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

Desenvolvimento de peptídeos miméticos a um inibidor de Fosfolipases A_2 por *Phage Display* com potencial para aplicações terapêuticas

Discente: Kellen Cristina Torres Costa

Orientador(a): Vivian Alonso Goulart

Co-orientador(a): Emília Rezende Vaz

UBERLÂNDIA – MG

2022

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

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ATA DE DEFESA - PÓS-GRADUAÇÃO

Programa de Pós-Graduação em:	Genética e Bioquímica				
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Data:	Vinte e oito de julho de dois mil e vinte e dois	Hora de início:	14:00h	Hora de encerramento:	18:10H
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Nome do Discente:	Kellen Cristina Torres Costa				
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Aos vinte e oito dias do mês de julho de dois mil e vinte e dois, às 14:00 horas, reuniu-se via web conferência pela Plataforma *Cisco Webex*, em conformidade com a Portaria nº 36, de 19 de março de 2020 da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES, Resolução de nº 06/2020 e Resolução nº 19/2022 do Conselho de Pesquisa e Pós-graduação pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Genética e Bioquímica, assim composta: Drª. Léia Cardoso de Sousa, Drª. Larissa Prado Maia, Drª. Fabiana de Almeida Araújo Santos, Dr.ª Carla Cristine Neves Mamede e Dr.ª Vivian Alonso Goulart, orientador (a) do (a) candidato (a) e demais convidados presentes conforme lista de presença. Iniciando os trabalhos o (a) presidente da mesa, Dr.ª Vivian Alonso Goulart apresentou a Comissão Examinadora e o (a) candidato (a), agradeceu a presença do público, e concedeu o (à) Discente a palavra para a exposição do seu trabalho. A duração da apresentação do (a) Discente e o tempo de arguição e resposta foram conforme as normas do Programa de Pós-graduação em Genética e Bioquímica. A seguir o (a) senhor (a) presidente concedeu a palavra, pela ordem sucessivamente, aos examinadores, que passaram a arguir o (a) candidato (a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais. Em face do resultado obtido, a Banca Examinadora considerou o candidato (a):

APROVADO (A).

Esta defesa de Tese de Doutorado é parte dos requisitos necessários à obtenção do título de Doutor. O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU. Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

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Discente: Kellen Cristina Torres Costa

COMISSÃO EXAMINADORA

Presidente: Profa. Dra. Vivian Alonso Goulart (Orientadora)

Examinadores:

Profa. Dra. Larissa Prado Maia

Profa. Dra. Léia Cardoso de Sousa

Profa. Dra. Carla Cristine Neves Mamede

Profa. Dra. Fabiana de Almeida Araújo Santos

Data da Defesa: 28/07/2022

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

Vivian Alonso Goulart

Profa. Dra. Vivian Alonso Goulart

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Sempre começo qualquer agradecimento mencionando Deus. Acredito piamente que Ele é o motivo maior de estarmos todos aqui. É a fé Nele que me faz acreditar que depois de qualquer tempestade virá o sol.

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“Quem acredita sempre alcança.”
(Renato Russo)

Lista de Abreviaturas

AdPLA$_2$: Fosfolipases A$_2$ de adipócitos específicos

BID-PLA$_2$: Fosfolipase A$_2$ da serpente Bothrops leucurus

Bleu TX-III: Fosfolipase A$_2$ da serpente *Bothrops leucurus*

BpPLA2-TXI: Fosfolipase A$_2$ da serpente *Bothrops pauloensis*

Ca^{2+}: Íons cálcio

cPLA$_2$: Fosfolipase A$_2$ citosólica

IL-10: Interleucina 10

IL-1β: Interlecucina 1β

IL-6: Interlecucina 6

iPLA$_2$: Fosfolipase A$_2$ independente de Ca^{2+}

kDa: Kilodaltons

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

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PBMC: Células mononucleares de sangue periférico

PBS: Tampão fosfato-salino

PDB: Banco de dados de proteínas

PhD: Phage Display

PLA$_2$s: Fosfolipases A$_2$

sPLA$_2$ - Fosfolipase A$_2$ secretada

LyPLA2: Fosfolipases A$_2$ lisossomais

PAF-AH: Fator de plaquetas ativador de acetilhidrolases

γCdcPLI: Inibidor nativo de fosfolipase A$_2$ da serpente *Crotalus durissus terrificus*

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Sumário

Apresentação

A peçonha das serpentes é considerada a mais complexa dentre as peçonhas do reino animal envolvendo uma ampla mistura de componentes e propriedades. Dentre os compostos orgânicos e inorgânicos destacam-se as fosfolipases A_2 (PLA_2s), uma grande família de enzimas que hidrolisam fosfofolipídeos na posição sn-2 das membranas liberando ácidos graxos com importantes funções biológicas.

Diversos tipos de PLA_2s têm sido relacionadas com inúmeras patologias, dentre elas doenças inflamatórias e câncer. As citocinas são proteínas que regulam reações imunológicas e inflamatórias, atuando como importantes biomarcadores de saúde. Diversos cânceres têm sido associados a uma alta expressão de PLA_2, dentre eles, o câncer de mama. Dessa forma, moléculas capazes de interagir com as PLA_2s despertam enorme interesse já que possuem potencial terapêutico tanto para desordens inflamatórias quanto para neoplasias.

A metodologia de *Phage Display* fornece a possibilidade de selecionar peptídeos ligantes contra uma molécula alvo. Os peptídeos são moléculas atrativas uma vez que são pequenos e capazes de mimetizar ligantes naturais adquirindo funções de interesse. As análises *in silico* têm facilitado a descoberta de novas abordagens terapêuticas, reduzindo algumas limitações que os métodos tradicionais *in vivo* e *in vitro* possuem.

Este trabalho desenvolveu 2 peptídeos: peptídeo 1- C2PD e peptídeo 2 - PepSeq9. Ambos gerados pela tecnologia de *Phage Display* com atividades inibitórias contra PLA_2 em que o inibidor nativo de PLA_2 da serpente *Crotalus durissus* collilineatus (γCdcPLI) foi usado para eluição competitiva. Dessa forma, foram gerados peptídeos miméticos do inibidor da PLA_2. Os peptídeos C2PD e PepSeq9 foram sintetizados com a finalidade de analisá-los como possíveis agentes terapêuticos em desordens inflamatórias e no câncer de mama, respectivamente.

Posteriormente ao *Phage Display*, foram feitas análises *in silico* dos dois peptídeos para verificar similaridade com o inibidor e interação com a PLA_2. Foi feita ainda, uma modelagem por bioinformática do peptídeo PepSeq9 com o intuito de modificar e aprimorar sua sequência, seguida de uma cromatografia de afinidade para verificar sua ligação à PLA_2, já que o peptídeo PepSeq9 teve sua sequência modificada.

O teste de atividade fosfolipásica foi executado com ambos os peptídeos para análise de sua capacidade de inibição da enzima. Uma avaliação citotóxica do peptídeo C2PD foi feita em células mononucleares de sangue periférico (PBMC). Após confirmar que o peptídeo não causou efeito sob PBMC, importantes citocinas (IL-6, IL-1β e IL-10) foram dosadas pós-tratamento com o C2PD para que sua capacidade de modular a resposta inflamatória fosse analisada.

A avaliação citotóxica do peptídeo PepSeq9 foi feita em células normais de rim de macaco (Vero) e em células tumorais de mama (MDA-MB-231).

O presente trabalho está dividido de acordo com os seguintes capítulos:

Capítulo I: Fundamentação teórica a qual aborda uma revisão geral acerca das PLA_2s, seus inibidores e doenças relacionadas, além da metodologia de *Phage Display* e análises *in silico*.

Capítulo II: Apresenta o manuscrito intitulado "Phospholipase A_2 inhibitor mimetic peptide selection with therapeutical potential for inflammatory responses through phage display technology", cujos objetivos foram:

- Selecionar peptídeos miméticos ao inibidor de PLA_2 (γCdcPLI) através da técnica de *Phage Display*. O peptídeo C2PD foi sintetizado e sua capacidade de inibir a atividade fosfolipásica e modular respostas inflamatórias foi analisada.

Capítulo III: Apresenta o manuscrito intitulado "Development of phospholipase A_2 binding peptide by combining phage display-derived sequence motifs and docking analysis with potential cancer therapeutics", cujos objetivos foram:

- Analisar por bioinformática peptídeos gerados por *Phage Display* com a finalidade de selecionar o peptídeo mais similar ao inibidor e com a melhor interação com a PLA_2, além de modificar a sequência para posterior síntese. A capacidade do PepSeq9 de inibir a atividade fosfolipásica e sua toxicidade sob células tumorais de mama foi analisada.

Capítulo I

Fundamentação Teórica

1. Fosfolipases A_2 de peçonhas de serpentes

A peçonha de serpente é uma rica fonte de princípios ativos capazes de induzir uma gama imensa de efeitos tóxicos e farmacológicos (GUTIÉRREZ et al., 2009), sendo secretada por glândulas especializadas (MENEZES et al., 2006).

Para a serpente, a função da peçonha é a captura de alimento, pois auxilia a imobilização e início da digestão da presa. Outro ponto essencial é que a presença da peçonha fornece uma defesa contra agressores (MENEZES et al., 2006; SANTOS et al., 1995).

Entre os componentes orgânicos, têm-se as proteínas, carboidratos, lipídeos, aminas biologicamente ativas, nucleotídeos, aminoácidos e peptídeos. Já as substâncias inorgânicas são exemplificadas pelo cálcio, cobre, ferro, potássio, magnésio, sódio, fósforo, cobalto e zinco (KUDO e MURAKAMI, 2002).

Inúmeras proteínas encontradas em peçonhas de serpentes têm despertado interesse na área médico-científica devido à alta seletividade para seus alvos moleculares. Fosfolipases A_2, metaloproteases, e desintegrinas têm mostrado grande potencial como agentes terapêuticos (ZAMBELLI et al., 2017).

As fosfolipases A_2 (PLA_2s) são uma família de enzimas distribuídas de forma ampla na natureza (KINI, 2003), sendo identificadas e caracterizadas em tecidos de mamíferos e peçonha de serpentes e artrópodes (GARCIA-DENEGRI et al., 2010). De modo específico, catalisam a hidrólise de fosfolipídios na ligação éster do carbono 2 (Fig. 1), liberando ácidos graxos livres, como o ácido araquidônico e os lisofosfolipídeos (KUDO e MURAKAMI, 2002). O ácido araquidônico é um precursor de lipídeos bioativos, tais como prostaglandinas, leucotrienos e tromboxanos atuantes em diversas funções, dentre elas, resposta imune, inflamação e percepção da dor. Vale ainda destacar, que as PLA_2s também estão relacionadas com o metabolismo, reprodução, remodelamento da membrana para a sinalização e defesa antimicrobiana (LOMONTE e RANGEL, 2012).

