expression. The presence of a trimethoxyaromatic ring in the PL structure, which may favor an interaction with tubulin, motivated Western blotting analyzes, which demonstrated that PL inhibited endogenous α-tubulin protein synthesis in all experimental conditions. Our results showed pro-proliferative effects of the peptide Ac_{2-26} and, on the other hand, antiproliferative and pro-apoptotic of PL. Proinflammatory cytokine levels confirmed the anti-inflammatory role of Ac_{2-26} in LPS-activated cells, reducing MCP-1 and IL-8 levels within 24 hours. PL treatment reduced MCP-1 levels in LPS-activated cells, and increased IL-8, including in the treatment associated with Ac_{2-26}/PL.Taken together, the results with HUVEC cells indicate that PL inhibits proliferation through the activation of cellular apoptosis, regulated by the inhibition of α-tubulin polymerization and elevated IL-8 cytokine levels. PL and Ac_{2-26} showed anti-inflammatory actions, and the Ac_{2-26}/PL interaction appears to be neutral in relation to the anti-inflammatory properties of PL.

Keywords: HUVEC; annexin A1; lipopolysaccharide; inflammation; proliferation, apoptosis, piplartine, piperlongumine.

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Lista de abreviações

Ac_{2-26} – peptídeo da região N-terminal da proteína anexina A1
ANXA1 – proteína Anexina A1
ARPE-19 – linhagem de células do epitélio pigmentar da retina
Bcl-2 – regulador de apoptose BCL-2
Boc-1 – inibidor 1 não específico de FRP
Boc-2 – inibidor 2 não específico de FPR
$cPLA_2$ – fosfolipase citosólica A_2
EBNs – extratos bioativos naturais
EIU – uveíte induzida por endotoxina
FPR – receptor de peptídeos formilados
HIF-2 – fator de transcrição induzível de hipoxina 2
HUVEC – linhagem de células endoteliais da veia umbilical humana
IL – interleucina
IKK – ativação da quinase IκB
LPS – lipopolissacarídeo
LXA4 – lipoxina A4
MAPKs – proteínas quinaes ativadas por mitógenos
MCP-1 – proteína quimioatrativa de monócitos 1
NF-κβ – fator nuclear kappa beta
NO – óxido nítrico
PL – piplartina (piperlongumina)
ROS – espécies reativas de oxigênio
SAA – amiloide sérica A

Apresentação

Os resultados dessa tese de Doutorado originaram o trabalho intitulado "*Biological and physical approaches on the modulatory functions of potential piplartine (=piperlongumine) and annexin A1 interactions*" (CAPÍTULO 1) que foi submetido para a revista *Journal of Inflammation Research* (ANEXO 1). Nesse manuscrito foram incluídas as análises relacionadas com as células HUVEC (linhagem de células endoteliais de veias umbilicais humanas) e HEp-2 (linhagem de células tumorais derivadas de carcinoma epidermoide de laringe). Os resultados com a linhagem HEp-2 foram utilizados para complementar os estudos com a piplartina (PL) e, em associação com o Laboratório de Marcadores Moleculares e Bioinformática, coordenado pela Profa. Dra. Eloiza Helena Tajara, da Faculdade de Medicina de São José do Rio Preto (FAMERP).

Paralelamente ao trabalho de Doutorado, colaborei na investigação ~~foi~~ desenvolvida com linhagens de células de câncer de mama e melatonina. O artigo intitulado "*Effect of Melatonin in Epithelial Mesenchymal Transition Markers and Invasive Properties of Breast Cancer Stem Cells of Canine and Human Cell Lines*" foi publicado na *PLOS ONE* (ANEXO 2).

As investigações discutidas nesses trabalhos foram anexadas na íntegra.

Sumário

1 Introdução

As células endoteliais participam de processos inflamatórios apresentando resposta adaptativa específica ao estresse que consiste na formação de espécies reativas de oxigênio (ROS), principalmente a produção de óxido nítrico (NO) (CRISTINA DE ASSIS et al., 2002; AL-SOUDI et al., 2017), o que pode acarretar lesão e/ou disfunção dessas células. Uma entre várias complicações chave associadas com o choque séptico, é mediada, pelo menos em parte, pelo lipopolissacarídeo (LPS) (DAUPHINEE; KARSAN, 2006).

Estudos têm demonstrado que o LPS, uma endotoxina presente na membrana externa de bactérias gram-negativas (RAETZ; WHITFIELD, 2002; DAUPHINEE; KARSAN, 2006; LIU et al., 2014), é capaz de estimular e ativar várias células do corpo como os leucócitos, plaquetas, células endoteliais, epiteliais e dendríticas, assim como, células do sistema fagocitário mononuclear (monócitos e macrófagos) (LYNN, 1998; WANG; QUINN, 2010). Além disso, o LPS induz apoptoses no endotélio de diferentes tipos de tecidos, incluindo as células endoteliais de veias umbilicais humanas (HUVEC) (LIU et al., 2014) e microvasculares derivadas de pulmão normal humano (TREPELS; ZEIHER; FICHTLSCHERER, 2006; WANG et al., 2013). O LPS ativa diretamente o endotélio vascular e promove respostas dessas células, incluindo aumento na expressão de moléculas de adesão específicas e citocinas pró-inflamatórias, como IL-1, IL-8 e MCP-1 (proteína quimioatrativa de monócitos), o que resulta no recrutamento seletivo de leucócitos para o foco da inflamação (YOKOCHI et al., 1996; ECHEVERRÍA et al., 2013; ZITVOGEL et al., 2017).

Nos últimos anos tem ocorrido um avanço no entendimento dos mecanismos anti-inflamatórios que operam no organismo para suprimir a reação inflamatória, (KAMAL; FLOWER; PERRETTI, 2005; NORLING; PERRETTI, 2013). O organismo, seguido de um insulto inflamatório, constrói uma rápida e coordenada resposta: os leucócitos são ativados e transmigrados para o tecido circundante, em direção ao local da inflamação (BEUTLER, 2004; COOPER et al., 2012; NOURSHARGH; ALON, 2014). As primeiras células que atravessam a barreira endotelial são os neutrófilos, no entanto, devido à ação dos leucócitos extravasados ser altamente prejudicial ao organismo, é crucial que o processo inflamatório seja mantido sob controle e de maneira tempo-dependente (KAMAL; FLOWER; PERRETTI, 2005, LIM et al., 2018).

Os recentes avanços nos mecanismos da inflamação e a descoberta de vários mediadores endógenos anti-inflamatórios têm propiciado novas investigações sobre possibilidades terapêuticas para o tratamento de processos inflamatórios, em substituição das atuais, devido aos seus efeitos colaterais. Particularmente, um mediador endógeno anti-inflamatório com potencial terapêutico é a proteína Anexina A1 (ANXA1), que pode representar a descoberta de uma droga inovadora (PERRETI; DALLI, 2009; STUQUI et al., 2015; PRATES et al., 2015; DE PAULA-SILVA et al., 2016; MOLÁS et al., 2017; CARDIN et al., 2017).

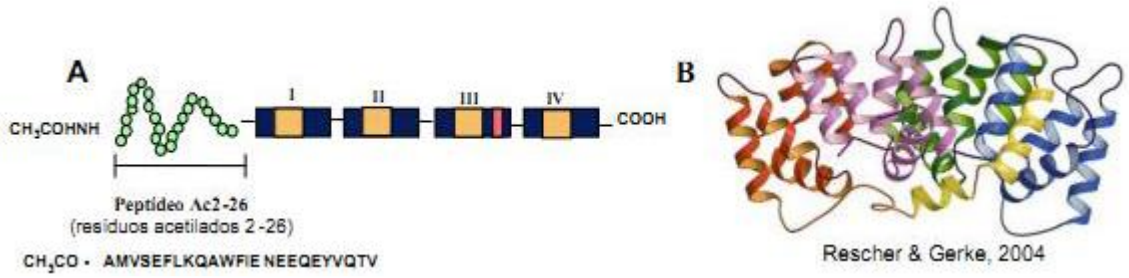
1.1 Proteína Anexina 1

A ANXA1 é uma proteína de 37 kDa formada por 346 aminoácidos. Primeiro de 13 membros da família das Anexinas que possuem características estruturais semelhantes, incluindo a presença de sítios de ligação ao cálcio (GERKE; CREUTZ; MOSS, 2005).

Estruturalmente, as anexinas compreendem dois domínios, uma pequena região N-terminal, que varia em comprimento e composição e, um domínio central formado por quatro a oito repetições de uma sequência de 70 a 80 aminoácidos, altamente conservada (MUNN; MUES, 1986; LIM; PERVAIZ, 2007; LIZARBE et al., 2013). Dois sítios de ligação ao cálcio são expressos na região convexa de cada repetição. Essa região faz face à membrana celular, enquanto a região côncava, com o domínio N-terminal, permanece livre para interagir com as proteínas citosólicas (RESCHER; GERKE, 2004). O domínio N-terminal é único para cada membro da superfamília, confere as atividades e funções específicas das anexinas e contém sítios para processos pós-traducionais, tais como, fosforilação, glicosilação e proteólise (PERRETTI; FLOWER, 2004; SOLITO et al., 2006; PERRETTI; D'ACQUISTO, 2009; LIZARBE et al., 2013).

Uma representação esquemática da estrutura primária e o arranjo tridimensional da ANXA1 são mostrados na Figura 1.

Figura 1 – **Estrutura da Anexina A1. (A)** Representação esquemática da estrutura primária da proteína anti-inflamatória anexina 1, com destaque do sítio ativo anti-inflamatório (peptídeo Ac2-26). **(B)** Ilustração do arranjo tridimensional dessa proteína.



FONTE: RESCHER, U.; GERKE, V. Annexins--unique membrane binding proteins with diverse functions. **J Cell Sci**, v. 117, n. Pt 13, p. 2631-9, 2004.

Uma importante característica da ANXA1, que também é compartilhada por outros membros da família, é a habilidade para alterar a conformação durante a ligação aos cátions de cálcio (GERKE; CREUTZ; MOSS, 2005; PERRETTI; D'ACQUISTO, 2009). Na presença de concentrações de cálcio ≥ a 1mM a ANXA1 é reestruturada, o que permite uma ligação fosfolipídica, em particular aos ácidos fosfolipídicos, por meio das regiões do domínio central (ROSENGARTH; GERKE; LUECKE, 2001). Essa interação ocorre concomitantemente ao rearranjo da região N-terminal, o que expõe seus aminoácidos ao ambiente extracelular e gera a forma ativa da proteína (ROSENGARTH; GERKE; LUECKE, 2001; LECONA et al., 2008; PERRETTI; D'ACQUISTO, 2009).

A ANXA1 é amplamente distribuída no organismo e pode ser encontrada em diversos tipos celulares como: células endoteliais, células epiteliais dos pulmões, intestino, estômago, mama, rins e sinoviócitos (SAMPEY; HUTCHINSON; MORAND, 2000; ALLCOCK et al., 2001; OLIANI et al., 2001; OLIANI; PERRETTI, 2001; GIL et al., 2006; PERRETTI; D'ACQUISTO, 2009; ARAUJO et al., 2010), e também em células estromais, como os fibroblastos (TAGOE et al., 2008). No entanto, a expressão da ANXA1 tem sido observada especialmente em células relacionadas aos processos de defesa, como neutrófilos (PERRETTI et al., 2000; OLIANI et al., 2001; SOLITO et al., 2003a; STUQUI et al., 2015); mastócitos (OLIANI et al., 2000, OLIANI et al., 2008; SOUZA et al., 2017), eosinófilos (OLIANI; DAMAZO; PERRETTI, 2002), monócitos (GOULDING; LUYING; GUYRE, 1990; SOLITO et al., 2001) e linfócitos (D'ACQUISTO et al., 2007; D'ACQUISTO et al., 2008).

A descoberta de que a atividade biológica da ANXA1 poderia ser reproduzida pelos primeiros aminoácidos da porção N-terminal da proteína (peptídeo Ac_{2-26}) (CIRINO et al., 1993), ou por alguns peptídeos menores (PERRETTI et al., 2001), estimulou a prática do uso dessas moléculas em modelos experimentais de inflamação aguda (SOLITO et al., 2003b; GAVINS et al., 2007), crônica (YANG; HUTCHINSON; MORAND, 1999; GIBBS et al., 2002) e sistêmica (DAMAZO et al., 2005).

O nosso grupo de pesquisa tem, ao longo dos anos, investigado o efeito da ANXA1 nos processos alérgicos (SENA et al., 2006), na biologia tumoral (RODRIGUES-LISONI et al., 2006; SILISTINO-SOUZA et al., 2007; GASTARDELO et al., 2014; PRATES et al., 2015) e, principalmente, nas inflamações experimentais (OLIANI et al., 2001; GASTARDELO et al., 2009; MIMURA et al., 2012; GIROL et al., 2013; STUQUI et al., 2015; GIMENES et al., 2015; DE PAULA-SILVA et al., 2016). A ANXA1, endógena ou exógena, regula as atividades das células imunes inatas, em particular o processo de geração de mediadores pró-inflamatórios e de transmigração (LIM; PERVAIZ, 2007; PERRETTI; D'ACQUISTO, 2009). Esses efeitos garantem que níveis suficientes de ativação celular sejam alcançados, mas não excedidos, o que proporciona a homeostase do processo inflamatório.

Outro aspecto importante na biologia da ANXA1 tem sido o estudo do seu mecanismo de ação na superfície celular. A observação que a ANXA1 e os seus peptídeos miméticos são ligados a uma classe específica de receptores transmembrana acoplados a proteína G, os receptores para peptídeos formilados (nFPR) (WALTHER; RIEHEMANN; GERKE, 2000) revolucionou o campo de pesquisa da ANXA1. Este estudo fundamental permitiu novas investigações nas relações funcionais e moleculares entre a ANXA1 e a família de receptores FPR (PERRETTI; DALLI, 2009).