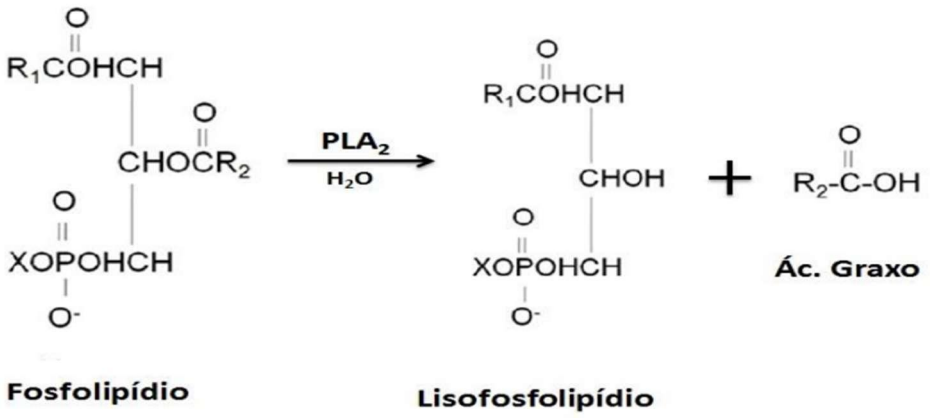

Figura 1. Hidrólise de um fosfolipídio por PLA_2. Quebra da ligação sn-2 do fosfolipídio e consequente liberação do lisofosfolipídio e ácido graxo.
Fonte: Adaptado de KINI, 1997.

Dentre as PLA_2s há uma divisão em seis grupos:

1- PLA_2s secretadas ($sPLA_2$);
2- PLA_2s citosólicas ($cPLA_2$);
3- PLA_2s Ca^{2+} independentes ($iPLA_2$);
4- PLA_2s fator de plaquetas ativador de acetilhidrolases (PAF-AH);
5- PLA_2s lisossomais ($LyPLA_2$)
6- PLA_2s de adipócitos específicos ($AdPLA_2$) (QUACH et al., 2014).

As PLA_2s secretadas são encontradas em secreções de vertebrados e invertebrados, possuem massa molecular entre 13-19 kDa, apresentam um resíduo de histidina junto ao de aspartato no sítio ativo e requerem a presença do íon Ca^{2+} para a catálise (DENNIS et al., 2011).

As PLA_2 de peçonhas de serpentes são do tipo secretada pertencentes aos grupos I (Elapidae/Hydrophiidae) e II (Crotalidae/Viperidae), dependendo de sua estrutura primária e padrão de pontes dissulfeto (SIX e DENNIS, 2000). As PLA_2 do grupo II são classificadas como Asp49 e Lys49, sendo a primeira ativa cataliticamente. Ao longo da evolução, as PLA_2 do tipo Lys49 perderam a capacidade de hidrolisar fosfolipídios devido a substituição do resíduo de aspartato por um resíduo de lisina na posição 49 (SOARES et al., 2003). Mesmo sendo inativas cataliticamente, as PLA_2s Lys49 demonstram toxicidade sobre membranas, o que não é bem elucidado (ÂNGULO et al., 2005; LOMONTE et al., 2003). Já as

PLA_2s Asp49 possuem em seu sítio catalítico um ácido aspártico altamente conservado na posição 49 que é diretamente envolvido na ligação de Ca^{2+}, seu cofator essencial (WARD; AZEVEDO-JR; ARNI, 1998) e, correspondem ao tipo de PLA_2s utilizadas em todas as etapas deste trabalho.

1.1. Inibidores de Fosfolipases A_2

Inibidores de PLA_2 isolados do sangue de serpentes são importantes para proteger esses animais contra suas próprias toxinas (LIMA et al., 2011). Sua síntese provavelmente ocorre no fígado, órgão conhecido como o principal produtor de proteínas plasmáticas, sendo então destinados à corrente sanguínea (LIZANO; DOMONT, PERALES, 2003). Os inibidores de PLA_2 estão bem descritos na literatura, sendo classificados como α, β e γ dependendo de seus aspectos estruturais (OHKURA et al., 1999; FORTES-DIAS et al., 2014; GIMENES et al., 2014; INOUE et al., 1991).

De forma geral, os inibidores da classe α são proteínas globulares constituídas por mais de duas subunidades isoméricas. Sua característica principal é a similaridade sequencial com o domínio de reconhecimento de carboidrato (CRD), o que provavelmente lhes conferem a capacidade de reconhecimento e neutralização das PLA_2s (LINOUE et al., 1991; LIZANO; DOMONT, PERALES, 2003). Os inibidores do tipo β também são constituídos por subunidades proteicas apresentando múltiplas repetições de domínios sucessivos ricos em leucina e quatro sítios de N-glicosilação por subunidade e, preferencialmente, inibem PLA_2s básicas de peçonhas de serpentes (OKUMURA et al., 1998).

Já os inibidores de PLA_2s do tipo γ (γPLI) são glicoproteínas ácidas, apresentando o maior número de subunidades proteicas, com massa molecular de 90-130 KDa (OHKURA et al., 1994; PERALES et al., 1995). Eles possuem regiões ricas em prolina (DUNN e BROADY, 2001), além de contar com a presença de dois conjuntos de repetições intramoleculares ricos em cisteína, denominados motivo *Three-fingers* (OHKURA et al., 1999; ESTEVAO-COSTA et al., 2008). Estas características estruturais auxiliam o reconhecimento da região de ligação do Ca^{2+} das PLA_2s, o que faz com que esse tipo de inibidor tenha um alto espectro de inibição em todos os grupos de PLA_2s (IA, IIA e IIIA), o que não acontece com os inibidores tipo α e β (DUNN e BROADY, 2001; GIMENES et al., 2014).

Os inibidores do tipo γ ainda possuem um sítio de glicosilação bem conservado na posição 157, que foi mostrado em diferentes inibidores purificados do soro/plasma de diferentes espécies de serpentes como de *Crotalus durissus terrificus (FORTES-DIAS, 1991)*, *Python reticulatus* (THWIN et al., 2002), *Crotalus durissus collilineatus* (GIMENES et al., 2014).

Dependendo das diferenças encontradas nas estruturas e nos perfis de inibição destes inibidores, alguns autores propõem uma sub-divisão dos inibidores da classe γ em duas subclasses: γPLI-I e γPLI-II, em que o primeiro grupo teria heterodímeros na sua estrutura quaternária, podendo ser encontrados em serpentes das famílias Elapidae, Hydrophidae e Colubridae. Enquanto a subclassificação γPLI-II seria constituída por apenas monômeros, mais facilmente encontrados nas famílias Crotalidae e Viperidae (LIZANO et al., 2003).

Perales e colaboradores (1995) demonstraram o mecanismo de neutralização do efeito citotóxico de um inibidor do tipo γ através da crotoxina proveniente do soro de *Crotalus durissus terrificus*. Foi mostrado que o inibidor atuou na supressão tanto da atividade da crotoxina quanto também na sua toxicidade *in vivo*.

O inibidor γCdcPLI foi isolado de *Crotalus durissus collilineatus* e caracterizado por Gimenes e colaboradores (2014), e foi demonstrada sua capacidade de inibir a atividade catalítica e citotóxica de PLA_2s isoladas de peçonha de serpentes. No presente trabalho, utilizamos o inibidor γCdcPLI na metodologia de *Phage Display* para a obtenção de peptídeos miméticos do inibidor.

Uma vez que diversos tipos de PLA_2 têm sido associadas a inúmeras patologias (artrite reumatóide, inflamação pulmonar, distúrbios neurológicos e cardiovasculares, câncer), há uma busca incessante por inibidores potentes e seletivos de PLA_2s (MAGRIOTI e KOKOTOS, 2010).

2. Fosfolipases A_2 e sua associação com desordens inflamatórias

As PLA_2s têm sido relacionadas a inúmeros eventos inflamatórios, como aumento da permeabilidade microvascular, formação de edema, recrutamento de leucócitos e liberação de mediadores inflamatórios (TEIXEIRA et al., 2003).

De forma geral, a inflamação pode ser entendida como uma reação natural a danos, injúrias ou lesões teciduais frente à presença de um corpo estranho, trauma (mecânico, químico ou térmico), infecções, reações imunológicas e necrose

tecidual (SHERWOOD e TOLIVER-KINSKY, 2004). Na ocorrência de uma lesão à estrutura da membrana, os fosfolipídios são degradados pela ação da PLA_2, presente nos leucócitos e plaquetas, e ativadas pelas citocinas pró-inflamatórias (PRASHER et al., 2019). Com a finalidade de manter a homeostasia, o organismo tem essas reações como resposta protetora com a função de bloquear, inativar ou eliminar o agente causador (WALLACH et al., 2014).

De acordo com o grau de intensidade, a inflamação é classificada como inflamação aguda autolimitada, de curta duração ou inflamação crônica. A segunda ocorre no caso de doenças crônicas de longa duração, que podem surgir em decorrência da infiltração direta e abundante de células imunes mononucleares, como monócitos, macrófagos, linfócitos e células plasmáticas, bem como a produção de citocinas inflamatórias em um processo de cascata (AHMED et al., 2017).

A redução dos agentes pró-inflamatórios, assim como a liberação de mediadores químicos anti-inflamatórios juntamente com a remoção de detritos celulares, são mecanismos importantes para o reparo da inflamação (VANE e BOTTING, 1987). Quando se tem uma lesão tecidual, a liberação desses mediadores inflamatórios e a degradação dos fosfolipídios de membrana ativam a produção de ácido araquidônico (PRASHER et al., 2019). Além do ácido araquidônico, os eicosanoides (prostaglandinas e tromboxanos) e as citocinas constituem os principais mediadores químicos envolvidos na resposta inflamatória (TEIXEIRA et al., 2003).

As prostaglandinas estão diretamente relacionadas com a geração da resposta inflamatória, cuja produção é exacerbada pela ativação da cascata do ácido araquidônico, oriundo da degradação fosfolipídica. Os eicosanoides, uma vez produzidos, auxiliam no aparecimento e progressão das respostas da inflamação aguda, dessa forma, as prostaglandinas terão importantes papeis em diversos processos fisiológicos e patológicos, tais como vasodilatação ou vasoconstrição, contração ou relaxamento da musculatura brônquica ou uterina, hipotensão, aumento do fluxo sanguíneo renal, resposta imunológica, hiperalgesia, regulação da atividade quimiotática celular, entre outros (COUTINHO et al., 2009). A figura 2 mostra um esquema ilustrativo da síntese de prostaglandinas, ressaltando a importância da família de enzimas ciclooxigenase (COX).