A família FPR apresenta divergência evolutiva significante em espécies de mamíferos, com notável expansão diferencial de genes em murinos (GAO et al., 1998). Em camundongos a família dos genes FPR é complexa, está localizada no cromossomo 17 e compreende sete membros. Enquanto, em humanos, apenas três tipos de receptores foram encontrados e incluem, FPR1, FPR2/ALX (também conhecido como FPRL-1) e FPR3 (antigamente designado com FPRL-2) (YE et al., 2009). Os receptores FPR1 e FPR2 de roedores (anteriormente designados FPR-

RS1 e FPR- RS2) correspondem, respectivamente, ao FPR1 e FPR2/ALX em humanos, com os quais compartilham homologias de 77 e 76% (DUFTON; PERRETTI, 2010).

Embora os FPRs sejam classicamente conhecidos por agirem como receptores quimiotáticos, regulando a migração leucocitária, eles são expressos em populações celulares diversas e ativados por uma variedade de ligantes endógenos e exógenos que provocam respostas biológicas diferentes, tais como lipoxina A4 (LXA4), amiloide sérica A (SAA) e ANXA1 (LE; OPPENHEIM; WANG, 2001; DUFTON; PERRETTI, 2010).

A ANXA1 ativa somente o receptor FPR2/ALX (HAYHOE et al., 2006), já o peptídeo Ac_{2-26}, *in vitro*, ativa todos os três receptores da família FPR (RESCHER et al., 2002; ERNST et al., 2004). No entanto, tais respostas são bloqueadas por antagonistas não seletivos dos receptores FPRs, os antagonistas Boc1 e Boc2 (STENFELDT et al., 2007), por meio da inibição do fluxo de cálcio induzido pela ANXA1 em PMNs (LA et al., 2001).

Investigações realizadas em nosso laboratório mostraram que o tratamento com o peptídeo Ac_{2-26} reduziu a proliferação das células Hep-2 (linhagem celular derivada de carcinoma epidermoide da laringe), um efeito que foi bloqueado pelo Boc2 e alta expressão da ANXA1/FPR2. Desta forma, a ANXA1 pôde contribuir para a regulação do crescimento tumoral e metástase, por meio de mecanismo mediado pelo FPR2/ALX. (GASTARDELO et al., 2014). No modelo de uveíte induzida por endotoxina (EIU) em ratos (GIROL et al., 2013) e com células do epitélio pigmentar da retina (ARPE-19), ativadas pelo LPS, a inibição da liberação de mediadores inflamatórios ocorreu após ativação dos receptores FPR2 pela ANXA1 endógena translocada e o peptídeo mimético Ac_{2-26}.

O papel anti-inflamatório da ANXA1 está parcialmente relacionado à sua capacidade de inibir a atividade pró-inflamatória da fosfolipase citosólica A_2 ($cPLA_2$) e, consequentemente, a hidrólise de fosfolipídios de membrana e a liberação de ácido araquidônico. A superexpressão do $cPLA_2$ e a indução da metabolização do ácido araquidônico resultam em altos níveis de eicosanoides, como a prostaglandina E_2, que são frequentemente observados em tumores (NAKANISHI; ROSENBERG, 2006). Portanto, uma combinação de abordagens anti-inflamatórias que visam o

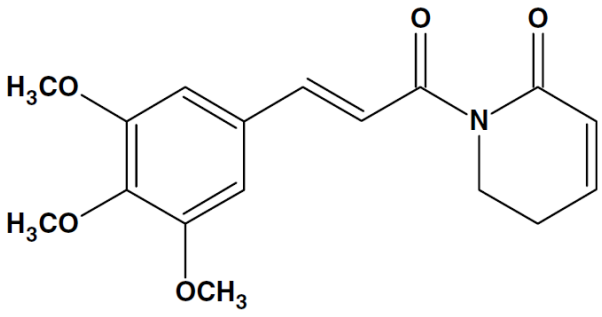
microambiente tumoral, utilizando medicamentos tumoricidas mais sofisticados e seletivos podem levar a futuras intervenções terapêuticas no câncer.

1.2 Extratos bioativos naturais

Os extratos bioativos naturais (EBNs) como os terpenos e flavonoides têm demonstrado muitas propriedades, entre elas, redução da inflamação, estimulação do sistema imune, modulação de enzimas detoxificantes e metabolismo de esteroides, (BEZERRA et al., 2013). Entre os EBNs destacamos a piplartina {piperlongumina (PL), 5,6-di-hidro-1-[(2E)-1-oxo-3 (3,4,5-trimetoxifenil)-2-propenil]-2(1H) piridinona}, o componente biologicamente ativo das espécies de *Piper longum L.* (*Piperaceae*), que apresenta grande importância econômica e medicinal, em particular, propriedades anti-inflamatórias e anticâncer (GERSCH et al., 2012).

Atal e Banga, dois químicos indianos, isolaram pela primeira vez em 1961 a piplartina. Posteriormente, em 1963, caracterizaram sua estrutura química pela presença de dois sistemas de anéis ligados por uma cadeia de radicais carbonilos α, β-insaturados (ATAL; BANGA, 1961, 1962, 1963). Um dos anéis é aromático e substituído com três grupos metoxi, enquanto o segundo é um anel do tipo piperidinona contendo um alceno conjugado, vide Figura 2. A PL é estruturalmente semelhante a um número de compostos antiproliferativos alvo da tubulina (DUCKI et al 2009).

Figura 2 – Estrutura química da piplartina. {5,6-dihydro-1-[1-oxo-3-(3,4,5-trimethoxyphenyl)-2-propenyl]- 2(1H)pyridinone}.



Fonte: BEZERRA, D. P. et al. Piplartine induces inhibition of leukemia cell proliferation triggering both apoptosis and necrosis pathways. **Toxicol In Vitro,** v. 21, n. 1, p. 1-8, 2007.

Estudos sobre as atividades farmacológicas da PL incluem citotoxicidade (LIN et al., 2007; BEZERRA 2008a; JYOTHI et al., 2009; RAJ et al., 2011; GOLOVINE et

al.,2013), genotoxicidade (BEZERRA et al., 2008b, 2009), antitumoral (BEZERRA et al., 2006, 2008a; RAJ et al., 2011), anti-angiogênica (RAJ et al., 2011), antimetastática (RAJ et al., 2011), anti-agregação plaquetária (IWASHITA et al., 2007; FONTENELE et al., 2009; LEE et al., 2010), antibacteriano (NAIKA et al., 2010) e antifúngica (NAVICKIENE et al., 2000; SILVA et al., 2002).

A atividade antitumoral da PL envolve parada do ciclo celular, ativação de caspases e proteínas quinases ativadas por mitógenos (MAPKs), *down-regulation* de proteínas anti-apoptóticas como a Bcl-2 e NF-κB (KONG et al., 2008; GINZBURG et al., 2014; LIU et al., 2014), assim como citotoxicidade contra linhagens de células tumorais. Essa citotoxicidade foi observada em uma gama micromolar em células tumorais, mas não em células normais (RAJ et al., 2011). Diante dessas terapias antitumorais, os microtúbulos, componentes chave do citoesqueleto, desenvolvem importante papel no ciclo celular, por isso a descoberta e desenvolvimento de moléculas que atuam sobre a tubulina, interferindo na dinâmica da sua polimerização, tem sido interessante na descoberta de novas terapias do câncer (PEREZ, 2009; DUMONTET; JORDAN, 2010). No entanto, não há relatos do mecanismo de ação da PL sobre a tubulina nas células HUVEC.

Na literatura existem várias publicações relacionadas com a PL, contudo, há poucos estudos focando a atividade anti-inflamatória e os mecanismos da PL e seus análogos. Recentemente, Sun e colaboradores (2015) estudaram a atividade anti-inflamatória de seis análogos baseados na PL, com diferentes características estruturais. Os pesquisadores identificaram um análogo da PL com um átomo de nitrogênio a menos (PL-ON) como um potencial agente anti-inflamatório, por meio do estudo da via do NF-κB, dependente da ativação da quinase IκB (IKK).

Estudos têm utilizado drogas anticâncer em combinação com a PL a fim de reduzir os mecanismos de resistência em clínicas de quimioterapia. Jyothi e colaboradores (2009) investigaram o tratamento concomitante da PL com diferuloylmetano (curcumina), um agente anti-inflamatório e anticâncer. Esse tratamento aumentou a citotoxicidade induzida pela PL e a atividade antitumoral de drogas quimioterápicas.

Investigações mais antigas têm atribuído aos compostos das pimentas e outros EBNs, um efeito indireto no endotélio vascular, músculo liso e mastócitos (BÍRÓ et al., 1998), ou direto na integridade das junções oclusivas e na função de alguns

transportadores de membrana, resultando no aumento da permeabilidade vascular e do fluxo de sangue na mucosa intestinal (HASHIMOTO et al., 1997; JENSEN-JAROLIM et al., 1998). No entanto, suas ações na linhagem celular HUVEC não são conhecidas.

1.3 Interação anexina 1 e piplartina

Um dos maiores desafios no desenvolvimento de drogas é a inibição ou a ativação do alvo sem interferir nas funções celulares normais. As ferramentas de Bioinformática têm auxiliado nesse processo e se tornando parte integral das pesquisas acadêmicas e industriais (PITCHAI et al., 2011).

Os fármacos são ligantes que não somente se encaixam no sitio de ligação da proteína alvo como também são absorvidos, transportados, distribuídos para o sítio correto, idealmente estáveis à metabolização, não tóxicos, livres de efeitos colaterais e quimicamente estáveis. Portanto, o processo de busca de um composto e otimização de suas propriedades não é simples (BREDA, 2006). Os progressos recentes em ciências básicas e tecnologia estão mudando a maneira pela qual a busca de fármacos é conduzida. O uso de microarranjos de DNA e abordagens proteômicas, tem facilitado a identificação de genes e proteínas alvos, e aberto inúmeras oportunidades nesta área. Da mesma forma, podem ser citados os avanços em métodos de química combinatorial, *screening* de grande alcance e, ocupando uma posição central, as técnicas computacionais que permitem a análise de uma grande quantidade de dados (SCHNEIDER, 2000).

A metodologia QSAR (*quantitative structure-activity relationships*) auxilia no desenho dessas modificações, checando a confiabilidade dos modelos (ROY; MITRA, 2011; NANDI; BAGCHI, 2012). O reconhecimento molecular é fundamental para que ocorram interações entre dois compostos e, portanto, a base de todos os processos biológicos. Muitas propriedades físicas e químicas afetam estas interações e, consequentemente, o reconhecimento molecular (CIPRIANO; PHILLIPS; GLEICHER, 2012).

Atualmente, a interação entre a proteína ANXA1, descrita por Flower e Blackwell (1979), e outras moléculas tem sido investigada pelo nosso grupo, por distintas técnicas de *screening* molecular ou computacional, tais como *phage display* e *docking* molecular. As análises *in silico* mostraram que, entre outras moléculas da PL, há um terpeno que parece interagir com a lisina 9 da ANXA1, na região

correspondente ao peptídeo N-terminal Ac_{2-26}, próxima, portanto ao triptofano 12. Contudo, o entendimento fisiológico desta interação, se positiva ou negativa, com relação aos efeitos anti-inflamatórios da AnxA1 não é conhecido, o que abre um novo e estimulante campo para pesquisas.

No presente estudo, investigamos evidências biofísicas e químicas das interações entre PL e ANXA1 e seus potenciais efeitos na proliferação celular, migração e apoptose, respostas inflamatórias e expressão gênica de mediadores inflamatórios.

2.1 Objetivos gerais

O objetivo do presente trabalho foi investigar, *in vitro*, a ação da piplartina (PL) e do peptídeo mimético da proteína anti-inflamatória Anexina A1 (Ac_{2-26}) na linhagem de células endoteliais de veias umbilicais humanas (HUVEC).

2.2 Objetivos específicos

- Parâmetros termodinâmicos da interação física entre Ac_{2-26} e PL.
- Nas células HUVEC induzidas pelo LPS e/ou A_{c2-26}, LPS e/ou PL e LPS e/ou Ac_{2-26}/PL, avaliamos os seguintes aspectos:
    - ✓ proliferação celular*;*
    - ✓ viabilidade celular;
    - ✓ dosagens da quimiocina MCP-1 e das citocinas pró-inflamatórias IL-8 e IL-1β;
    - ✓ expressão da proteína endógena α-tubulina.

3 Capítulo 1

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Biological and physical approaches on the modulatory functions of potential piplartine (=piperlongumine) and annexin A1 interactions

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Abstract

Chronic inflammation provides a favorable microenvironment for tumorigenesis, which opens opportunities for targeting cancer development and progression. Several proteins involved in inflammatory process have a dual role in cancer, acting as pro or anti-tumorigenic factor, depending on the context. Piplartine (PL) is a biologically active alkaloid from long pepper, which exhibits anti-inflammatory and antitumor activity. In the present study, we investigated physical and chemical interactions of PL with anti-inflammatory compounds and their effects on cell proliferation, migration and apoptosis, and on gene expression of inflammatory mediators. Molecular docking data and physicochemical analysis suggested that PL shows potential interactions with six anti-inflammatory drugs in current clinical use and with Annexin A1 (ANXA1), an endogenous anti-inflammatory mediator with therapeutic potential in cancer. Apparently, treatment of normal or neoplastic cells with PL alone or with ANXA1 reduces cell proliferation or viability and increases apoptosis. PL also inhibits tubulin expression and cell migration, as well as modulates expression of MCP-1 chemokine, IL-8 cytokine and several genes involved in inflammatory processes. These results support the anti-inflammatory and antitumoral properties of piplartine, which are not affected by a potential interaction with annexin A1.

Keywords: piplartine, annexin A1, inflammation, neoplasms, tubulin, pepper, gene expression.

3.1. Introduction

Several epidemiological evidences indicate that persistent infection and chronic inflammation are predisposing factors for cancer, as well documented for the cervix after HPV infection [1] and stomach in the presence of *Helicobacter pylori* [2]. S*upporting this idea are data from* experimental studies of tumor initiation and promotion, such as the ones using exogenous inducers of localized inflammation to promote cancer in mouse skin [3,4], or xenotransplants of colonic adenoma cells that only induce tumors if introduced into the host together with an inducer of inflammation [5].

Mediators of inflammation, such as cytokines, chemokines, growth factors and free radicals, can provide a favorable microenvironment that fosters genome instability, survival, proliferation and migration, and thus contribute to all cancer development stages, from initiation and promotion to metastatization (reviewed by [6]. This link between inflammation and tumorigenesis raises the possibility of therapeutic interventions that target inflammation for cancer prevention and treatment, especially because anti-inflammatory agents show a modest toxicity compared to conventional chemotherapy [7].

An endogenous anti-inflammatory mediator with therapeutic potential in cancer is annexin A1 (ANXA1), a 37 kDa glucocorticoid–inducible protein that is involved in several biological processes, such as cell proliferation [8], survival, apoptosis [9], migration and invasion [10,11]. Considering its involvement in such biological processes, it is to conclude that ANXA1 may directly participates in tumor initiation and progression [12]. Indeed, an altered expression of ANXA1 [13], has been observed in several tumors and may induce oncogenic pathways involving MAPK and PI3K signaling. Abnormal expression is also found for their cognate partners, the formyl-peptide receptors (FPRs), a family of seven transmembrane G-protein-coupled receptors that, besides ANXA1, bind many proteins and peptides, and may have opposite effects depending on the ligand [14].

It has been shown that intracellular ANXA1 activates nuclear factor κB (NF-κB), which stimulates breast cancer cell invasion through increased expression of target genes [15]. Nuclear ANXA1 also appear to be involved in heavy metal-induced mutagenesis [16] and is a predictor of decreased overall survival in oral squamous cell carcinoma [17]. Similarly, externalized ANXA1/FPR1 overexpression is associated with

metastasis in gastric cancer [18,19], tumor growth and invasion in gliomas [20], and unfavorable prognostic factors in breast cancer [21]. Otherwise, our group showed that nuclear, cytoplasmic ANXA1 and ANXA1/FPR2 are downregulated in dysplastic, primary tumor or metastatic laryngeal carcinoma [22-24], suggesting that the role of ANXA1 in tumorigenesis is context-dependent.

The anti-inflammatory role of ANXA1 is partially due to its ability to inhibit the proinflammatory activity of cytosolic phospholipase A_2 ($cPLA_2$) and, consequently, the hydrolysis of membrane phospholipids and release of arachidonic acid. Overexpression of $cPLA_2$ and induction of arachidonic acid metabolization result in high levels of eicosanoids, such as prostaglandin E_2, which are frequently observed in tumors [25]. These data support the use of aspirin and others non-steroidal anti-inflammatory drugs for cancer prevention, which although effective are associated with several adverse side effects [26]. It is therefore important to search for new medications that control inflammation and show a more acceptable safety profile.

Piplartine (PL), also known as piplartine [5,6-dihydro-1-[(2E)-1-oxo-3-(3,4,5-trimethoxyphenyl)-2-propenyl]-2(1H)-pyridinone)], is a plant-derived small molecule, which has been the subject of reports from members of our group [27-30]. PL is a biologically active alkaloid/amide from long pepper (*Piper longum*) with many reported pharmacological properties, including anti-inflammatory and antitumor activity by induction of oxidative stress, and low toxicity [31,32]. The presence of a trimethoxyaromatic ring in PL structure may favor its interaction with tubulin [33] and its role as a microtubule-destabilizing agent with antiproliferative effects [34]. Similar to annexin A1, PL is involved in the NF-κB and MAPK signalings [35-37].

In the present study, we investigated physical and chemical evidences of PL and ANXA1 interactions and their potential effects on cell proliferation, migration and apoptosis, and inflammatory responses, as well as on gene expression of inflammatory mediators.

3.2 Materials and methods

3.2.1 Drugs

The annexin A1-derived peptide Ac_{2-26} (Ac-AMVSEFLKQAWFIENEEQEYVQTVK) [38] was obtained from Thermo Scientific (Waltham, MA, USA) and used to prepared 1 mg/mL or 323,677 µM stock solution in

diluted in dimethyl sulfoxide (DMSO). Piplartine ($C_{17}H_{19}NO_5$; CAS number 20069-09-4) was isolated as previously described (Bezerra et al., 2005) and used to prepare a 5 mg/mL or 16,9 mM stock solution in dimethyl sulfoxide (DMSO) or in absolute ethyl alcohol. Bacterial lipopolysaccharide (LPS, *Escherichia coli* O127:B8, Sigma Chemical Co. Poole, Dorset, UK), a component of the surface membrane of most Gram-negative bacteria and potent stimulator of immune cells, was purchased from Sigma Aldrich (Dorset, UK) and diluted in 10% MEM-Earle medium (Cultilab, Campinas, SP, Brazil), in the concentration of 10 µg/mL.

3.2.2 Molecular Docking

Human proteins related to inflammatory and neoplastic processes were selected by a literature mining in the PubMed library using medical subject heading (MeSH) terms of interest. Fourteen proteins with defined three-dimensional structures in the Protein Data Bank (PDB, http://www.rcsb.org/pdb/home/home.do) were selected to molecular docking studies, including annexin A1. Piplartine and nine anti-inflammatory drugs in current clinical use that meet the Lipinski rules [39] were used as ligands. Their structures were obtained from the Zinc Database (http://zinc.docking.org/).

Proteins were prepared using AutoDock Tools (version 1.4.5, http://autodock.scripps.edu/) with water molecules deleted, and hydrogen atoms and Gasteiger charges added. Docking of ligands to protein was performed using AutoDock Vina for Linux [40]. The grid box was defined to include the binding site of the proteins, and the ligands were docked sequentially. Pymol software (https://pymol.org/2/) was used to display the proteins with the ligand binding site.

3.2.3 UV-Vis Absorbance and Fluorescence Spectroscopy

The UV-Vis absorption and fluorescence spectroscopic studies were performed to investigate the ligand-annexin A1 interactions.

In UV-Vis spectroscopic analysis, spectra were recorded at room temperature between 200 and 500 nm (integration time of 0.333 s) on a Cary 3E UV–Vis spectrophotometer (Varian, Palo Alto, CA, USA) equipped with tungsten and deuterium lamps and using quartz cuvettes with an optical pathlength of 10 mm.

Fluorimetric titrations were carried out in 10 mm quartz cuvettes on an ISS-PC1 spectrofluorimeter (Champaign, IL, USA) with a Neslab RTE- 221 thermostat bath, running on Vinci software. Excitation wavelengths were fixed at 280 and 295 nm in order to excite phenylalanine and tryptophan residues, respectively. Excitation and

emission slits widths were set to 8 nm. The emission spectra were in the range of 295 to 500 nm with a 1.0 nm resolution step and the spectra were obtained by averaging 10 successive accumulations. Aliquots of piplartine were titrated in 5.0 μM annexin A1-derived peptide solution. The measurements were performed at temperatures of 288, 298 and 308 K. Piplartine concentration varied from 0 to 15.6 μM, approximately, with increments of 1.2 μM at 288, 298 and 308 K. In all experiments, the final volume of ethyl alcohol in the buffer was ≤ 0.6%, and the fluorescent signal intensity was corrected for the background fluorescence and inner filter effects[41].

3.2.4 Cell culture and drug treatments

Two cell lines derived from normal or neoplastic tissues were used in the present study, the HUVEC cell line, obtained from human umbilical vein endothelial cells (American Type Culture Collection/ATCC, Manassas, VA, EUA, CRL-2873 TM), and the HEp-2 cell line (ATCC, CCL-23), originally established from an epidermoid carcinoma of the larynx. HUVEC cells were grown in MEM-Earle medium (Cultilab), supplemented with 10% fetal bovine serum (FBS, Cultilab), 0.5% antibiotic/antimycotic (Invitrogen Corp., Carlsbad, CA USA). HEp-2 cells were cultured in Dulbecco's Modified Eagle Medium – DMEM (Cultilab), supplemented with 10% FBS, 10mM *non-essential amino acids* (Gibco, Carlsbad, CA, USA*),* 2mM L-glutamine), 1mM *sodium pyruvate* (Sigma Aldrich, St. Louis, MO, USA) 0.1% antibiotic/antimycotic (Sigma Aldrich). Both cell lines were grown at 37 C in a humidified atmosphere with 5% CO2 and the cell medium was changed at 72 h intervals. When the cells became 70-80% confluent, they were trypsinized and subcultured in the same medium.

To investigate whether piplartine in the presence of annexin A1 plays a role in inflammatory and neoplastic processes, HUVEC and HEp-2 cells were treated with the peptide $Ac_{2-26,}$ piplartine or both, or LPS. Peptide Ac_{2-26} and PL were dissolved in DMSO or absolute ethyl alcohol 1 mg/mL of Ac_{2-26} and 5 mg/mL of PL. Before use, stock solutions were diluted into fresh medium containing 10% FBS and added to cell cultures at a final concentration of 1 μM $Ac_{2-26,}$ 10 μM PL, 10 μg/mL LPS. For the negative *control, an equal volume of solvent (DMSO)* was added to cultures. Cell proliferation, viability, migration and invasion assays were performed and the results were compared with those obtained in control cells.

Peptide Ac_{2-26}, PL and LPS effective concentrations were determined by experiments previously performed (data not shown).

3.2.5 Cell proliferation assay

HUVEC cells were seeded at density of $1.5x10^4$ cells/well. After adherence, the cells were treated with 1 μM peptide Ac_{2-26}, 10 μM PL, 1 μM/10 μM Ac_{2-26}/PL, 10 μg/mL LPS. Cell growth was observed daily under inverted *phase-contrast microscope* (Olympus CKX41, Tokyo, Japan). The cells were trypsinized at the times of 8, 24, 48 and 72 h and counted using a cell counter (Countess Automated Cell Counter, Invitrogen, UK).

3.2.6 Cell viability assay

HUVEC cells were seeded at density of $1.5x10^5$ cells/flask. After adherence, the cells were treated with culture medium, 1 μM peptide Ac_{2-26}, 10 μM PL, Ac_{2-26}/PL, or 10 μg/mL LPS for 72 h. The cells were then trypsinized and centrifuged at 1,500 rpm for 5 min at 4ºC. The pellet was washed with PBS twice, and the cells were counted by a cell counter. Cells were resuspended with 1X binding buffer (FITC Annexin V Apoptosis Detection Kit I, BD Pharmigen, San Diego, CA, USA) to a concentration of $1x10^6$ cells/mL. An aliquot of 100 μL was incubated with 5 μL FITC Annexin V and 5 μL propidium iodide (PI, BD Pharmigen) for 15 min, at room temperature in the dark. Then, 400 μL of 1X binding buffer was added and the cells were analyzed immediately by a flowcytometer (Guava EasyCyte Flowcytometer, Millipore, Hayward, CA, USA).

The cell viability assay of HEp-2 cell line was performed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay/MTS (Promega, Madison, MI, USA). Cells were seeded in 96-well plates *at density* of $5x10^3$ cells/well in 200 uL complete medium, according to the manufacturer's instructions. One hour before experiment, 10 uL/well CellTiter 96 AQueous One Solution was added to a plate, and cells were incubated for 1 h at 37 C in a humidified atmosphere with 5% CO2 and analyzed on an TP-Reader NM (ThermoPlate, Equipar, Curitiba, PR, Brazil) at 490 nm to establish baseline readings. On remaining plates, cells were treated with 1 μM peptide Ac_{2-26}, 5, 10 or 20 μM PL and Ac_{2-26}/PL. Successive readings were performed on every 24 h from plating to 48 h and 72 h post seeding to establish growth curves. Each experiment was performed in three replicate wells and independently repeated two times.

3.2.7 Cell migration and invasion assays

Quantitative evaluation of in vitro migration and invasion assays was performed using HEp-2 cells and a BD BioCoat^{TM} Migration/Invasion Chambers (BD Biosciences, San Jose, USA). The cells (5×10^4 suspended in 300 mL serum-free DMEM) were seeded in the upper compartment of an 8-μm Boyden chamber and treated with 20 μM PL or negative control. Migration and invasion assays were carried out with uncoated or coated polycarbonate filter, respectively. The bottom chamber was filled with 500 mL medium with 10% FBS. After incubation for 72 h, the nonmigrated cells on the upper surface of the filter were carefully removed. Cells that migrated to the lower surface of the insert were fixed with 0.4% *formaldehyde* for 20 min and stained with 4',6-diamidino-2-phenylindole (DAPI, Uniscience, São Paulo, SP, Brasil). Images of five fields were captured with an inverted fluorescence microscope using Axiovision software (Release 4.8, Carl Zeiss, Oberkochen, Germany). Cells were quantified by ImageJ software (http*://imagej*.nih.gov/ij*/*). Assays were carried out in triplicate.

3.2.8 Real time PCR

Total RNA was extracted for HEp-2 cells using TRIzol (Life Technologies, Grand Island, NY, USA) and concentration was determined by Nanodrop ND-1000 (Thermo Scientific). RNA was reverse transcribed into complementary DNA (cDNA) using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). A PCR array (TaqMan® Array Human Inflammation 96-well plate, Fast, Thermo Fisher Scientific) was screened *according to the* manufacturer's instruction on an ABI Prism 7500 Fast Real-Sequence Detection System (*Applied Biosystems*). The results were analyzed using DataPCR array Data Assist software (version 3.01, Thermo Fisher Scientific). Differentially expressed genes were imported into DAVID, a database for annotation, visualization and integrated discovery [42,43] and Benjamini-Hochberg method was used to control false discovery rate (FDR) [44]. The genes were annotated for gene ontology and pathways using the whole human genome as background. Ingenuity Pathway Analysis (IPA) software (Qiagen, Redwood City, CA, USA) was also used to identify relevant canonical pathways, diseases and biological functions over-represented in differentially expressed gene.