Existem 2 tipos de COX: COX 1 e COX 2 (HERSCHMAN, 1996). A COX 1 é expressa na maioria dos tecidos mediando a produção de prostaglandinas que controlam funções fisiológicas normais. Já a COX 2 é indetectável em grande parte dos tecidos normais sendo induzida por estímulos pró-inflamatórios, além de aumentar a síntese de prostaglandinas em inflamações e em tecidos neoplásicos (SUBBARAMAIAH, 1996).

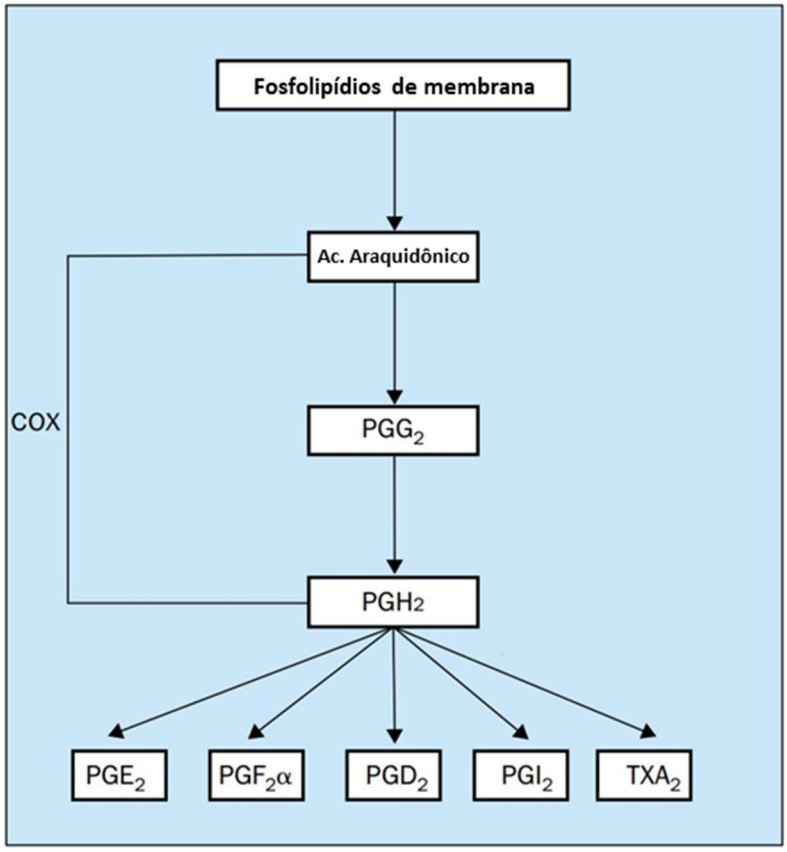

Figura 2: Biossíntese de prostaglandinas.
Reação chave catalisada pela COX: o oxigênio molecular é inserido em ácido araquidônico para produzir um intermediário instável, prostaglandina G2 (PGG2), que é rapidamente convertida em prostaglandina H2 (PGH2) pela atividade da peroxidase da COX. Prostaglandina (PG), Tromboxano (TX).
Fonte: Adaptado de DANNENBERG et al., 2001.

As citocinas são proteínas que medeiam e regulam reações imunológicas e inflamatórias, podendo ser liberadas pelos monócitos e macrófagos, linfócitos T, linfócitos B e mastócitos, além de células endoteliais, fibroblastos e adipócitos (CALDER, 2015). Além disso, as citocinas são responsáveis pela comunicação célula-célula, sendo moléculas auxiliares em todas as fases do processo

inflamatório (AREND e GABAY, 2004) e importantes biomarcadores de saúde (BURSKA e BOISSINOT, 2014).

Por estarem diretamente relacionadas ao contexto geral de resposta imune, a quantificação de citocinas possui um enorme valor na medicina clínica e biológica já que seus níveis fornecem informações sobre os processos fisiológicos e patológicos, podendo ser úteis em diagnósticos e tratamentos (LIU et al., 2021).

Dependendo do seu papel, as citocinas podem ser classificadas como pró-inflamatórias ou anti-inflamatórias (SPRAGUE e KHALIL, 2009). O primeiro grupo contém citocinas como interleucina 1 β (IL-1β), interleucina 6 (IL-6), fator de necrose tumoral α (TNF-α), que acarretam reações inflamatórias estimulando células imunocompetentes, como os linfócitos. Por outro lado, a interleucina 10 (IL-10) é um dos exemplos de citocinas anti-inflamatórias sendo essenciais na inibição da inflamação e supressão de células imunes (BOSHTAM et al., 2017). A IL-10 possui função imuno-regulatória capaz de inibir a expressão de enzimas e mediadores pró-inflamatórios como TNF- α, IL-1β e prostaglandinas (STREETZ et al., 2001; HERNÁNDEZ-CRUZ et al., 2008). Além disso, também tem efeito anti-inflamatório em eosinófilos, basófilos e mastócitos, atuando fortemente no controle e regulação da alergia e asma (ALDABBAGH, 2013).

A principal atuação das citocinas pró-inflamatórias se dá na comunicação aos tecidos adjacentes, sinalizando quando ocorre uma infecção ou injúria (CHENG et al., 2014). Assim, suas propriedades imunes agem contra a invasão de bactérias e outros microrganismos (DINARELLO, 2000).

A IL-6 é considerada a maior mediadora para as respostas de inflamação (MUÑOZ-CARRILLO et al., 2018), sendo capaz de interferir nas respostas imune antígeno específicas, além de estimular a produção de proteínas e atrair eosinófilos para o local (CHEN et al., 2006).

A IL-1β corresponde a uma citocina pró-inflamatória atuante em processos importantes como: ativação de linfócitos, estímulo de macrófagos, adesão de macrófagos às células endoteliais, regulação da síntese de prostaglandinas além da liberação de quimiocinas (RUCAVADO et al., 2002; CUNHA et al., 2008). A IL-1β é capaz de ativar células como os astrócitos e micróglias, ativando a liberação de outras citocinas como IL-6 e IL-18, implicando na alteração funcional e na integridade neuronal (SAADI et al., 2020). Um estudo mostrou que a IL-1β, IL-6 e

IL-12 exercem papel importante no desenvolvimento de doenças autoimunes humanas (GHOLIJANI et al., 2019).

O excesso de citocinas pró-inflamatórias induz um quadro de inflamação crônica, o que pode causar diversas desordens no organismo. Todo o contexto da inflamação vem sendo descrito como sendo parte importante e fator determinante na severidade de diversas patologias como as doenças neurodegenerativas, câncer e diabetes, além dos processos inflamatórios crônicos exemplificados pela artrite e outras doenças autoimunes e infecciosas (KABEL, 2014; RODNEY et al., 2018; ETIENNE et al., 2021). Assim, sempre haverá a necessidade de pesquisas com foco em alvos moleculares da inflamação com a finalidade de beneficiar milhares de pessoas em todo o mundo.

3. Fosfolipases A_2 e sua associação com câncer

Fatores extrínsecos como tabagismo, consumo excessivo de álcool, má alimentação, e características intrínsecas ao indivíduo como pré-disposição genética, disfunções hormonais e danos no reparo do DNA, podem contribuir para o aparecimento de tumores (SCHNEIDER et al., 2014). Entretanto, diversos estudos já demonstraram que a expressão de PLA_2s pode estar associada tanto com o início, quanto com a progressão de cânceres, tais como gastrointestinal, câncer de próstata e colorretal (WANG et al., 2013; LI et al., 2013; MIRTTI et al., 2009). Entretanto, a atuação das PLA_2s no câncer é controversa, pois essas enzimas podem ser pró-tumorigênicas em alguns tecidos e tumorigênicas em outros (CORMIER et al., 1997; LAYE e GILL, 2003). Essa dualidade por ser vista em trabalhos que abordam PLA_2s com efeitos antitumorais (AZEVEDO et al., 2019; AZEVEDO et al., 2016), assim como pesquisas com inibidores de PLA_2s capazes de matar células cancerosas (WANG e DUBOIS, 2007; DONNINI et al., 2011; PUCER et al., 2013).

De acordo com Hanahan (2022) as células cancerosas se diferenciam das células normais em diversos aspectos, sendo alguns primordiais como: sustentação de sinalização proliferativa, evasão da ação de supressores de crescimento tumoral, resistência à morte celular, capacidade de induzir ou acessar a rede vascular (através da angiogênese ou associação de vasos de tecidos normais), invasão e metástase. Além disso, duas novas características foram recentemente

incorporadas, uma vez que as duas juntas compõem as habilidades necessárias para o crescimento e progressão tumoral, são elas: inflamação promotora de tumor e instabilidade genômica e mutação. Todos os marcadores tumorais estão ilustrados na figura 3.

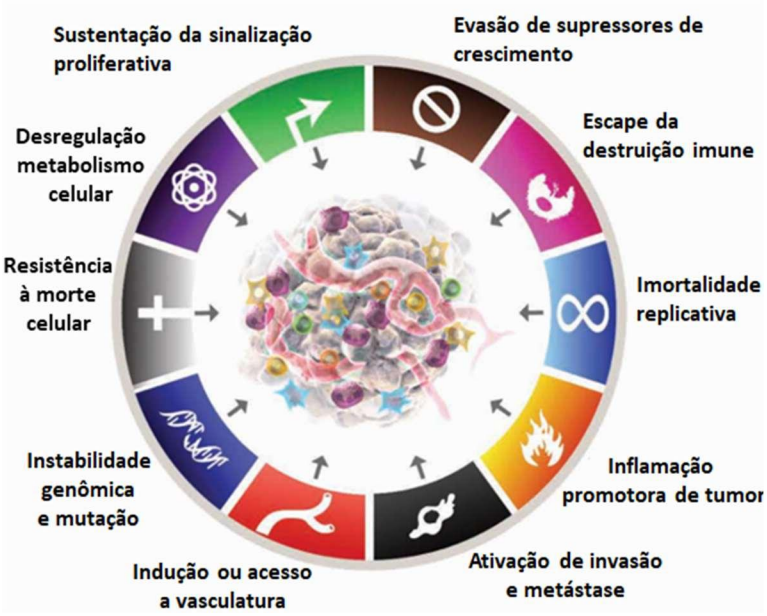

Figura 3: Marcadores de progressão tumoral.
Fonte: Adaptado de HANAHAN, 2022.

Alterações genéticas fazem parte do processo de desenvolvimento tumoral (BOYD, 2014). Dois grupos de genes atuam na regulação dessas alterações genéticas, os genes de supressão tumoral e os genes definidos como protoncogenes. Os genes de supressão tumoral inibem o crescimento celular, já os protoncogenes atuam de forma contrária, sendo responsáveis pelo crescimento celular (ELENBAAS et al., 2001; HOSNY et al., 2016).