3.2.9 MCP-1 chemokine and IL-1β and IL-8 cytokines

MCP-1 chemokine and IL-8, IL-1β cytokines in the supernatant of the culture medium of HUVEC cells were quantified using ELISA immunoassays (BD Biosciences, San Diego, CA, USA). Cytokine concentrations were determined by a microplate reader (TP-Reader NM ThermoPlate, Equipar), according to the manufacturer's instructions. Concentrations were expressed as mean ± standard error of mean (SEM) of cytokine concentrations (pg/mL).

3.2.10 Western blotting Assays

HUVEC cells were lysed by syringe passage in ice-cold RIPA buffer (1% NP-40, 0.25% sodium deoxycholate, 150mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 50 mM Tris-HCl pH 7.4) supplemented with a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, USA). Proteins from the cell lysates were separated by electrophoresis using a 15% SDS-PAGE polyacrylamide gel, and then transferred to nitrocellulose membrane (Millipore, Bedford, MA, USA). The blotted membrane was stained with a 10% solution of Ponceau red (Sigma Aldrich) and blocked with 3% TBS-T-milk (140 mM NaCl, 20 mM Tris-HCl pH 7.4, 0.1% Tween-20, 3% milk powder) for 1 h and washed three times with TBS buffer. Thereafter, membranes were incubated with the antibodies rabbit anti-α-tubulin (1/5000, Sigma-Aldrich) or rabbit anti-β-actin mAb (1/5000, Cell Signaling Technology, Inc.) in 3% TBS-T milk overnight at 4°C. The membranes were then washed three times with TBS buffer and incubated with secondary anti-rabbit and anti-mouse antibodies (1: 1000, Jackson Immuno Research, USA) in 3% TBS-T-milk for 1 h, and washed three times with TBS-T buffer.

The blots were visualized and developed with the enhanced chemiluminescence (ECL) method. Levels of β-actin were used as endogenous control.

3.2.11 Statistical analysis

For samples with normal distribution, analysis of variance (ANOVA) was used for repeated measures followed by the Bonferroni and test. The Kruskal-Wallis test followed by Dunn’s test was used for samples with non-normal distribution. Results were shown as mean ± standard error of mean (SEM). Statistical significance was defined as $p < 0.05$.

3.3 Results

The present study investigated the action of PL on two cell lines derived from normal or neoplastic tissues, HUVEC and HEp-2, respectively, as well as the potential effect of PL and ANXA1 interactions on biological processes related to inflammation and cancer.

3.3.1 Molecular Docking

Fourteen human proteins related to inflammatory and neoplastic processes with defined three-dimensional structures in PDB were submitted to molecular docking studies. Piplartine and nine anti-inflammatory drugs that meet the Lipinski rules were used as ligands. The data showed that PL exhibits physicochemical properties similar to those of the anti-inflammatory drugs, including binding free energy (**Table 1**).

Docking method was validated by redocking cocristallized ligand to MAP kinase 1 (MAPK 1). The results showed that the redocked ligand pose is similar to the cristallized pose, with a positional root mean square deviation (RMSD) below the tolerance level and that, therefore, the protocol is reliable to predict the binding conformation of ligands. The potential interactions obtained by the docking pose of PL with Annexin A1 are located in the sequence that corresponds to Ac_{2-26} peptide. Apparently, Ac_{2-26} peptide binds to piplartine via two hydrogen bonding interactions at lysine 9 but not at tryptophan 12 (**Figure 1**). Six anti-inflammatory drugs in current clinical use (acetaminophen, ketorolac, naproxen, nimesulide, piroxicam, resveratrol) also showed potential interactions with Annexin A1 in the same sequence (**Supplementary File 1 – Figure S1**). PL and ANXA1 were thus selected for subsequent experiments.

3.3.2 UV-Vis Absorbance and Fluorescence Spectroscopy

The UV-Vis absorption and fluorescence spectroscopic studies were performed to investigate the piplartine-annexin A1 interactions. Fluorescence emission data (**Supplementary File 2 – Figure S2**) were plotted in double logarithmic frame (**Supplementary File 3 – Figure S3**) to obtain energy values involved in Ac_{2-26} peptide and piplartine interaction[45] and the van't Hoff analysis was performed to determine the thermodynamic parameters. According to the sequence of Ac_{2-26}, the presence of three endogenous fluorescence probes enhances the probability of

checking distinct regions of the peptide undergoing interaction. Two phenylalanine (F) probes at positions 6 and 12, and one tryptophan (W) at position 11 favorably expand the search in two sectors of the peptide. Free energy changes were monitored by the changes in emission spectra during titration. Excitation wavelengths of 280 nm and 295 nm excited F and W probes, respectively. At both excitation wavelengths, the enthalpy and entropy variations were positive ($\Delta H>0$ and $\Delta S>0$) and the Gibbs free energy variation was negative ($\Delta G<0$) as shown in **Figure 2**, suggesting that the interaction of PL with the annexin A1-derived peptide Ac2-26 occurs spontaneously. Excitation wavelength was fixed at 280 and 295 nm in order to excite phenylalanine and tryptophan residues, respectively.

3.3.3 Ac_{2-26} and PL modulate proliferation, viability and apoptosis

The cytotoxic effects of LPS, Ac_{2-26} and PL on cell proliferation, viability, apoptosis and necrosis were investigated using normal tissue-derived endothelial cells (HUVEC) and a carcinoma-derived cell line (HEp-2). The results indicated that HUVEC treated with LPS and LPS + Ac_{2-26} did not alter the cell proliferation rate, maintaining a pattern of growth similar to control cells. Differently, treatment with Ac_{2-26} alone significantly increased cell proliferation over 72 h, but showed no effect on cell viability, apoptosis and necrosis. Contrary to these findings, treatment with PL alone, or in combination with LPS and/or Ac_{2-26} for 48 or 72 h, decreased cell proliferation (**Figure 3**), and increased apoptosis when combined with LPS **(Figure 4)**.

To validate whether Ac_{2-26} and/or PL influence carcinoma cell survival, HEp-2 cells were treated with PL or PL + Ac_{2-26} and MTS assays were read at 24, 48 and 72 h. Both treatments reduced cell viability in a dose-dependent manner when compared to control cells exposed to vehicle (DMSO) alone in the same conditions of treated cells (ANOVA $p<0.05$) (**Figure 5**).

3.3.4 PL inhibits migration of carcinoma cells

In order to investigate whether PL could affect metastasis of carcinoma cells, the effects of PL on cell migration and invasion in MTS assays were analyzed. As can be observed in **Figure 6A**, PL suppressed cell migration ability of HEp-2 treated with PL for 72 h compared to control cells exposed to DMSO or grown in complete medium. The numbers of migratory cells were reduced to almost zero in drug-treated group (ANOVA $p<0.001$). Differently, PL treatment reduced invasion in Hep-2 cells, but not significantly (ANOVA $p>0.1$) (**Figure 6B**).

3.3.5 PL inhibits α-tubulin expression

Western blotting data demonstrated absence of tubulin in HUVEC cells treated with piplartine (PL, PL/Ac_{2-26}, LPS + PL, LPS + Ac_{2-26}/PL), suggesting that PL directly or indirectly affects cytoskeleton reorganization, as observed in migratory assays. As expected, the endogenous control showed similar immunoreactivity in all experimental conditions (**Figure 7**).

3.3.6 PL modulates chemokine and cytokine expression

The effects of LPS, Ac_{2-26} and PL on chemokine and cytokine expression were investigated using HUVEC cells. The cells were grown in different experimental conditions and the supernatants were collected for the determination of MCP-1 chemokine and IL-8 and IL-1β cytokines (**Figures 8A-F**) after 24 and 72 hours. After 24 hours, a significant increase in the levels of the proinflammatory MCP-1 chemokine (**Figure 8A**) was observed in the LPS treated cells, and only LPS and PL groups increase IL-8 levels after 24h (**Figure 8B**). Ac_{2-26} peptide did not alter significantly the MCP-1and IL-8 levels compared to controls (**Figures 8A and 8B**). No significant differences between groups in relation to IL-1β levels were detected (**Figure 8C**).

At the 72-hour time point, no effect of LPS in modulating cytokines was observed. The levels of MCP-1 (**Figure 8D**) and IL-8 (**Figure 8E**) were high in control. Treatment with PL, alone or in combination with LPS and/or Ac_{2-26}, induced a reduction of MCP-1 concentration. Ac_{2-26} peptide did not alter significantly the MCP-1and IL-8 levels. Regarding IL-8 and IL-1β levels, no significant differences were observed in all groups studied (**Figures 8E and 8F**).

3.3.7 PL modulates expression of genes involved in inflammatory processes

To explore the role of PL in inflammatory processes, expression of 92 genes related to inflammatory response were quantified in treated carcinoma cells and compared to untreated control cells. A gene ontology analysis using DAVID tools was performed. As expected for a PCR array targeting genes involved in inflammatory processes, the highest ranked molecular functions for differentially expressed genes after PL treatment were phospholipase C, leukotriene receptor, phosphatidylinositol phospholipase C, receptor signaling protein, MAP kinase, G-protein coupled peptide receptor and signal transducer activities (*Benjamini*-Hochberg method, p-values<0.05). Twenty-five genes showed decreased expression compared to

untreated cells, particularly genes encoding phospholipases, leukotrienes and interleukin receptors, serine/threonine kinases, and TNF-activated receptor. Among the 18 genes with increased expression in PL-treated cells are the ones that encode phospholipase A2, cytokine and interleukin receptors, endopeptidases and the transcriptional regulator TNF (**Table 2**). Two major canonical pathways were identified by the Ingenuity Pathway Analysis (IPA) for the set of differentially expressed genes were Vitamin C Antioxidant Action and Eicosanoid Signaling (p values $5.27E^{-15}$, $6.10E^{-15}$, respectively). The five upstream regulators of these pathways with lower p values were TNF, IL1B, IL4, LPS and CD40 (p values of overlap = $6.09E^{-15}$ to $1.20E^{-10}$), and the major diseases and biological functions were Cardiovascular Disease, Response Inflammatory Disease, Immune Disease, Inflammatory Disease and Respiratory Disease (p values = $7.43E^{-20}$ to $1.21E^{-05}$).

3.4 Discussion

In the present study, the role of piplartine, a natural substance extracted from *Piper longum* pepper, was evaluated for its activity as a ligand of the endogenous protein annexin A1 by favoring or attenuating its anti-inflammatory effects. PL was also analyzed *in vitro* for its biological functions linked to inflammation and tumor development.

Docking analysis of PL with annexin A1 showed that potential interactions between these compounds are located in the sequence corresponding to Ac_{2-26} peptide and that, therefore, the peptide can be used in functional experiments together with PL.

Computational and spectroscopy tools used in conjunction with each other were essential to evaluate physical aspects of the Ac_{2-26}-PL interaction. The peptide presents endogenous fluorophores, which helped the titration experiments. The responses of P6, P12 and W11 residues were very similar though in different positions of the peptide. The thermodynamic parameters confirmed a favorable interaction between Ac_{2-26} and PL, due to negative Gibbs free energy calculation. These results confirmed the docking analysis of PL with annexin A1 showing that potential interactions between these compounds are located in the sequence corresponding to Ac_{2-26} peptide and that, therefore, the peptide can be used in

functional experiments together with PL. Such results suggest that the molecular structures and charge distribution in both compounds are appropriate for stereochemical recognition, which was sustained by considering the role of enthalpy and entropy contributions. The data also showed that the energy balance is dictated by entropy rather than enthalpy. No covalent bonds are formed, and the complex probably is stabilized by weak interactions, indicating that electrostatic interactions may have a key role in the formation of the complex.

In vitro analyses were conducted to explore whether piplartine and/or Ac_{2-26} alter proliferation, viability, apoptosis and necrosis in cells derived from normal or neoplastic tissues and, consequently, affect inflammatory responses. The results confirmed that Ac_{2-26} significantly increases cell proliferation. In fact, previous studies of our group [46] have already shown that the peptide induces cell proliferation in HUVEC cells, both under basal conditions and after stimulation by VEGF. Other studies using inflammation and tumor models evidenced that ANXA1 may stimulate proliferation [20] and migration [47] or even inhibit proliferation [48], probably depending on the context of the experiment, cell type or tissue.

The treatment with PL, alone or together with LPS or Ac_{2-26}, showed antiproliferative effect on the normal endothelium- and carcinoma-derived cell lines, with a significant induction of late apoptosis in HUVEC cells and decrease in viability in HEp-2 cells. These results indicate that PL abrogates the proliferative effect of annexin. PL also suppressed cell migration ability of neoplastic cells but was not able to significantly reduce invasion.

Previous studies of our group had already observed that PL is able to reduce leukemic cell survival regulating cell death by caspase-dependent apoptosis and/or necrosis, an effect that appears to be selective for tumor cells [28] and not related to cell membrane damage [27]. Bezerra and collaborators also observed the PL increases antitumor activity of chemotherapeutic drugs, in both *in vitro* and *in vivo* experimental models [29], and induces G2/M cell cycle arrest, probably due to its genotoxicity [30]. In addition, the authors commented that the compound is cytotoxic to tumor cell lines and that this may be due to the presence of two α, β-unsaturated carbonyl radicals [29]. Other groups have expanded the knowledge of the biological properties of PL and suggested, inter alia, that PL inhibits hypoxia inducible factor-2 (HIF-2) transcription [49] and modulates redox and ROS homeostasis [50,51].

The presence of a trimethoxyaromatic ring in PL structure, which may favor an interaction with tubulin [33], and the fact that PL may be considered a microtubule-destabilising agent with antiproliferative effects [34] provide evidence that PL affect tubulin polymerization. Microtubules are composed of protofilaments containing repeating α/β-tubulin heterodimers and contribute to cell shape and chromosome segregation. During the course of the cell cycle, interphasic microtubules generate mitotic and meiotic spindles, which allows the correct distribution of the chromosomes at cell division [52]. Microtubule dysfunction may result in chromosomal instability, mitotic arrest and cell death [53]. The results of the present study, besides the antiproliferative and antimigratory activities of PL, showed that the treatment of a normal cell line with this compound, alone or together with LPS or Ac_{2-26}, inhibited the expression of α-tubulin and, therefore, suggest that PL may have a metastasis-inhibitory effect, directly or indirectly linked to cytoskeleton reorganization and migratory features.

The results obtained in the present study showed that LPS increases pro-inflammatory MCP-1 chemokine and IL-8 cytokine expression in endothelium-derived cells, an expected result considering the LPS properties [54,55]. Piplartine, alone or in combination with LPS and/or Ac_{2-26}, also induced high levels of IL-8 levels, but reduced the MCP-1 levels, with no effect on annexin performance. A recent study observed that high IL-8 levels may result in low capillary activity not related to cell viability [56]. IL-8 is a member of the CXC chemokine family that regulates endothelial cell migration, proliferation and angiogenesis [57], and high levels of IL-8 reduce capillarization in *in vitro* and in *ex vivo* systems [56]. Thus, we can conclude that piplartine effects on cell proliferation may be in part due to PL-mediated IL-8 expression.

To a better understanding on the role of PL in modulating inflammatory mediators associated with tumorigenesis, the expression of 92 genes were analyzed in carcinoma cells. Among the genes that showed altered expression after PL treatment are receptors and enzyme linked to leukotriene biosynthesis (LTA4H, LTB4R, LTB4R2), prostaglandin (PTGER2), interleukin (IL2RB) and adrenergic (ADRB1) receptors, G-protein coupled receptors (BDKRB1, HRH1, TBXA2R), membrane receptors involved in cell adhesion (ITGB1), regulators of inflammatory response (TNFSF1A), or members of signaling pathways (A2M, MAPK1, MAPK8, MAPK14, PDE4B, PDE4D, PLCB3, PLCE1, PLCG1, PLCG2), transcriptional

regulators (NFKB1, NR3C1) and calcium channel subunits (CACNB2, CACNB4). The genes of two phospholipases (PLA2G2A and PLA2G5), tumor necrosis factor (TNF), interleukin and cytokine receptors (CD40, IL1RL1, IL2RA and IL1R2) and leukotriene (CYSLTR1) had high expression after treatment.

The altered expression of these genes confirms the action of piplartine as a substance capable of regulating the synthesis or activity of important members of the inflammatory cascade. Leukotrienes (LTs) and prostaglandins (PGs) are two good examples. These compounds are representatives of the class of eicosanoid lipids derived from the arachidonic acid released from the membrane by phospholipase A2 and subsequently oxygenated by the lipoxygenase (LOX) and cyclooxygenase (COX) pathways, respectively [58]. Both leukotrienes and prostaglandins act on homeostasis and inflammation through G protein-coupled receptors and can be blocked by non-steroidal anti-inflammatory drugs [59]. Prostaglandins are synthesized by most cells and act in an autocrine and paracrine manner, whereas leukotrienes, both cysteine (cys-LTs) and LTB4, are generated by inflammatory cells after stimulation triggered by exogenous factors or events of intracellular phosphorylation [60,61].

Proinflammatory signaling initiated by leukotriene LTB4 through its BLT1 and BLT2 receptors (encoded by *LTB4R* and *LTB4R2* genes) has been associated with various diseases, such as asthma, rheumatoid arthritis, atherosclerosis, abdominal aortic aneurysm, multiple sclerosis, and cancer. Signaling by cys-LT receptors, in turn, has emerged as a key component of vascular inflammation, with an important role in the pathogenesis and progression of cardiovascular diseases (reviewed by [62]. Prostaglandin PGE2 and its cognate receptors (EP1-4, encoded by *PTGER1-4*) are also involved in many processes, including vascular permeability, cell proliferation and cell migration. (reviewed by [58]. In vitro studies have shown that the signaling pathway triggered by prostaglandins and the EP2 receptor in the presence of lipopolysaccharides is associated with both anti-inflammatory effects and proinflammatory effects, and that these responses depend on the type of stimulus, but also on the immune cell in which they were activated (reviewed by [63]).

Prostaglandins have been extensively studied and high levels of PGE2 have already been observed in several subtypes of cancer. As a mediator of inflammation, PEG2 is involved in tumor growth and progression and, together with LTB4, can stimulate signaling pathways involving phospholipases (PLCs), phosphodiesterases

(PDEs), cyclic nucleotides, inositol triphosphate (IP3), calcium channels, phosphatidylinositol 3 (PI3K) and MAPK kinases, resulting in different events, such as proliferation, angiogenesis, migration, invasion and survival [64,65]. Several of these effectors showed altered gene expression in HEp-2 cells after piplartine treatment.

Proinflammatory proteins were also inhibited by PL, such as those acting as interleukin receptors and linkers (encoded by the *A2M*, *IL2RB* and *TNFRSF1A* genes), transcription factors (encoded by the *NFKB1* and *NR3C1* genes), phospholipases C, all of which are known promoters of inflammation. Phospholipases C catalyze the hydrolysis of phosphatidylinositol 4,5 bisphosphate in two secondary messengers, diacylglycerol and inositol 1,4,5-triphosphate. Cui et al. [66] observed that the elevation of *PLCE1* expression in patients with esophageal carcinoma is associated with lymph node metastasis and staging of the lesion. The silencing of this enzyme in bladder carcinoma cells led to a reduction in the levels of metalloproteinases and the Bcl-2 apoptosis regulator, consequently decreasing invasion activities [67].

Most results on gene expression analysis support the anti-inflammatory and antitumoral properties of piplartine. The explanation of PL-mediated induction of proinflammatory genes, may lie on the simultaneous dual activity pro and anti-inflammatory of some proteins or on negative feedback mechanisms. This dual activity depends on the cellular context and is observed for several inflammatory cytokines, such as TNF (tumor necrosis factor). TNF plays a central role in the pathogenesis of some inflammatory diseases and has been extensively studied by mediating important biological processes - cell proliferation, survival, and death (37, 38). Deregulation of such processes is a characteristic of inflammation and cancer. The signal transduction pathway of TNF is complex. Responses to this factor are triggered by the activation of one of its receptors, TNFR1 and TNFR2 (encoded by the *TNFRSF1A* and *TNFRSF1B* genes, respectively). The extracellular domains of these receptors are homologous and have similar affinity for TNF, but the cytoplasmic regions are distinct and activate signaling events with different biological effects. While TNFR1 contains the death domain, TNFR2 does not. Thus, depending on the context, receptor activation may promote proliferation or apoptosis [68,69].

The TNFR1 receptor showed reduced levels following treatment with piplartine, and TNFR2 showed no expression change. This result indicates a decreased TNFR1 / TNFR2 ratio compared with control, which might alter the cell fate, but no definitive

conclusions can be drawn at the moment. These antagonistic effects evidence the complexity of the inflammatory process and show that the development of efficient control mechanisms is still a great challenge.

3.5 Conclusions

In conclusion, we can assume that PL and other anti-inflammatory compounds show potential interactions with the same peptide sequence of annexin A1. This interaction appears to be neutral regarding the anti-inflammatory and antitumoral properties of PL, including its effect on cell proliferation, viability, apoptosis and migration, of both normal and neoplastic cells. As an anti-inflammatory agent, PL is able to regulate the expression of MCP-1 chemokine, IL-8 cytokine and genes that act in several immune signaling pathways and inflammatory diseases. In addition, the data confirm an inhibitory effect of PL on tubulin expression and consequently on cytoskeleton organization. Considering the role of the inflammatory context in promoting tumor initiation, the present study reinforces the potential of piplartine as a therapeutic immunomodulator for cancer prevention and progression.

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3.7 Disclosure

The authors declare that they have no conflict of interest.

3.8 Author contributions

CFZ: designed and performed experiments with HUVEC cells, acquisition of data, analysis and interpretation of data, drafting the manuscript. TH: participated in the design of the study, performed experiments with HEp-2 cells, identified the

metabolic pathways and associated ontologies, carried out the analysis and interpretation of the data, and preparation of the manuscript. APG: analysis and interpretation of data, revising the manuscript critically for important intellectual content. All authors agree to be accountable for all aspects of the work. NJFS performed molecular docking and contributed to data analysis and interpretation. DPB and ERS participated in the design of the study and revised the manuscript. MLC conceived and participated in the design of the study, performed UV-Vis Absorbance and Fluorescence Spectroscopy experiments and revised the manuscript. EHT and SMO: conceived and coordinated the design of the study, obtained funding for the project, evaluated the results, contributed to drafting the manuscript and supervised the process. All authors critically revised the manuscript, checked the accuracy of the data, and approved the version to be published.

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3.10 Tables and Figures

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Table 1. Lipinski parameters. Physicochemical properties of *PL and common a*nti-inflammatory drugs in current clinical use.

Ligand	Zinc code	Molecular weight	Log P	Number of H-bond donors	Number of H-bond acceptors
Acetaminophen	18274777	151.165	0.68	2	3
Dexamethasone	03875332	392.467	2.06	3	5
Diclofenac sodium	00001281	295.145	4.57	1	3
Ketoprofen	00002272	253.277	3.59	0	3
Ketorolac	00002279	254.265	2.20	0	4
Naproxen	00105216	229.255	3.38	0	3
Nimesulide	04617749	307.307	2.81	0	7
Piroxicam	12466469	331.353	0.88	2	7
Resveratrol	00006787	228.247	0.79	3	3
PL	00899053	317.341	1.62	0	6

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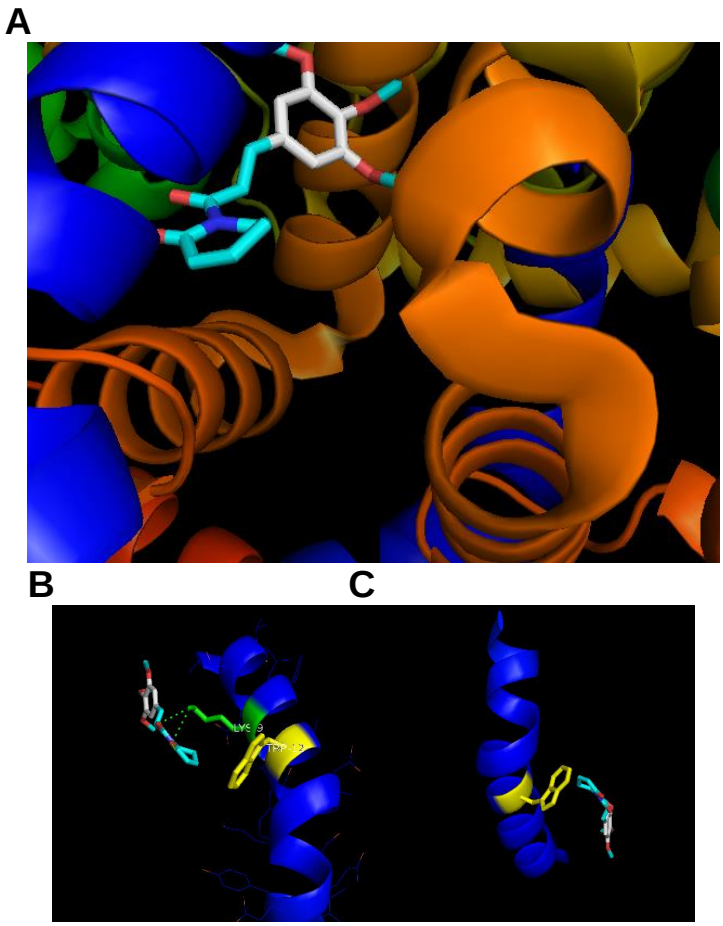

Figure 1. The docked pose of PL onto Ac2-26 peptide. (**A**) Secondary structure of the annexin A1 is represented in orange, peptide in dark blue and PL as sticks. (**B, C**) Apparently, $Ac_{2\text{-}26}$ peptide binds to PL via two hydrogen bonding interactions (dotted lines) at lysine 9 (in green) but not at tryptophan 12 (in yellow).

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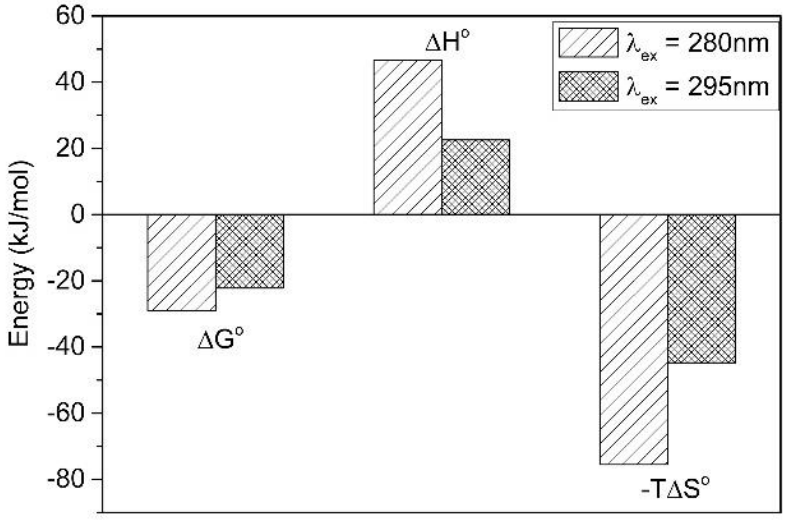

Figure 2. Energy contributions to the interaction between the Ac_{2-26} and PL at diferente excitation wavelengths (280 nm and 295 nm). Monitoring the microenvironment of the interaction around the aromatic tryptophan residue. ΔG=Gibbs free energy changes, ΔH=enthalpy changes, ΔS=entropy changes, T=temperature.