Iniciação, promoção e progressão são fases existentes para a instalação de um tumor. Na iniciação, há ativação dos genes e proteínas oncogênicas que são os responsáveis pela multiplicação desordenada destas células (TANAKA et al., 2013; ARRUDA MACEDO et al., 2015). Na fase de promoção tem-se primordialmente a expansão celular, que resulta na geração de tumores potencialmente malignos. Além disso, pode estar presente o processo de

angiogênese, favorecendo a nutrição das células tumorais (ALMEIDA et al., 2005; VINCENT e GATENBY, 2008; ARRUDA MACEDO et al., 2015).

Por fim, na etapa de progressão ocorre a malignização das células tumorais, pois o microambiente está preparado para o processo de progressão celular, que irá ocorrer na presença de etapas importantes como migração, invasão, adesão à parede vascular, extravasamento e difusão em um novo tecido hospedeiro (ALMEIDA et al., 2005; VINCENT e GATENBY, 2008; TANAKA et al., 2013; ARRUDA MACEDO et al., 2015).

Diversos estudos relatam a alta expressão das PLA_2s em diversos tipos de cânceres, dentre eles, o câncer colorretal, gastrointestinal, cânceres de próstata e mama (WENDUM et al., 2005; PATEL et al., 2008; MIRTTI et al., 2009; LIM et al., 2010; WANG et al., 2013; LI et al., 2013). Alguns trabalhos mostraram que a COX 2 e PLA_2s secretadas estão envolvidas no desenvolvimento de tumores colorretal (HAO et al, 1999; SANO et al, 1995). Além disso, pesquisas evidenciaram que inibidores seletivos de COX 2 não só previnem a tumorigênese, como também suprimem o crescimento de tumores estabelecidos, incluindo cabeça e pescoço, colorretal, tumores de estômago, pulmão, mama e próstata (SHENG et al., 1997; SAWAOKA et al., 1998; STOLINA et al., 2000; ALSHAFIE et al., 2000).

Jingkun Qu e colaboradores (2018) demonstraram que pacientes com câncer de mama, principalmente em estágios tardios da doença, possuem alta atividade de $sPLA_2$ no plasma, independentemente de idade e uso de cigarro ou álcool. Dessa forma, os pesquisadores abordam a possibilidade da atividade fosfolipásica plasmática ser um biomarcador relevante para esse tipo de câncer.

O câncer de mama é o tipo de câncer que apresenta maior prevalência entre as mulheres em todo o mundo, e depois do câncer de pulmão, é o responsável pelo maior número de mortalidade entre as pessoas (TORRE et al., 2015). Existem diversos estudos em andamento com estratégias vacinais promissoras para o tratamento do câncer de mama (BURKE et al., 2019). No entanto, ainda se faz necessário o desenvolvimento de agentes terapêuticos mais eficazes para melhorar o prognóstico de pacientes (LI et al., 2014).

O câncer de mama é classificado em quatro subtipos moleculares principais: Luminal A, Luminal B, HER-2 (receptor 2 do fator de crescimento epidérmico humano) e triplo-negativo. O luminal A é o tipo mais frequente e apresenta alta

expressão de receptores hormonais de estrogênio e progesterona, com baixos índices de proliferação celular, enquanto o luminal B possui um índice maior de proliferação de células. O subtipo HER-2 é negativo para expressão de receptores e possui elevada expressão do gene pró tumoral Her-2, promovendo mais facilmente o processo metastático. O câncer de mama triplo-negativo possui um prognóstico desfavorável quando comparado a outros subtipos de cânceres de mama, devido ao seu alto potencial metastático e alta taxa de recidiva. Além disso, não apresenta expressão de nenhum dos marcadores para prognósticos, o que dificulta o tratamento (WANG et al., 2019; SARRIO et al., 2008; KAO et al., 2011; VOSS et al., 2011; TOMAO et al., 2015).

Estudos anteriores avaliaram aspectos que ligam câncer e inflamação, tais como a atuação de células inflamatórias (BALKWILL e MANTOVANI, 2002), mediadores (WANG e DUBOIS, 2006; ZLOTNIK, 2006) e vias de sinalização no câncer (KARIN e GRETEN, 2005). A partir do momento que uma inflamação se torna crônica, é possível que haja o desenvolvimento de um tumor (VENDRAMINI-COSTA e CARVALHO, 2012; GUREL et al., 2014). Células e mediadores do sistema imune inato são detectados em grande parte dos cânceres. Uma das razões para este fato é que as alterações oncogênicas nas células levam à indução de vias inflamatórias em células pré-malignas e malignas. Dessa forma, não somente a inflamação pode causar o câncer, mas o câncer pode também causar inflamação (RAKOFF-NAHOUM, 2006).

Células inflamatórias e mediadores no microambiente tumoral coordenam milhares de respostas inflamatórias, tanto em células malignas, quanto em células não malignas. A infiltração das células imunes nos tumores pode ter dupla função: liderar uma resposta antitumoral ou promover ativamente a tumorigênese, inibindo uma resposta imune protetora. Assim, a composição do microambiente inflamatório do tumor tem uma influência fundamental no resultado da doença (COUSSENS e WERB, 2002).

Dentro desse contexto, moléculas capazes de modular a resposta inflamatória que tenham as PLA_2s como alvo, são de enorme interesse para a área médico-científica, já que possuem potencial terapêutico tanto para doenças inflamatórias quanto para neoplasias. Assim, buscamos nesse trabalho o

desenvolvimento de peptídeos ligantes de PLA_2s para posterior análises de alguns desses potenciais.

4. *Phage Display* e sua importância na biotecnologia

A técnica de *Phage Display* foi descrita por George Smith no ano de 1985. É considerada uma ferramenta muito eficiente e bastante utilizada para identificação de novos peptídeos ou exposição de biomoléculas na superfície de bacteriófagos filamentosos (SMITH e PETRENKO, 1997; JACOBSSON et al., 2003). Bacteriófagos ou fagos são vírus que infectam bactérias gram-negativas usando como receptor o pilus sexual (RUSSEL, 1991). Tais vírus são formados por uma fita simples de DNA envolta por uma capa protéica constituída por cinco proteínas - pIII, pVI, pVII, pVIII e pIX (Fig. 4) (FUKUNAGA e TAKI, 2012). A maior parte dos peptídeos são expressos nas proteínas pIII e pVIII, sendo a pIII a mais comum. Existem 5 cópias da pIII e 2700 cópias da pVIII aproximadamente (RUSSEL, 1991). Segundo Paschke (2006), nesta técnica o gene codificador do peptídeo ou proteína de interesse é fusionado a um dos genes destas duas proteínas do capsídeo sem que haja modificação na função delas. Porém, a representatividade da pIII é baixa quando comparada à pVIII, sendo assim, as bibliotecas de peptídeos sintéticos fusionados na pIII são mais apropriadas para descoberta de ligantes com alta afinidade.

De acordo com Smith (1985) a pIII está relacionada com a infectividade do fago, já que a região amino-terminal dessa proteína se liga ao pilus F da bactéria durante a infecção, enquanto a região carboxi-terminal se encontra na parte interna do vírus participando de sua morfogênese. Dessa forma, a utilização do bacteriófago M13 (Figura 3) permite a realização de um *screening* de peptídeos ligantes a molécula alvo de modo mais específico (PLOSS et al., 2014). De forma geral, esses fagos são utilizados como vetores de DNA em técnicas de engenharia genética por meio da infecção da bactéria hospedeira padrão, *Escherichia coli* (BARBAS et al, 2001).

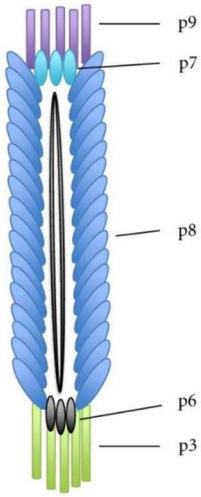

Figura 4: : Ilustração representativa do bacteriófago filamentoso M13 ilustrando as proteínas do capsídeo viral: pIX, pVII, pVIII, pVI e pIII.
Fonte: FUKUNAGA e TAKI, 2012.

A seleção de sequências com base na afinidade de ligação do fago a uma molécula alvo é realizada por um processo de seleção *in vitro* denominado *biopanning* (PARMLEY e SMITH, 1988). *Biopanning* ou seleção biológica se refere ao processo de seleção de ligantes específicos de uma molécula alvo por afinidade (EHRLICH; BERHOLD; BAILON, 2000). O alvo é imobilizado em um suporte sólido como placas ELISA, microesferas magnéticas ou de afinidade, resinas e membranas. Os fagos que não se ligarem ao alvo serão eliminados através de lavagens, enquanto os fagos específicos permanecem ligados para posterior eluição. O *pool* de fagos específicos é amplificado para os ciclos posteriores de *biopanning* (ciclos de ligação, eluição e amplificação) para o enriquecimento do conjunto de fagos com sequências específicas contra o alvo. Assim, após três ou quatro passagens, os clones individuais são caracterizados por sequenciamento de DNA, western blotting ou ELISA (SMITH, 1985). A figura 5 mostra um esquema representativo da seleção de peptídeos por *Phage Display*.

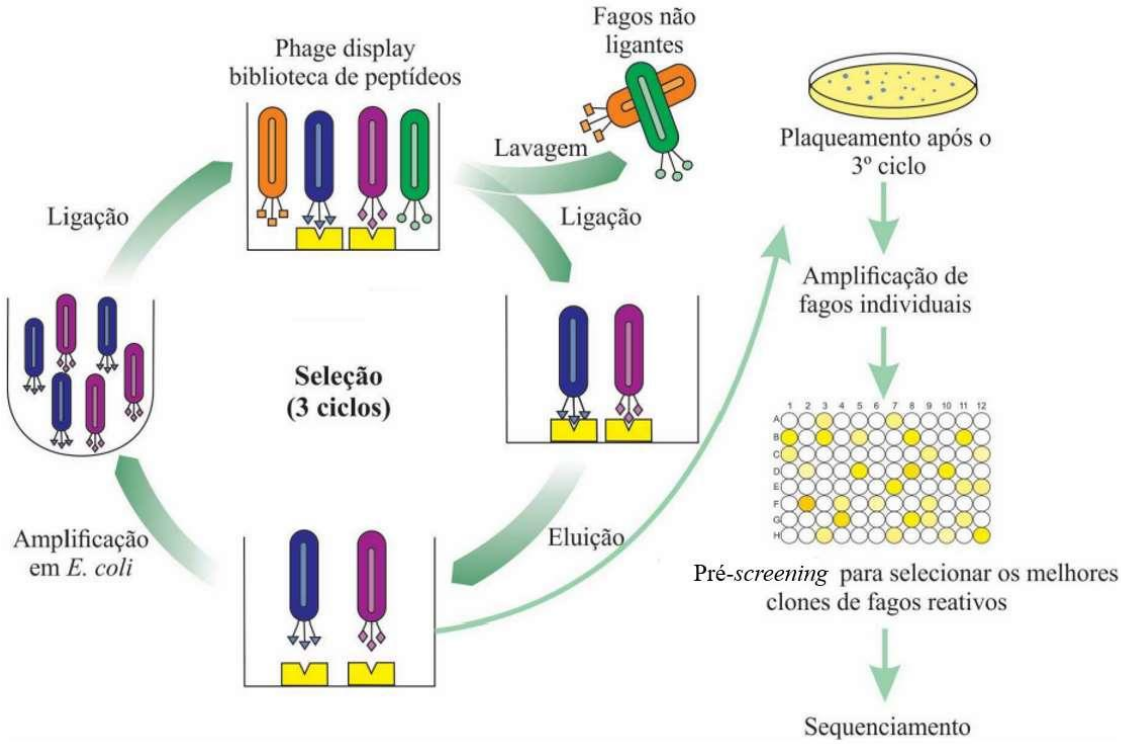

Figura 5. Esquema representativo da seleção de peptídeos por *Phage Display*. A molécula alvo é imobilizada em suporte sólido, como microplacas, para posterior incubação com a biblioteca de peptídeos. Os fagos não ligantes à molécula alvo são descartados por lavagens sucessivas e os fagos ligantes são eluídos e amplificados para o próximo ciclo da seleção. Após o 3° ciclo, o eluato é plaqueado e os clones individuais são amplificados. Em seguida, é feito um pré-*screening* para selecionar os melhores clones de fagos reativos para análise de sequenciamento.
Fonte: Adaptado de SOUZA, 2019.

Bibliotecas de peptídeos têm sido utilizadas na determinação de epítopos aos quais os anticorpos se ligam. Sabe-se que os anticorpos reconhecem sequências de peptídeos baseados apenas em três ou quatro resíduos conservados, dessa forma, é possível a determinação da região de uma proteína que está sendo reconhecida por um anticorpo através da técnica de *Phage Display* (SCOTT e SMITH, 1990). O sucesso de tal técnica depende da complexidade da biblioteca, o que significa que quanto maior a diversidade de clones dentro da biblioteca, maior a probabilidade de conter sequências que ligarão a um determinado alvo com maior afinidade (NOREN e NOREN, 2001).

Os peptídeos vêm sendo foco na área da ciência por serem pequenos e diversificados estruturalmente. Além disso, eles possuem especificidade, podendo ser selecionados sem dificuldade. Uma enorme vantagem dessas moléculas se dá pelo fato de serem capazes de mimetizar ligantes naturais adquirindo função de

agonista ou antagonista, além de interagir com complexos fisiológicos (NIXON et al., 2014).

A metodologia de *Phage Display* é essencial na área da biotecnologia devido a produção de bacteriófagos em larga escala, o que facilita a redução de custos para a fabricação de fármacos (WARNER et al., 2014). Tal metodologia tem sido um sucesso em diversas áreas como biologia celular e imunologia, além do desenvolvimento de vacinas e drogas (LIU et al., 2009; NAIM et al., 2008), contribuindo de forma efetiva para a humanidade.

No presente trabalho, para a seleção de peptídeos com alta afinidade aos receptores da PLA_2 foi utilizada uma biblioteca de peptídeos randômica comercial com uma diversidade de aproximadamente 2 bilhões de clones/cada. Após essa seleção, o inibidor nativo da PLA_2 (γCdcPLI) foi usado para eluição competitiva para que peptídeos miméticos com atividades inibitórias a PLA_2 fossem selecionados.

5. Análises *in silico*

Para se estudar moléculas é fundamental o entendimento acerca de suas funções. A compreensão molecular da função requer o conhecimento da estrutura, porém, a determinação da estrutura tridimensional de uma molécula é um processo extremamente complexo (REEB e ROST, 2018).

Os tradicionais métodos *in vitro* são responsáveis por grande parte das estruturas de proteínas encontradas. Devido aos diversos tipos de limitações como custo e manejo, muitas pesquisas têm se apropriado da bioinformática. A bioinformática é uma área interdisciplinar responsável pelo desenvolvimento de softwares para a compreensão de dados biológicos (LI et al., 2020).

Os métodos computacionais, denominados *in silico*, utilizam ferramentas para a identificação de moléculas contra uma proteína alvo, o que tem sido um facilitador na descoberta de novas abordagens terapêuticas (SINGH et al. 2021; HAJJI et al. 2022). No campo de desenvolvimento de drogas, as metodologias computacionais são vantajosas no cálculo de todas as combinações possíveis de forma interativa baseando-se em estudos biológicos (CHANG et al, 2019; SHENG et al., 2018).

De forma geral, essas análises *in silico* usam algoritmos capazes de encontrar estruturas de proteínas com a menor energia (GIBAS e JAMBECK, 2001). Existem diversos tipos de softwares bem construídos e gratuitos que podem ser facilmente utilizados em instituições acadêmicas para diversas finalidades como: bancos de dados, modelagem molecular e simulação, predição de estruturas e de sítio de ligação, interação-molecular, entre outros (XIA, 2017).

No presente trabalho, utilizamos diversas ferramentas de análises *in silico*, como por exemplo os programas I-TASSER, Chimera e Patchdock para identificar a similaridade dos peptídeos miméticos gerados com o inibidor nativo da PLA_2 (γCdcPLI), assim como analisar a capacidade de interação destes com a enzima PLA_2 (PDB: 2QOG).

6. Referências

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Capítulo II

Phospholipase A_2 inhibitor mimetic peptide selection with therapeutical potential for inflammatory responses through phage display Technology

Phospholipase A_2 inhibitor mimetic peptide selection with therapeutical potential for inflammatory responses through phage display technology

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Highlights

- The performed Phage Display has created C2PD peptide.
- C2PD peptide had not harmful effect on PBMC.
- C2PD interacts with important PLA_2 amino acid residues, as PLA_2 inhibitor does.
- C2PD peptide was able to reduce the PLA_2 activity.
- C2PD promoted modulation of IL-6, IL-1β and IL-10 cytokines.

Abstract

Phospholipases A_2 (PLA_2s) are associated with inflammatory response, a complex process which cytokines are involved to. Chronic inflammatory response induced by the excess of pro-inflammatory cytokines can cause several disorders in the body. Therefore, the inhibition or regulation of these cytokines signaling pathways are considered a target treatment. Our aim was to select PLA_2 inhibitor mimetic peptides through Phage Display (PhD) with anti-inflammatory action. Mimetic peptides to PLA_2 inhibitor were selected using BpPLA2-TXI, a PLA_2 isolated from *Bothrops pauloensis* and γCdcPL, a PLA_2 inhibitor isolated from *Crotallus durissus collilineatus* which was used as a competitor during elution step. We have selected a peptide (C2PD) that seems to play an important role on modulation of IL-6, IL-1β and IL-10 cytokines. The synthetic C2PD peptide tested in PBMC significantly down-

modulated IL-6 and IL-1β and up-regulated IL-10 responses, besides reduced PLA_2 activity. These data suggest that this peptide may be useful for the treatment of inflammatory diseases because it displays anti-inflammatory properties and did not present cytotoxicity effect.

Keywords: inflammatory cytokines, Phospholipases A_2, γCdcPLI, Phage Display, peptides.

1. Introduction

Phospholipases A_2 (PLA_2s) constitute a family of proteins that catalyzes the hydrolize of phospholipids at the sn-2 position releasing fatty acids and lysophospholipids, in a calcium-dependent process [1]. PLA_2s can be divided in the groups I and II, whereby the second is subdivided into two classes Asp-49, which is catalytic and it displays an Asp residue at position 49 and Lys-49, which presents a Lys residue at position 49, with low or no catalytic activity [2].

PLA_2 is a key molecule in inflammatory response since it is responsible for releasing of arachidonic acid, the substrate for the biosynthesis of several lipid mediators of inflammation [3]. Therefore, this enzyme has been linked to numerous inflammatory events such as increased microvascular permeability, edema formation, leukocyte recruitment and release of inflammatory mediators [4].

Inflammation corresponds to a wide variety of physiological and pathological processes [5]. Inflammatory response is a natural reaction to tissue damage, injury, presence of a foreign body, performed to host defenses and it also mediates tissue repair and regeneration [6,7].

In addition to arachidonic acid, eicosanoids and cytokines are the main chemical mediators involved in the inflammatory response [8]. Cytokines are protein molecules released by monocytes and macrophages, T lymphocytes, B lymphocytes and mast cells [9], which help and participate in all phases of the inflammatory process [10]. The quantification of cytokines has an enormous value in clinical medicine as its levels provide information about pathological processes, which can be useful in diagnosis, treatment and monitoring of various diseases [11]. For the evaluation of molecules capable of regulating the immune response, numerous studies use human peripheral blood mononuclear cells (PBMC) to measure important cytokines [12,13].

The interleukin 6 (IL-6) is considered as a major mediator for immune response with a pleiotropic effect on inflammation and hematopoiesis [14]. The interleukin 1 β (IL-1β) is produced and secreted by a variety of cell types including monocytes and macrophages. It is an inflammatory cytokine responsible for exacerbating damage during chronic disease and acute tissue injury [15]. The interleukin 10 (IL-10) is an anti-inflammatory cytokine which is very important for host defense against various infections. This molecule inhibits the innate and adaptive immune response from leukocytes and limits the potential tissue damage caused by inflammation [16].

Since PLA_2s have been associated with several inflammatory effects [4], there is a growing search for molecules capable of inhibiting or reducing the activity of these enzymes. Several types of snake venom PLA_2 inhibitors are described in the literature [17–20].

The aim of this work was select by Phage Display (PhD) methodology PLA_2 inhibitor mimetic peptide able to modulate the inflammatory response, in addition to confirming its regulation in the phospholipase activity. We used the inhibitor γCdcPLI, isolated from *Crotalus durissus collilineatus* which was purificated and characterized by Gimenes et al. (2014) [20] for competitive elution and mimetic peptides with inhibitory activities against PLA_2s were selected. The C2PD peptide was able to reduce PLA_2 activity and it also modulated key cytokines involved in inflammation such as IL-6, IL-1β and IL-10, indicating that this novel molecule may have a therapeutic potential for inflammatory disorders which will be discussed herein.

2. Materials and Methods

2.1. Blood samples

Human peripheral blood mononuclear cells (PBMC) were obtained from twenty healthy volunteers using Ficoll-Paque PLUS (Sigma) following to manufactures protocol. The Ethics Committee on Human Research at the Federal University of Uberlândia (4.532.791) approved all procedures and all subjects enrolled in this study signed informed consent form.