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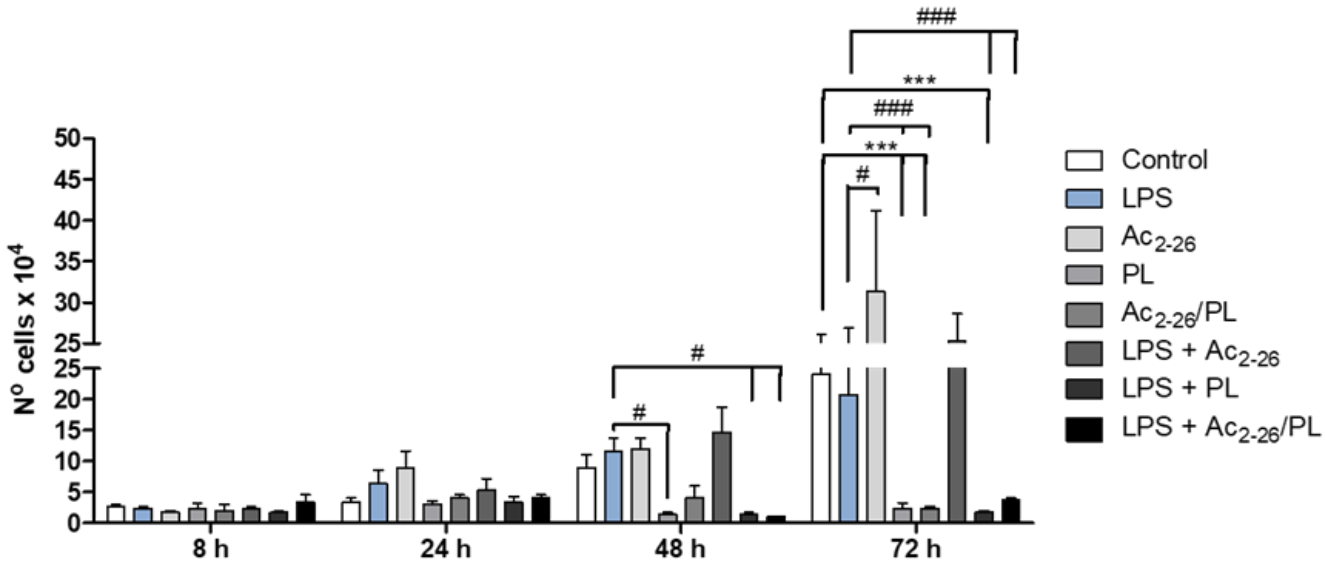

Figure 3. Ac_{2-26} increases and PL reduces endothelial cell proliferation. HUVEC cells were incubated with complete medium (control), and treated with LPS, Ac_{2-26} and/or PL for 8, 24, 48 and 72 h. Results are expressed as the means ± SEM of triplicates. (*** p <0.05 versus control; # p <0.05, ###p <0.001 versus LPS).

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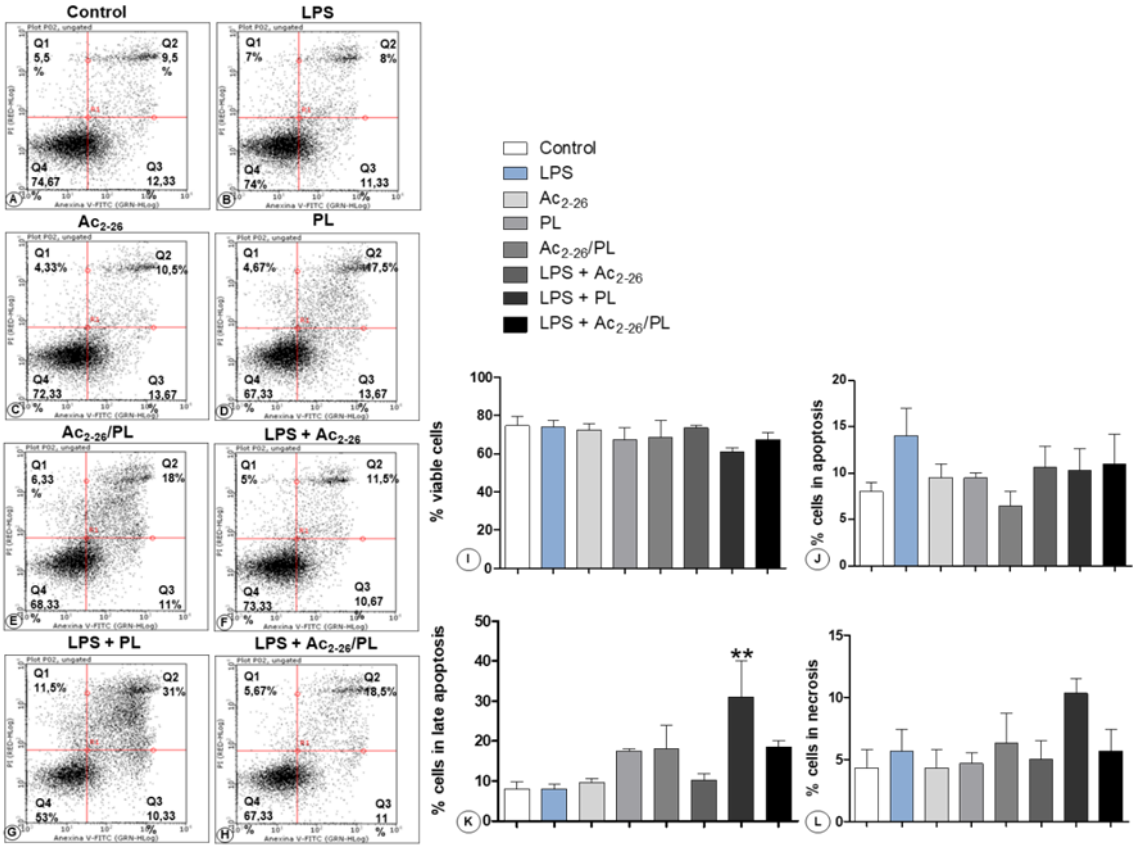

Figure 4. Effects of PL and Ac_{2-26} treatment on viable cells (Q4), apoptosis (Q3), late apoptosis (Q2) and necrosis (Q1). PL combined with LPS increases late apoptosis (Q2) compared to control cells (**G**). HUVEC cells were incubated with complete medium (control), LPS, Ac_{2-26} and/or PL for 72 h. Cell viability was measured by incubation with Annexin-V and/or PI and percentage of cells (%) evaluated by flow cytometry (**A-H**). (**I-L**) Results are expressed as the means ± SEM of three independent assays (n = 3). (** p <0.01 versus control).

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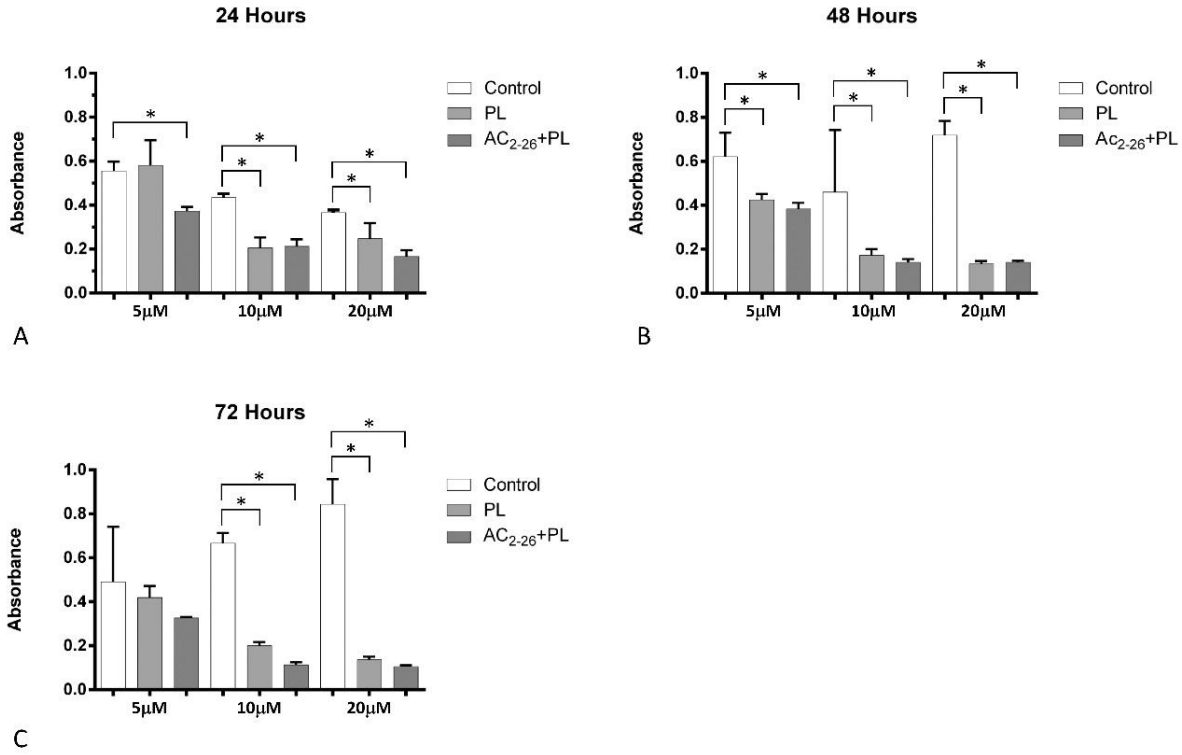

Figure 5. PL alone or combined with Ac_{2-26} reduces HEp-2 cell viability in a dose-dependent manner. HEp-2 cells were treated with PL alone or combined with A_{c2-26} for (**A**) 24, (**B**) 48 and (**C**) 72 h, and cell viability was measured via MTS assay. Assays were carried out in triplicate, and experiments were performed two times (ANOVA $p<0.05$).

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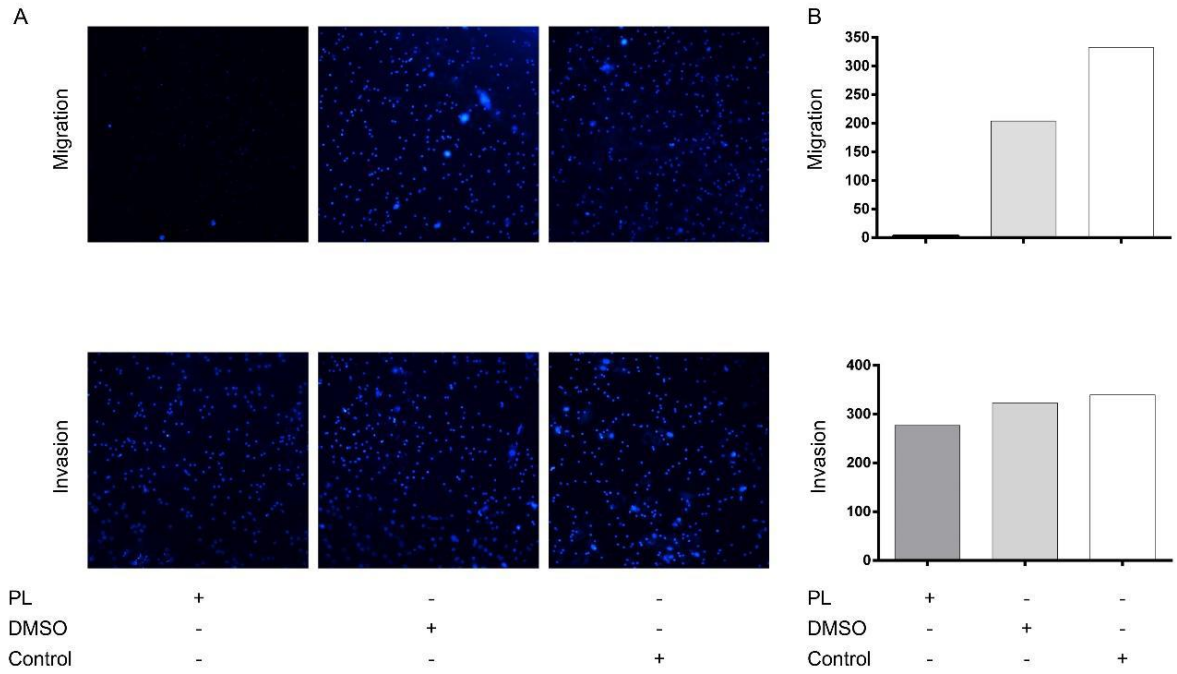

Figure 6. PL inhibits migration of carcinoma cells. (**A and B, top**): PL inhibited migration of HEp-2 cells treated with PL for 72 h compared to control cells) exposed to DMSO or grown in complete medium. The numbers of migratory cells were reduced to almost zero in drug-treated group (ANOVA $p<0.001$). (**A and B, bottom**: PL treatment of HEp-2 cells showed no effect on invasion compared to control cells exposed to DMSO or grown in complete medium (ANOVA $p>0.1$). Each experiment was performed in three replicate wells.