2.2. Phage Display

A combinatorial library containing billions of pooled peptides presented on phage particles was used, where 10^{10} different peptides can be screened simultaneously for the desired activity [21]. To select PLA_2 inhibitor mimetic peptides, $BpPLA_2$-TXI, a PLA_2 from *Bothrops pauloensis* [22], and a phage display 7-mer random peptide library (Ph.D.C7C, New England Biolabs) was used. The native PLA_2 inhibitor (γCdcPLI) from *Crotalus durissus collilineatus* was used for competitive elution and mimetic peptides with specific inhibitory activities against PLA_2 were selected (patent application BR1020160309468). The unbound phages were discarded by washing five times with PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, and pH 7.4). Phages that were bound to PLA_2 were competitively eluted with γCdcPLI. The eluted phages were amplified in *E. coli* ER2738 strain (New England Biolabs), purified by PEG-8000/ NaCl precipitation and used in the next round of selection. Three rounds of selection were performed for the enrichment of specific peptides. Titration was performed after each of the three rounds of selection to evaluate the rate of recovered phage clones.

2.3. DNA sequencing

A total of twenty-four phage clones were submitted for sequencing. For DNA extraction, an overnight culture of phages was suspended in a 100 µL of sodium-iodide buffer (10 mmol/L TrisHCl, pH 8.0, 1 mmol/L EDTA, 4 mol/L NaI) followed by precipitation with absolute ethanol. Phage DNA was centrifuged at 10,000 rpm for 10 minutes, washed with 70% ethanol, and resuspended in 30 µL of ddH_2O. Phage DNA quality was verified in 0.8% agarose gel electrophoresis prior to sequencing. For sequencing, a total of 50 ng of DNA was mixed with -96 M13 sequencing primer (5'-OH CCC TCA TAG TTA GCG TAA CG-3', Biolabs) and the sequencing mix (DYEnamic ET Dye Terminator Cycle Sequencing Kit, Amersham Biosciences). Sequences detection was performed in a MegaBace 1000 Genetic Analyzer (Amersham Biosciences) automatic capillary sequencer.

2.4. *In silico* analysis

DNA sequences were translated by ExPASy Translate tool (http://web.expasy.org/translate/). Chimera Program [23] was used for three-dimensional analysis by predicting the alignment of the peptide with the PLA_2 inhibitor (γCdcPLI) structure and it also to visualize all molecules.

Three-dimensional (3D) structural predictions of peptide and γCdcPLI were performed by PEP-FOLD 3 program (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/) and I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/), respectively. Peptide interactions with PLA_2 (PDB: 2QOG) were evaluated by the Pathdock program (http://bioinfo3d.cs.tau.ac.il/PatchDock/).

2.5. Peptide synthesis

The C2PD peptide was chemically synthesized by Synbio Technologies (NJ, USA). The peptide was constructed with 14 residues (ACESMKALACGGGS) following phage display manual [24].

2.6. Phospholipase A_2 activity

The enzymatic activity was assayed by potentiometric titration as described by de Haas et al. [25]. Phospholipase activity was measured using egg yolk as a substrate in the presence of 0.03 M sodium deoxycholate and 0.6 M $CaCl_2$. C2PD peptide was previously incubated with *Bl*D-PLA_2, a PLA_2 isolated from *Bothrops leucurus* [26], which was donated by Federal University of Belo Horizonte, at 37°C for 30 minutes. PLA_2 concentration was 5 µg and C2PD peptide was used at 100 µg (1:20) and 200 µg (1:40) concentrations. PLA_2 at 5 µg concentration without peptide incubation was used as positive control, while PBS was used as negative control. The phospholipase activity was calculated per amount of microequivalents of NaOH consumed per minute, by mg of protein.

2.7. Analysis of cell viability

PBMC from healthy donors were obtained after Ficoll density gradient centrifugation from heparinized blood samples. Nearly $1x10^4$ PBMC were maintained in RPMI-1640 medium (Gibco), supplemented with 10% fetal bovine serum and 1% gentamicin (Sigma-Aldrich) (complete medium) under standard culture conditions (37°C, 95% humidified air, and 5% CO_2). Cells were treated with the C2PD synthetic peptide at 1 ng, 5 ng, 10 ng, 50 ng and 100 ng per well for 24 and 48 hours in incomplete medium. Then, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Calbiochem, Darmstadt, Germany) solution (5 mg/mL) were added to each well, and the culture was further incubated for 4 h at

37°C. A total of 50 µL of N-dimethylmethanamide solution were added to each well followed by overnight incubation. The absorbance of each well was determined on a microplate reader at 592 nm. The relative cell viability (%) was calculated using the formula: % Viability = [(A592 –treated cells)/(A592 –untreated cells)] x 100. Negative control cells were treated with RPMI.

2.8. Cellular Stimulations and cytokine levels

Nearly $1x10^6$ PBMC were treated with the C2PD synthetic peptide at 1 ng, 5 ng, 10 ng, 50 ng and 100 ng per well for 1 hour in incomplete medium, followed by incubation with Lipopolysaccharides (LPS) (1 mg/mL) (Sigma-Aldrich) for 24 and 48 hours. After this, the cells were centrifuged, and the supernatant was stores at –80 until the cytokine measurement. After stimulus, pro- (IL-6 and IL-1β) and anti- (IL-10) inflammatory cytokines were quantified by commercial ELISA kit, according to the manufacturer's instructions (BD Biosciences).

2.9. Statistical analyses

Statistical analyzes and graphs were obtained by using the Prism 9.0 software (GraphPad 9 Software Inc.) ANOVA followed by Tukey and Kruskal–Wallis tests were used for the experimental analysis. $P < 0.05$ were considered statistically significant. All assays were performed in triplicates.

3. Results

3.1. Peptides selection

After Phage Display selection a total of 24 phage clones were submitted for sequencing where 13 presented valid sequences (Table 1).

Table 1. Sequences and frequency of the selected peptides.

Peptide	Sequences	Frequency (%)
C2PD	ESMKALA	1/13 (7,69)
D10PD	GLAPSHT	1/13 (7,69)
E11PD	HSHNVKS	1/13 (7,69)
F7PD	NPILKEA	1/13 (7,69)
A6PD	GPTAKYI	1/13 (7,69)
A9PD	TAKLTYS	1/13 (7,69)
A10PD	LNQSKFK	1/13 (7,69)
A11PD	DEATKKY	1/13 (7,69)
B5PD	TNARLTS	1/13 (7,69)
B9PD	SPIVKQA	1/13 (7,69)
C5PD	EQSPRSV	1/13 (7,69)
C6PD	NWMINKE	1/14 (7,14)
C10PD	GPTAKYI	1/14 (7,14)

3.2. Bioinformatics analysis

We have performed molecular docking between PLA_2-γCdcPLI and PLA_2-peptides. Three-dimensional alignments between peptides and γCdcPLI structure were also performed, where C2PD peptide and γCdcPLI presented an α-helix type structure partially aligned (Fig 1A, 1B, 1C). The predicted three-dimensional structure of C2PD peptide is shown in Fig 1D. Interestingly, both C2PD peptide and γCdcPLI interact with the Tyr28, Gly30, Gly32 and Asp49 of PLA_2 catalytic site (Fig 2A-B). For these reasons, the C2PD peptide was chosen for further investigations.

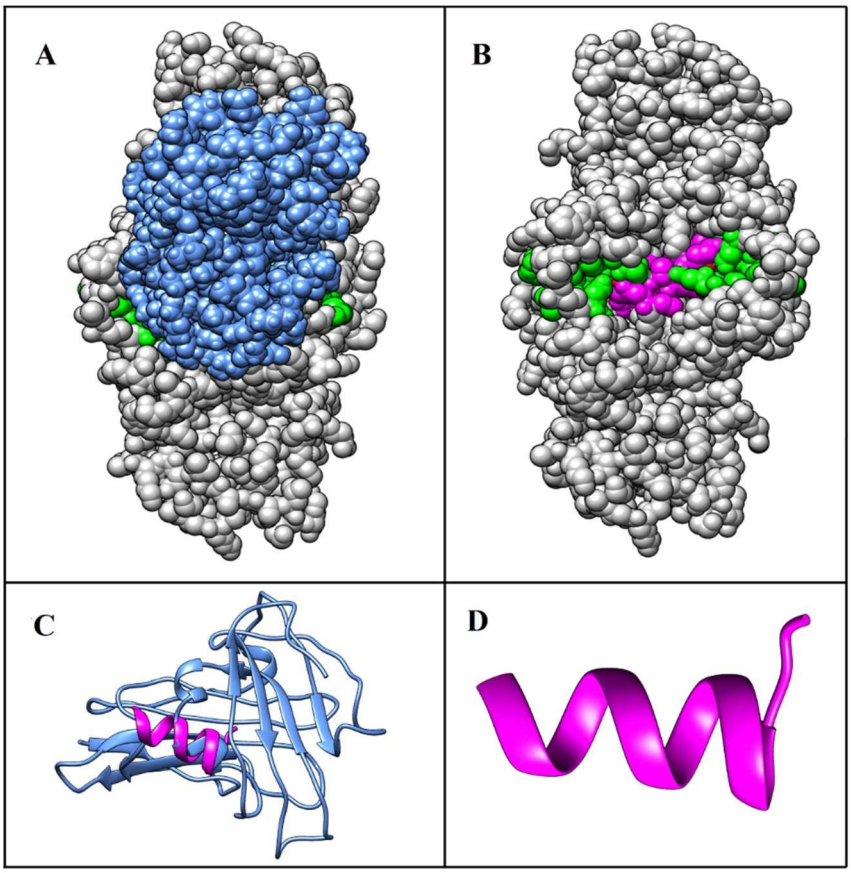

Fig 1. Three-dimensional analysis showing similarities between the C2PD peptide and the γCdcPLI molecule. (A) Molecular docking between PLA_2 and γCdcPLI. PLA_2 is shown in gray, PLA_2 catalytic site is shown in green, γCdcPLI is show in blue. (B) Molecular docking between PLA_2 and C2PD peptide. PLA_2 is shown in gray, PLA_2 catalytic site is shown in green, C2PD peptide is shown in magenta. (C) Three-dimensional alignment between C2PD peptide structure (magenta) and γCdcPLI structure (blue). (D) C2PD peptide α-helix structure.