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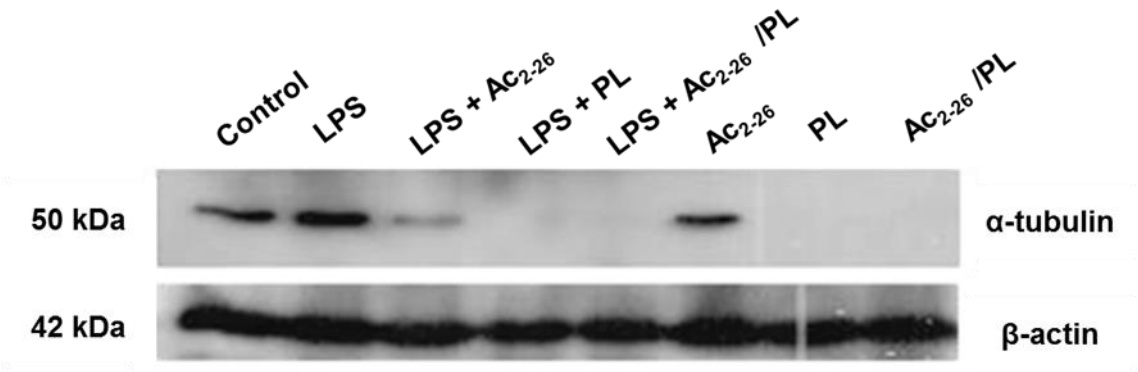

Figure 7. PL inhibits α-tubulin expression. Western blotting analysis of HUVEC cells treated with LPS, Ac_{2-26} and/or PL shows that PL inhibits α-tubulin (50 kDa) expression. β-actin (42 kDa) was used as an endogenous control.

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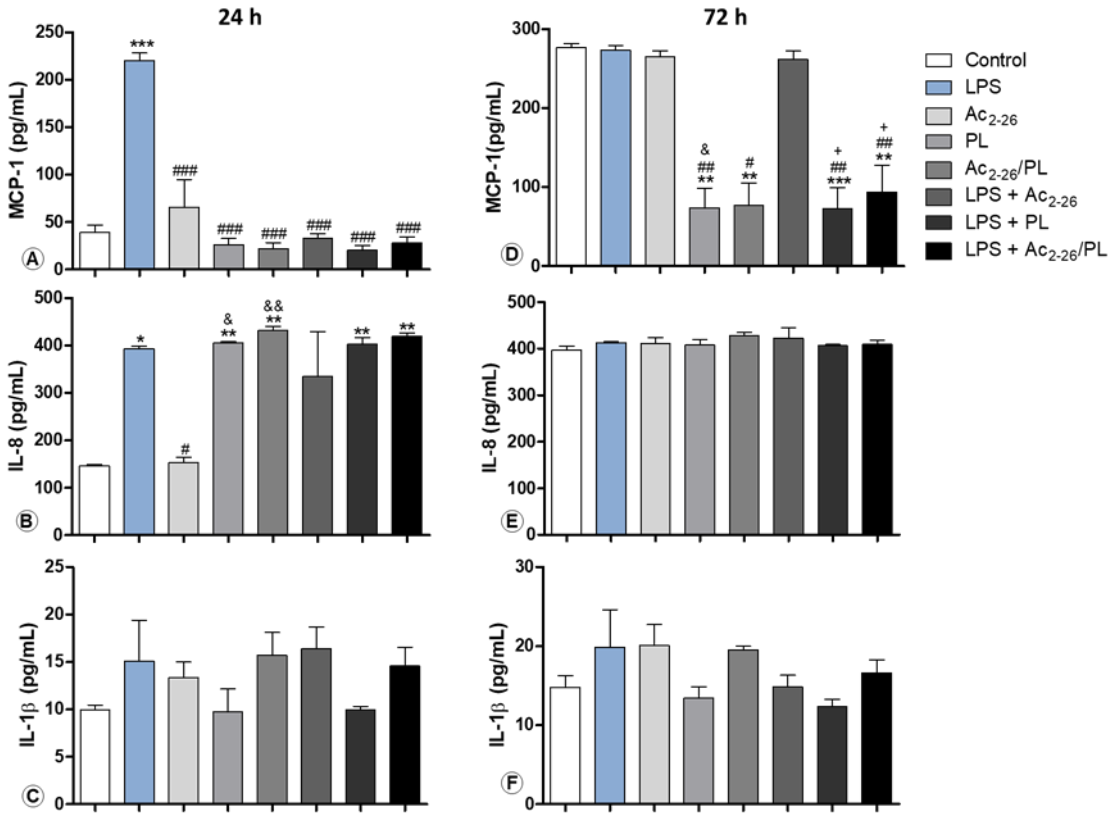

Figure 8. Ac_{2-26} and PL modulate chemokine and cytokine expression. Proinflammatory cytokines MCP-1 (**A and D**), IL-8 (**B and E**), IL-1β (**C and F**), were obtained from the supernatants of HUVEC cells, treated with LPS, Ac_{2-26} and/or PL for 24 and 72 h. The results are expressed as the means ± SEM of three independent assays (n = 3). (* p <0.05, ** p <0.01, *** p <0.001 versus control; # p <0.05, ## p <0.01 ### p <0.001 versus LPS).

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Table 2. PL modulates expression of genes involved in inflammatory processes. Differentially expressed [log2 (fold change]) genes identified by a PCR array (TaqMan® Array Human Inflammation 96-well plate) in HEp-2 cells treated with PL compared to untreated cells.

Gene symbol	Molecular function*	Fold change	Gene symbol	Molecular function*	Fold change
LTB4R2	leukotriene receptor	- 8.13	*TNF*	transcription regulator	7.89
LTB4R	leukotriene receptor	- 2.21	*IL2RA*	interleukin-2 receptor	6.00
PLCE1	phospholipase C	- 2.15	*IL1R2*	interleukin-1 receptor	5.90
PDE4D	phosphodiesterase	- 2.13	*PLA2G2A*	phospholipase A2	3.90
TBXA2R	thromboxane A2	- 2.12	*ITGAM*	glycoprotein binding	3.91
TNFRSF1A	TNF-activated receptor	- 2.11	*CYSLTR1*	cys-leukotriene receptor	2.98
PTGER2	prostaglandin receptor	- 2.06	*HTR3A*	serotonin receptor	2.97
IL2RB	interleukin-2 receptor	- 2.06	*ALOX12*	arachidonate 12- lipoxygenase	2.94
ITGB1	fibronectin binding	- 1.17	*ADRB2*	beta2-adrenergic receptor	2.91
MAPK8	serine/threonine kinase	- 1.16	*CD40*	signal transducer	2.87
MAPK14	MAP kinase	- 1.15	*IL1RL1*	cytokine receptor	2.87
MAPK1	serine/threonine kinase	- 1.15	*KLK2*	endopeptidase	2.87
BDKRB1	bradykinin receptor	- 1.13	*KLK3*	endopeptidase	2.87
HRH1	histamine receptor	- 1.12	*KNG1*	endopeptidase inhibitor	2.87
CACNB4	calcium channel	- 1.11	*PLA2G5*	phospholipase A2	2.87
PLCB3	phospholipase C	- 1.11	*NOS2*	nitric-oxide synthase	2.72
LTA4H	leukotriene-A4	- 1.11	*CES1*	hydrolase	1.91
A2M	interleukin-1/TNF	- 1.10	*KLK14*	endopeptidase	1.88
PLCG2	phospholipase C	- 1.10			
NFKB1	transcription fator	- 1.09			
NR3C1	transcription factor	- 1.09			
PDE4B	phosphodiesterase	- 1.07			
PLCG1	phospholipase C	- 1.06			
ADRB1	receptor signaling	- 1.05			
CACNB2	calcium channel	-1.04			

*According to Gene Ontology Consortium (http://geneontology.org/).

3.12 Supplementary files

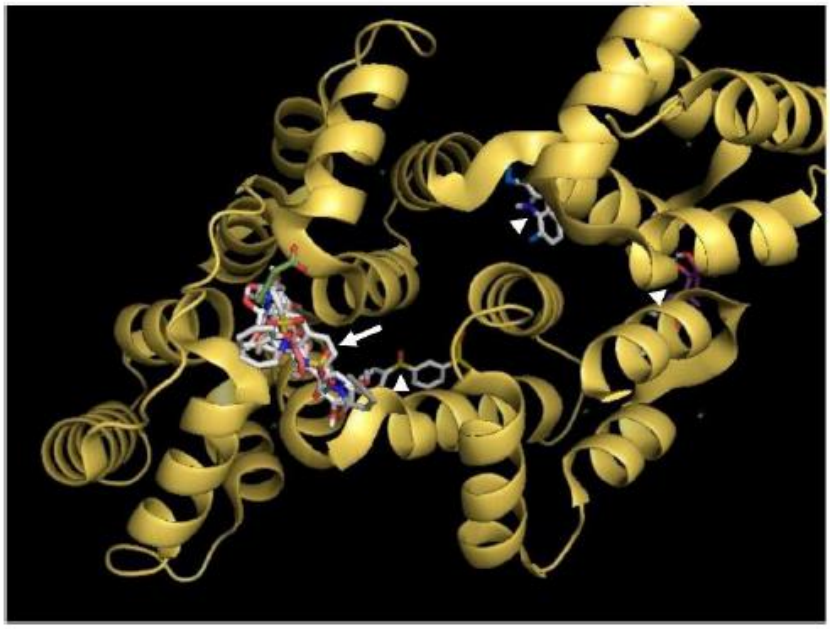

Figure S1. The docked pose of nine anti-inflammatory drugs in current clinical use onto Ac_{2-26}. Secondary structure of annexin A1 is represented in yellow, drugs and PL are shown as sticks. Arrow= acetaminophen, ketorolac, naproxen, nimesulide, piroxicam, resveratrol, PL; arrowheads= dexamethasone, diclofenac sodium, ketoprofen.

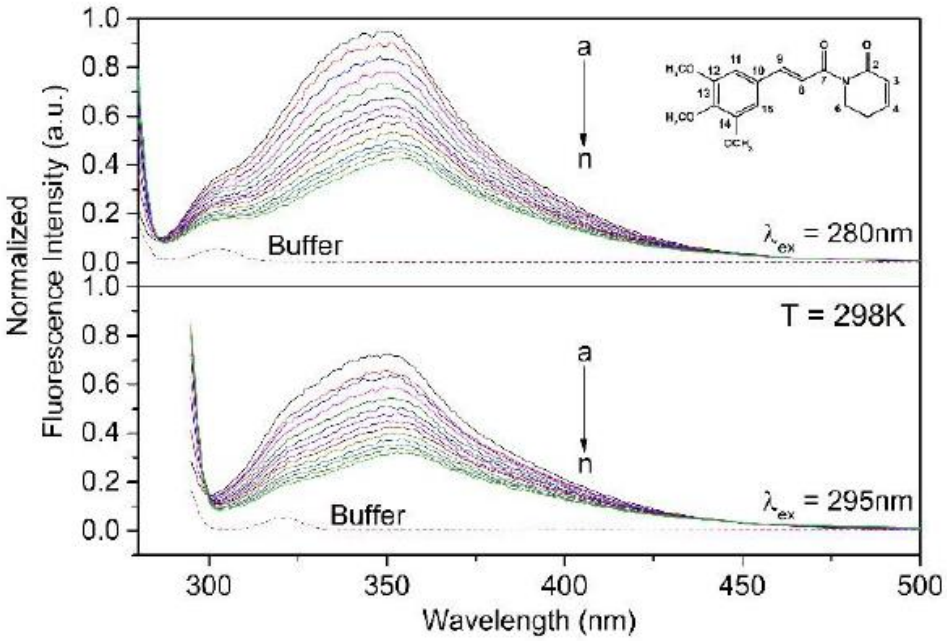

Figure S2. Emission spectra of the peptide (5.0 μM) in the absence (a) and presence of PL (b-n: 1.2-15.6μM) at 280 nm and 295nm excitation wavelengths. The arrow indicates the progress of titration, with increments of 1.2 μM (pH 7.0, T = 298 K). The dashed line represents the emission spectrum of phosphate buffer. Insert: chemical structure of PL.

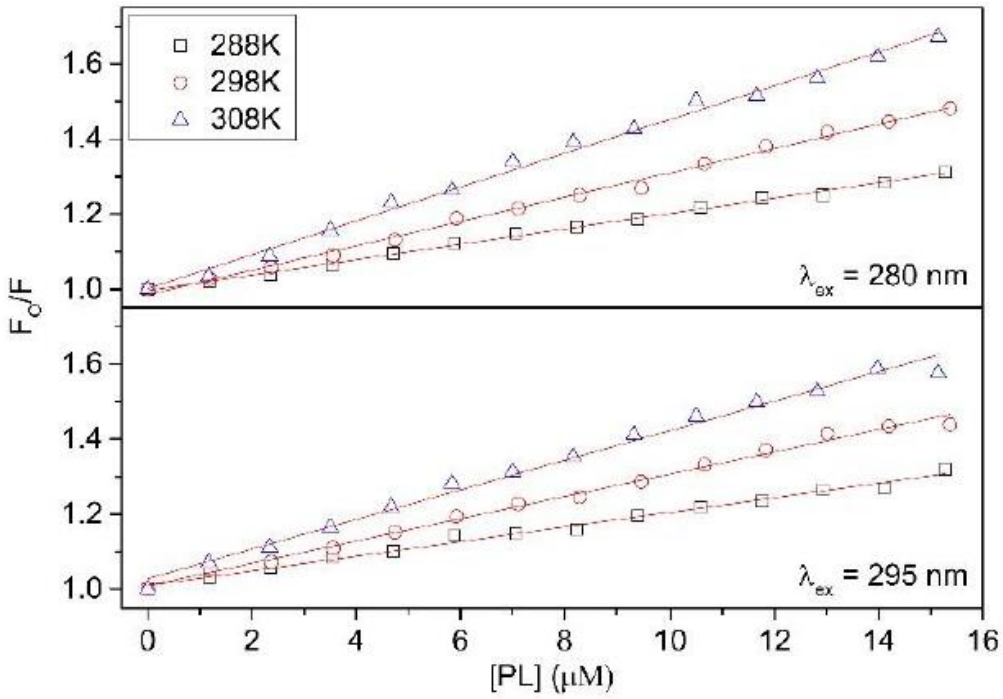

Figure S3. Stern-Volmer plots of fluorescence intensity quenching at 280 nm and 295 nm excitation wavelengths of the peptide $Ac_{2\text{-}26}$ by titration of PL at 288, 298 and 308 K, pH 7.0. Correlation coefficient ≥ 0.991.

4 Discussão

No presente estudo, o papel da piplartina (PL), substância natural extraída de *Piper longum pepper*, foi avaliado quanto à sua atividade como ligante da proteína endógena annexin A1 (ANXA1), favorecendo ou atenuando seus efeitos anti-inflamatórios. A PL foi também analisada *in vitro* por suas funções biológicas ligadas à inflamação e desenvolvimento tumoral.

As análises de ligação molecular, entre a PL e a ANXA1, demonstraram que as interações potenciais entre estes compostos estão localizadas na sequência correspondente ao peptídeo Ac_{2-26} da ANXA1, portanto, o peptídeo pode ser usado em experimentos funcionais em conjunto com PL.

Ferramentas computacionais e de espectroscopia, usadas em conjunto, foram essenciais para avaliar aspectos físicos da interação Ac_{2-26}-PL. O peptídeo apresenta fluoróforos endógenos, que ajudaram nas experiências de titulação. As respostas dos resíduos P6, P12 e W11 foram muito semelhantes, embora em posições diferentes do péptido. Os parâmetros termodinâmicos obtidos para esta interação, com uma mudança de energia livre de Gibbs negativa, apontaram para uma reação espontânea. Estes resultados confirmaram a análise de *docking* da PL com anexina A1 mostrando que interações potenciais entre esses compostos estão localizadas na sequência correspondente ao péptido Ac_{2-26} e que, por conseguinte, o péptido pode ser utilizado em experimentos em conjunto com PL. Tais resultados sugerem que as estruturas moleculares e distribuição em ambos os compostos são apropriados para o reconhecimento estereoquímico, que foi sustentada considerando o papel das contribuições de entalpia e entropia. Os dados também mostraram que o balanço energético é ditado pela entropia e não pela entalpia. Portanto, não são formadas ligações covalentes e o complexo provavelmente está estabilizado por interações fracas, indicando que as interações eletrostáticas podem ter um papel fundamental na formação do complexo.

Análises *in vitro* foram realizadas para investigar se piplartina e / ou Ac_{2-26} alteram proliferação, viabilidade, apoptose e necrose em células derivadas de tecidos normais ou neoplásicos e, consequentemente, afetam as respostas inflamatórias. Os resultados confirmaram que o Ac_{2-26} aumenta a proliferação celular. De fato, estudo anterior do nosso grupo de pesquisa (Lacerda et al., 2015) demonstrou que o peptídeo induz a proliferação em células HUVEC, tanto em condições basais, como após estimulação por VEGF. Outros estudos, usando inflamação e modelos tumorais, evidenciaram que ANXA1 pode estimular a

proliferação (YANG et al., 2011) e migração (BIZZARRO et al., 2012) ou mesmo inibir a proliferação (WAN et al., 2017), provavelmente dependendo do contexto experimental, tipo celular ou tecidual.

Por outro lado, o tratamento com a PL, sozinho ou em conjunto com LPS ou Ac_{2-26}, apresentou efeito antiproliferativo na linhagem celular HUVEC, com indução significativa de apoptose tardia. Estudos prévios têm demonstrado que a PL é capaz de reduzir a sobrevida de células leucêmicas, regulando a morte celular por apoptose e/ou necrose dependentes de caspase, um efeito que parece ser seletivo para células tumorais (BEZERRA et al., 2007) e não relacionado ao dano da membrana celular (BEZERRA et al., 2005).

Bezerra e colaboradores (2008) também observaram que a PL aumenta a atividade antitumoral de drogas quimioterápicas, tanto em modelos experimentais *in vitro* como *in vivo*, e induz parada de ciclo celular na fase G2/M, provavelmente devido à sua genotoxicidade. Estes pesquisadores verificaram que a citotoxicidade da PL, nas linhagens celulares tumorais, pode ser devido à presença de dois radicais carbonilo α, β-insaturados. Outros grupos expandiram o conhecimento das propriedades biológicas da PL e sugerem, inibição do fator de transcrição induzível de hipoxia 2 (HIF-2) (BOKESCH et al., 2011), e modulação da homeostase redox e ROS (RAJ et al., 2011; ADAMS et al., 2012).

Ainda, Kong e colaboradores (2008) demonstraram, em duas linhagens de células de câncer de próstata humana (PC-3 e LNCaP), que a inibição do crescimento celular, mediada pela PL, está relacionada com a parada do ciclo celular na fase G_2/M associado com a diminuição da regulação de cdc-2, e essa indução de apoptose está associada com o aumento da clivagem de procaspase-3 e poli (DPribose) polimerase (PARP). Mais recentemente, outros estudos mostraram os mecanismos de ação da PL na citotoxicidade de maneira dose e tempo-dependente, e efeito antiproliferativo seletivo para células tumorais (JYOTHI et al., 2009; BOKESCH et al., 2011; RAJ et al., 2011; ADAMS et al., 2012).

Os resultados do presente estudo, além das atividades antiproliferativas da PL, mostraram que o tratamento das células HUVEC com este composto, isoladamente ou em conjunto com LPS ou Ac_{2-26}, inibiu a expressão da proteína endógena α-tubulina e, portanto, sugerem que a PL pode ter um efeito inibidor de metástase, direta ou indiretamente ligada à reorganização do citoesqueleto e características migratórias. Yang e colaboradores (2014) demonstraram que a rede de microtúbulos

nas células HepG2 exibiram disposição e organização normais na ausência do tratamento com 1 μM de uma substância, denominada composto 8. Em contraste, após exposição por 24 horas desse composto, a polimerização de microtúbulos e formação do fuso mostrou anormalidades distintas, as células pararam na mitose e apresentaram fusos mitóticos anormais.

A presença de um anel trimetoxiaromático na estrutura da PL, o que pode favorecer uma interação com a tubulina (SALUM et al., 2013), e o fato de que a PL pode ser considerada um agente desestabilizador de microtúbulos com efeitos antiproliferativos (MEEGAN et al., 2017) fornecem evidências de que a PL afeta a polimerização da tubulina. Os microtúbulos são formados por protofilamentos contendo heterodímeros repetitivos de α/β-tubulina e contribuem para a forma celular e segregação cromossômica. No decorrer do ciclo celular, os microtúbulos interfásicos geram fusos mitóticos e meióticos, o que permite a distribuição correta dos cromossomos na divisão celular (ROOSTALU; SURREY, 2017). A disfunção dos microtúbulos pode resultar em instabilidade cromossômica, parada mitótica e morte celular (RIEDER; MAIATO, 2004).

As citocinas pró-inflamatórias, como IL-1β, IL-8 e a quimiocina MCP-1 são importantes para o recrutamento de leucócitos e reepitelização (GILLITZER & GOEBELER, 2001), mas em uma inflamação prolongada, pode ser prejudicial (ATIYEH et al., 2007). Desta forma, nossas avaliações mostraram que a ativação das células normais HUVEC com LPS aumentou a expressão da quimiocina pró-inflamatória MCP-1 e da citocina IL-8, após 24 horas. Resultado esperado, considerando as propriedades do LPS neste tempo experimental (MUNSHI et al., 2002; ANAND et al., 2009. No entanto, o tratamento com PL ou Ac_{2-26}/PL induziu altos níveis de IL-8, e reduziu MCP-1. Estudo *in vitro* mostra que a dosagem de MCP-1 foi diminuída nos fibroblastos derivados de quelóides, enfatizando o papel da MCP-1 no processo de cicatrização. Esta quimiocina é liberada principalmente pelos queratinócitos junto da lesão e recruta monócitos, macrófagos, linfócitos e, também, mastócitos (GILLITZER & GOEBELER, 2001; BARRIENTOS et al., 2008).

Estudo recente observou que altos níveis de IL-8 podem resultar em baixa atividade capilar não relacionada à viabilidade celular (AMIR et al., 2015). A IL-8 é um membro da família de quimiocinas CXC que regula a migração, proliferação e angiogênese das células endoteliais (LI et al., 2003) e altos níveis de IL-8 reduzem a capilarização em sistemas *in vitro* e *in vivo* (AMIR et al., 2015). Assim, podemos

sugerir que os efeitos antiproliferativos da PL podem ser em parte devido à expressão elevada de IL-8.

Em conclusão, podemos indicar que PL mostra interação potencial com a ANXA1, e essa interação parece ser neutra em relação às propriedades anti-inflamatórias da PL, incluindo o seu efeito na proliferação, viabilidade, apoptose e migração celular. Como um agente anti-inflamatório, o PL é capaz de regular a expressão de quimiocina MCP-1 e citocina IL-8. Esse quadro reforça o potencial da PL na prevenção e progressão do câncer e abre a possibilidade de que a PL module compostos anti-inflamatórios.

5 Conclusões

Os resultados obtidos com as células HUVEC, nas condições propostas, permitem concluir:

1. A interação potencial entre piplartina (PL) e anexina A1 (ANXA1) está localizada na sequência do peptídeo Ac_{2-26} da ANXA1, assim é possível usar em experimentos funcionais a associação Ac_{2-26}/PL
2. Os parâmetros termodinâmicos da interação PL/ANXA1 demonstram que a reação é espontânea ($\Delta G<0$) e coordenada por interações eletrostáticas (ligações fracas).
3. O tratamento com o peptídeo Ac_{2-26} induz proliferação celular em condições basais, no tempo de 72 horas.
4. A PL inibe a proliferação celular, em todos os tratamentos utilizados, incluindo associação com o peptídeo Ac_{2-26}.
5. A PL induz apoptose tardia nas células ativadas pelo lipopolissacarídeo (LPS).
6. Os tratamentos isolados e associados com Ac_{2-26}/PL diminuem os níveis da citocina pró-inflamatória MCP-1, no tempo de 24 horas.
7. O Ac_{2-26}, quando utilizado isoladamente, diminui os níveis de IL-8, enquanto a PL isolada ou Ac_{2-26}/PL aumentam os níveis dessa citocina.
8. O tratamento com a PL, em todos os grupos estudados, inibe a expressão da proteína endógena α-tubulina.

Associadas, essas conclusões permitem assumir que a PL mostra interação potencial com a ANXA1, o que parece ser neutro em relação às propriedades anti-inflamatórias da PL, incluindo o seu efeito na proliferação celular, viabilidade e apoptose. Como um agente anti-inflamatório, a PL é capaz de regular a expressão de quimiocina MCP-1 e citocina IL-8. Esse quadro reforça o potencial da PL na prevenção e progressão do processo inflamatório e câncer e abre a possibilidade de que a PL module compostos anti-inflamatórios.

Referências

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ANEXO 1

PLOS | ONE

RESEARCH ARTICLE

Effect of Melatonin in Epithelial Mesenchymal Transition Markers and Invasive Properties of Breast Cancer Stem Cells of Canine and Human Cell Lines

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Abstract

Cancer stem cells (CSCs) have been associated with metastasis and therapeutic resistance and can be generated via epithelial mesenchymal transition (EMT). Some studies suggest that the hormone melatonin acts in CSCs and may participate in the inhibition of the EMT. The objectives of this study were to evaluate the formation of mammospheres from the canine and human breast cancer cell lines, CMT-U229 and MCF-7, and the effects of melatonin treatment on the modulation of stem cell and EMT molecular markers: OCT4, E-cadherin, N-cadherin and vimentin, as well as on cell viability and invasiveness of the cells from mammospheres. The CMT-U229 and MCF-7 cell lines were subjected to three-dimensional culture in special medium for stem cells. The phenotype of mammospheres was first evaluated by flow cytometry ($CD44^{+}/CD24^{low/-}$ marking). Cell viability was measured by MTT colorimetric assay and the expression of the proteins OCT4, E-cadherin, N-cadherin and vimentin was evaluated by immunofluorescence and quantified by optical densitometry. The analysis of cell migration and invasion was performed in Boyden Chamber. Flow cytometry proved the stem cell phenotype with $CD44^{+}/CD24^{low/-}$ positive marking for both cell lines. Cell viability of CMT-U229 and MCF-7 cells was reduced after treatment with 1mM melatonin for 24 h ($P<0.05$). Immunofluorescence staining showed increased E-cadherin expression ($P<0.05$) and decreased expression of OCT4, N-cadherin and vimentin ($P<0.05$) in both cell lines after treatment with 1 mM melatonin for 24 hours. Moreover, treatment with melatonin was able to reduce cell migration and invasion in both cell lines when compared to control group ($P<0.05$). Our results demonstrate that melatonin shows an inhibitory role in the viability and invasiveness of breast cancer mammospheres as well as in modulating the expression of proteins related to EMT in breast CSCs, suggesting its potential anti-metastatic role in canine and human breast cancer cell lines.

ANEXO 2

Assunto: 7931572: Acknowledging Receipt

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Dear Dr. Caroline Zanon,

This is to inform you that your Research Article titled "Biological and physical approaches on the modulatory functions of potential piplartine (=piperlongumine) and annexin A1 interactions" by Caroline Zanon, Tiago Henrique, Ana Paula Girol, Nayara Contessoto, Nelson Silveira, Daniel Bezerra, Edilberto Rocha Silveira, José Maria Barbosa Filho, Marinonio Cornélio, Eloiza Tajara and Sonia M. Oliani has been submitted to Mediators of Inflammation by Eloiza Tajara, and it has been assigned the manuscript number 7931572.

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