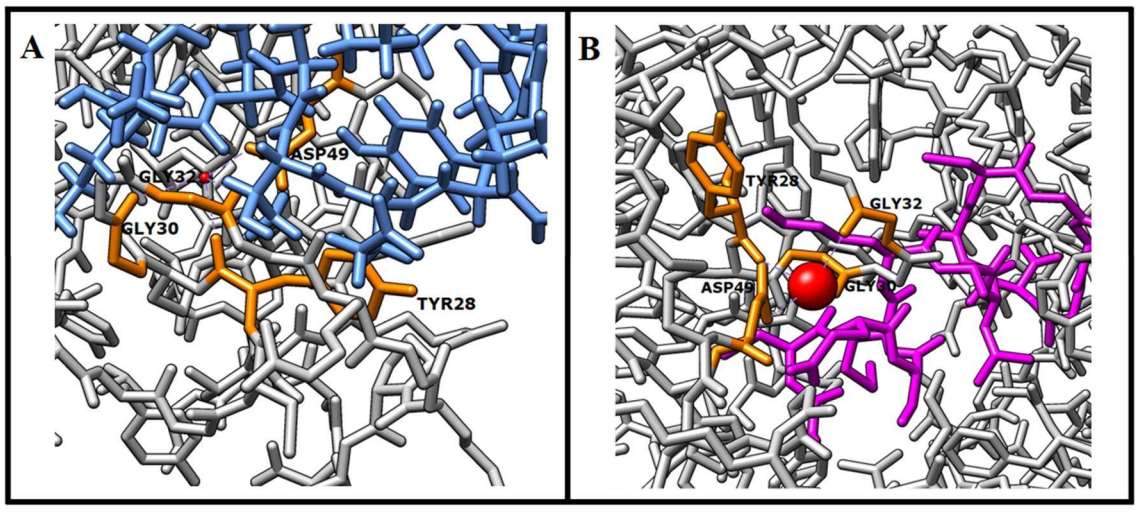

Fig 2. Three-dimensional analysis showing the interaction between PLA_2 with γCdcPLI and C2PD peptide. (A) PLA_2 is shown in gray, TYR28, GLY30, GLY32 and

ASP49 residues are shown in orange, calcium ion is shown in red, γCdcPLI is shown in blue. (B) PLA_2 is shown in gray, TYR28, GLY30, GLY32 and ASP49 residues are shown in orange, calcium ion is shown in red, C2PD peptide is shown in magenta.

3.3. The C2PD peptide reduces phospholipase A_2 activity

The results of enzyme activity assays (Fig. 3) confirmed that C2PD peptide was able to reduce the PLA_2 activity in almost 50% at 100 µg (1:20) and 200 µg (1:40) concentrations.

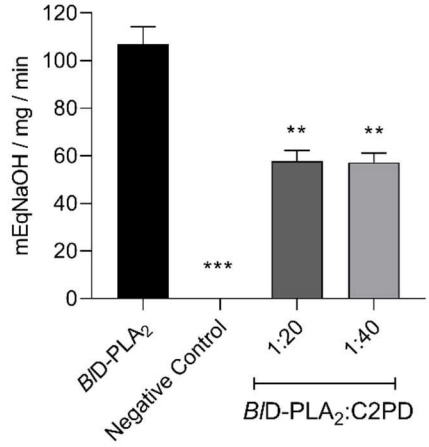

Fig 3. Phospholipase A_2 activity. C2PD peptide added to PLA_2 significantly reduced the enzyme activity at 100 µg (1:20) ($P < 0.005$) and 200 µg (1:40) ($P < 0.005$) concentrations when compared to control.

3.4. The C2PD peptide is non-cytotoxic and mediates an anti-inflammatory response

MTT assays was performed using PBMC to demonstrate that the C2PD peptide has no cytotoxicity effect. It was shown that the C2PD peptide tested at 1 ng, 5 ng, 10 ng, 50 ng and 100 ng concentrations did not affect cells viability and presented no significant differences from controls (Fig 4A-B).

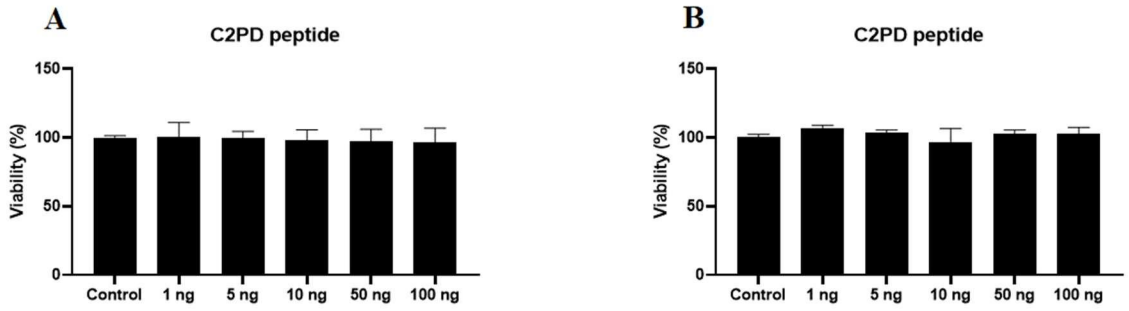

Fig 4. Effects of the synthetic peptide C2PD on PBMC in 24 hours (A) and 48 hours (B). The C2PD peptide showed no statistical difference when compared to control (PBMC without treatment) indicating that the tested concentrations did not have a cytotoxic effect.

To verify whether the C2PD peptide has the ability of modulating an immune response, we have stimulated PBMCs and measured IL-6, IL-1β and IL-10 production. The C2PD peptide presented no action on IL-6 (Fig 5A), IL-1β (Fig 5B) or IL-10 (Fig 5C) production in the absence of inflammatory stimulus in 24 hours. PBMC pretreated with the C2PD peptide for 1 hour followed by LPS stimulation for 24 hours presented significant decrease in IL-6 production at 5 ng ($P > 0.05$) and 10 ng ($P > 0.005$) concentrations when compared to LPS-treated cells (Fig 6A). There was no significant change in IL-1β production at the concentrations used (Fig 6B). The C2PD peptide at 1 ng ($P < 0.0005$), 5 ng ($P > 0.005$) and 10 ng ($P > 0.005$) concentrations resulted in a significant increase IL-10 production on the cells pretreated with the C2PD peptide followed by LPS stimulation for 24 hours when compared to controls (Fig 6C). When PBMC were pretreated with the C2PD peptide followed by LPS stimulation for 48 hours the C2PD peptide was able to modulate the responses of all cytokines used. It down-modulated IL-6 at 5 ng ($P > 0.005$), 50 ng ($P > 0.05$) and 100 ng ($P > 0.05$) concentrations when compared to controls (Fig 7A). The C2PD peptide was also able to decrease IL-1β production at 5 ng ($P > 0.05$) when compared to controls (Fig 7B). An up-regulating IL-10 was also confirmed after 48h-stimulus of PBMCs at 1 ng ($P > 0.005$) and 5 ng ($P > 0.05$) concentrations (Fig 7C). Similarly, the synthetic peptide presented no action on IL-6 (Fig 8A), IL-1β and IL-10 (Fig 8C) production in the absence of inflammatory stimulus.

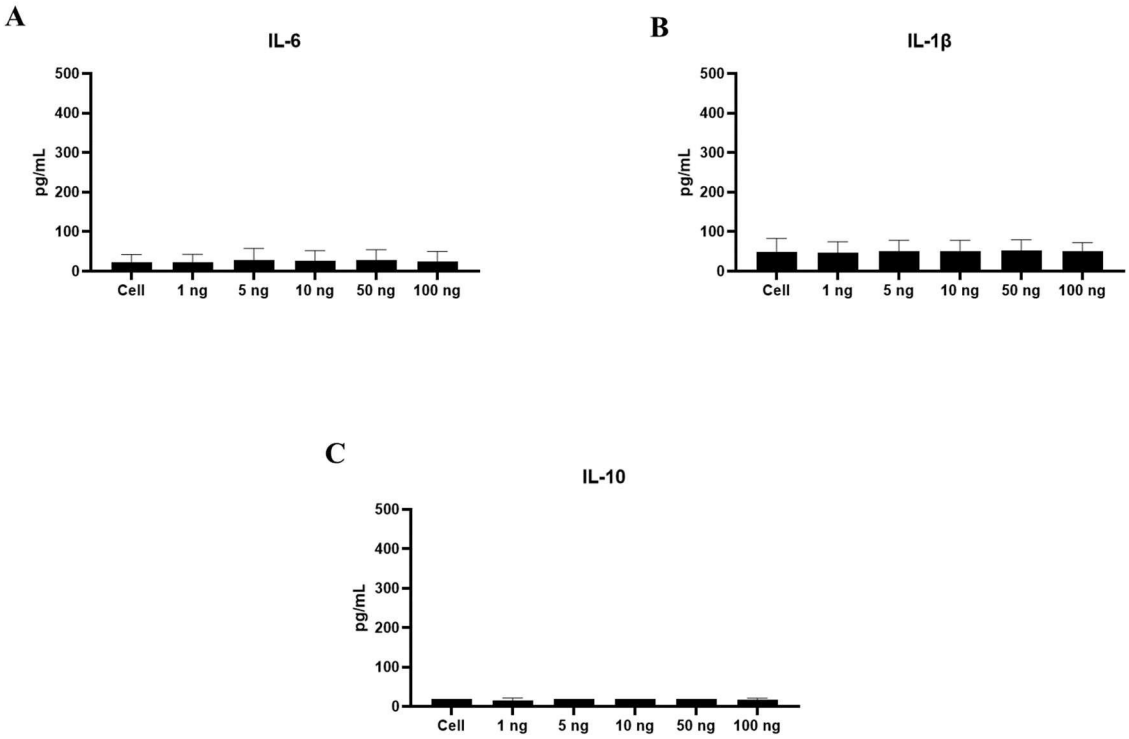

Fig 5. Analysis of cytokines production by PBMC in the absence of inflammatory stimulus after 24 hours. Synthetic peptide presented no action on IL-6 (A), IL-1β (B) and IL-10 (C) production in the absence of inflammatory stimulus.

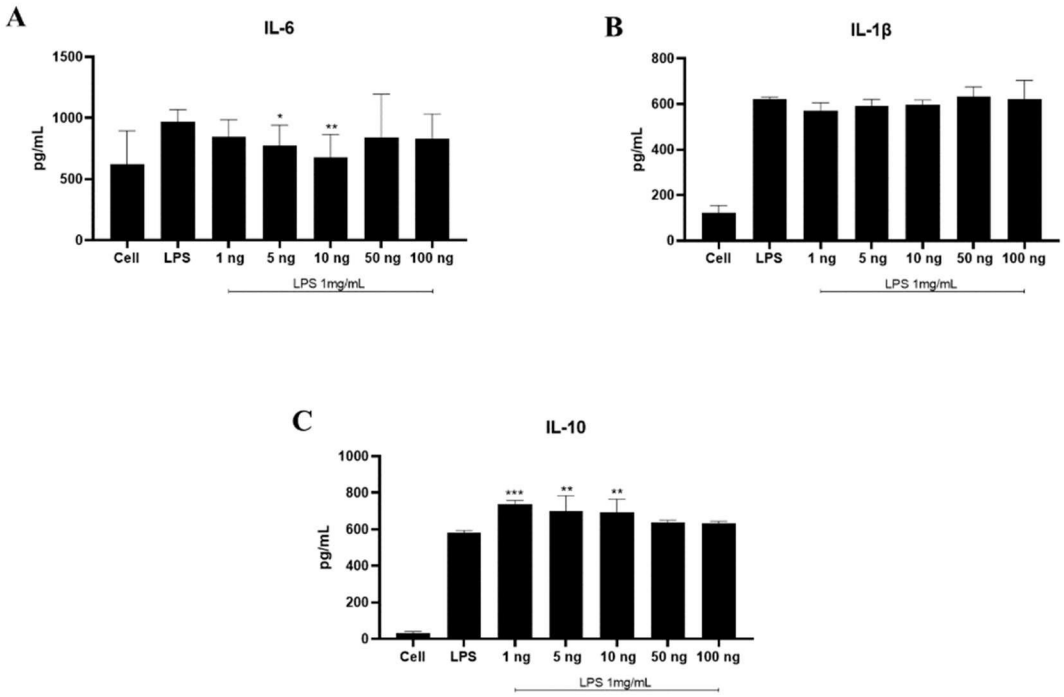

Fig 6. Analysis of cytokines production by PBMC after stimulation for 24 hours. Analysis of IL-6 (A), IL-1β (B) and IL-10 (C) cytokines released after PBMC stimulation with LPS. PBMC pretreated with the C2PD peptide at 5 ng ($P < 0.05$) and 10 ng ($P < 0.005$) was able to decrease

IL-6 production and resulted in a significant increase in IL-10 production at 1 ng ($P < 0.0005$), 5 ng ($P < 0.005$) and 10 ng ($P < 0.005$) when compared to control.

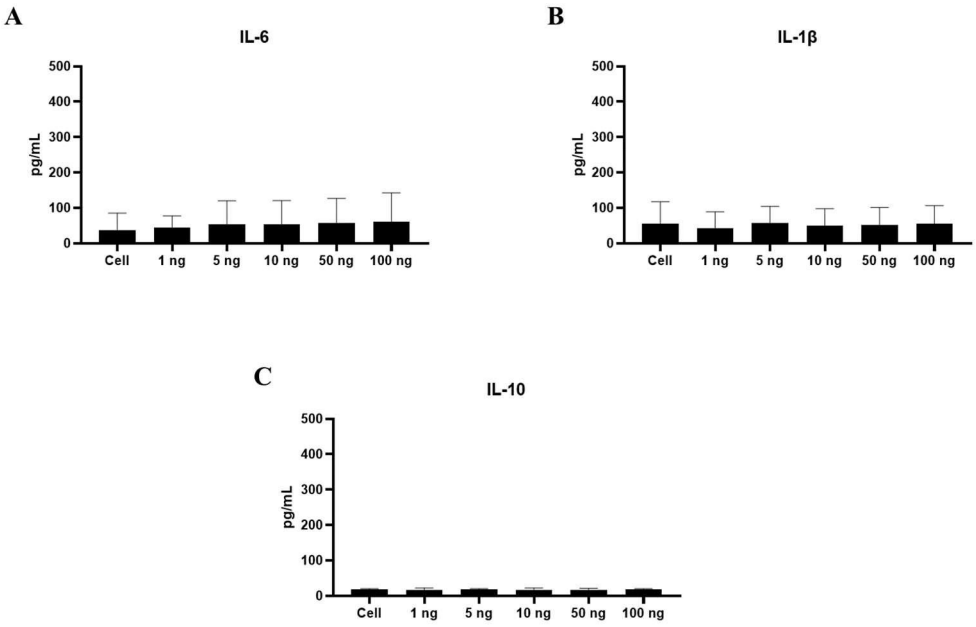

Fig 7. Analysis of cytokines production by PBMC in the absence of inflammatory stimulus after 48 hours. Synthetic peptide presented no action on IL-6 (A), IL-1β (B) and IL-10 (C) production in the absence of inflammatory stimulus.

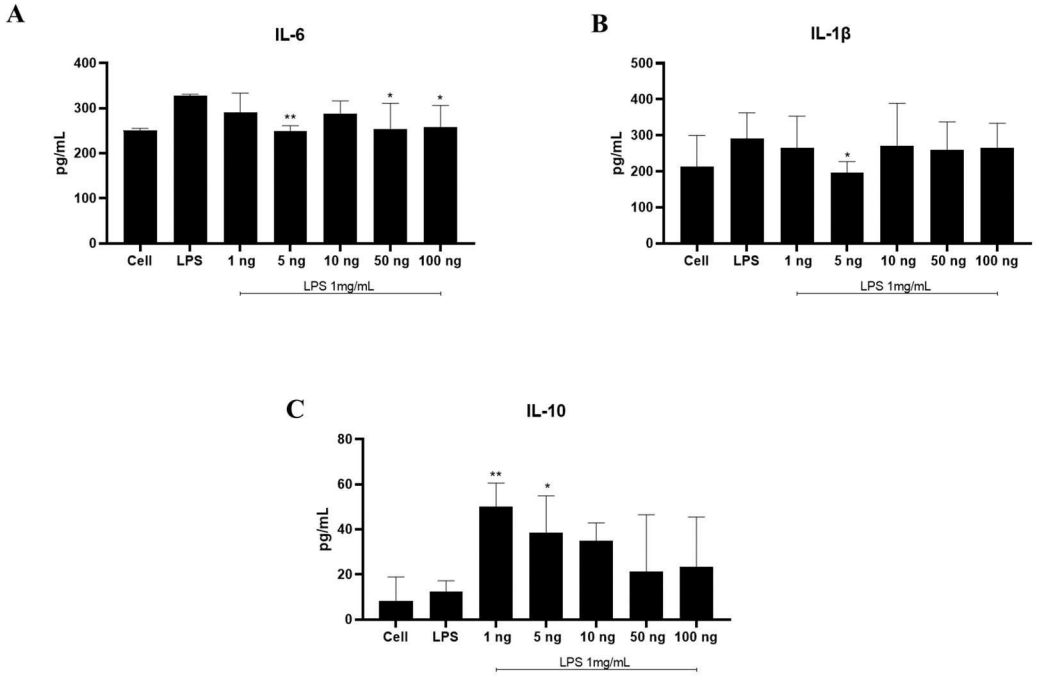

Fig 8. Analysis of cytokines production by PBMC after stimulation for 48 hours. Analysis of IL-6 (A), IL-1β (B) and IL-10 (C) cytokines released after PBMC stimulation with LPS. PBMC

pretreated with the C2PD peptide at 5 ng ($P < 0.005$), 50 ng ($P < 0.05$) and 100 ng ($P < 0.05$) was able to decrease IL-6. C2PD peptide was able to reduce IL-1β production at 5 ng ($P < 0.05$) and it also resulted in a significant increase in IL-10 production at 1 ng ($P < 0.005$) and 5 ng ($p < 0.05$) when compared to control.

4. Discussion

High levels of PLA_2 are detected in several inflammatory disorders in humans [27,28], therefore, the development of new molecules capable of interacting with this enzyme, and consequently, modulating the immune response, arouses great interest. Several PLA_2 inhibitors found in snake blood have been reported in the literature and they can be classified into 3 groups: α, β e γ [17–20]. These inhibitors are important for the protection of the snake against its own toxins [29], being able to inhibit the phospholipase activity [20], and consequently, the biological activities related to the enzyme [30].

PhD technology provides the selection of ligands peptides which can bind to a target sites, and it has been used to develop a series of new molecules with immunological action [31]. This study aimed to search for new bioactive peptides with the ability to mimic a PLA_2 inhibitor and analyze its potential in modulating cytokines production.

We use a competitive strategy for peptides elution, which led us to select mimetic peptides to the PLA_2 inhibitor. Even though the peptides were mimetic to a PLA_2 inhibitor obtained from *Crotallus durissus collilineatus*, the chosen peptide – C2PD, was able to reduce significantly the activity of *Bl*D-PLA_2, a PLA_2 isolated from *Botrhops leucurus*, which corroborates the findings of Gimenes et al. (2014) [20], where γCdcPLI was able to inhibit the effects induced by different PLA_2, a characteristic shared by other inhibitors of γ type [32,33]. Most of the secreted PLA_2s share highly conserved regions corresponding to the catalytic (DXCCXXHD) and Ca^{2+} binding sites (XCGXGG) [34], essential cofactor for typical PLA_2s activity in which Tyr28, Gly30, Gly32 and Asp49 are involved in ion coordination [35]. Higuchi et al. (2007) [36] and Cecilio et al. (2013) [26] isolated and partially characterized *Bl*D-PLA_2 and proved that the enzyme showed high-level homology with many PLA_2s from different snakes species, maintaining the conserved region of the catalytic site. Through *in silico* analysis it is possible to observe that γCdcPLI and the C2PD peptide interact with Tyr28, Gly30, Gly32 and Asp49 residues of the PLA_2, both close to the calcium ion, which may affect the PLA_2 activity.

The cytotoxicity assay showed that C2PD peptide presented no toxic effect on PBMC at any of the concentrations used in 24 and 48 hours, which means the peptide is safe because it does not induce cell death. An important result showed that C2PD peptide was able to modulate the immune response only in an inflammatory microenvironment. In the absence of the LPS stimulus, the C2PD peptide did not significantly affect the production of any of the cytokines in 24 and 48 hours, indicating again the safe use of the synthetic peptide. Besides, the C2PD peptide down-modulated IL-6 and IL-1β while up-regulated IL-10 responses during inflammatory stimuli, a regulatory profile important in the treatment of inflammatory diseases [37].

PLA_2s catalyze the hydrolysis of phospholipids releasing free fatty acids such as arachidonic acid, precursor of inflammatory factors [38]. When arachidonic acid cascade is activated by phospholipid degradation the production of prostaglandins is exacerbated [39]. Prostaglandins and cytokines are essential chemical mediators involved in the inflammatory response [4,40]. The use of PLA_2 inhibitors as a therapeutic strategy for the treatment of inflammatory diseases and tissue damage becomes necessary since it provides control of the activity of this enzyme, and consequently, the regulation of the mediators related to the inflammatory process [41].

The inflammatory and immune responses are regulated by a series of cellular and molecular events that involve several cytokines [42]. Thus, the role of cytokines in inflammatory processes is well documented [43,44]. The excess of pro-inflammatory cytokines induces a chronic inflammation process, which can cause several disorders in the body [45]. Studies showed that IL-1β and IL-6 play an important role in the development of human autoimmune diseases [46].

IL-6 is one of the main mediating cytokines for inflammation responses [47], as it can interfere with antigen-specific immune responses, in addition to stimulating acute phase proteins production and attracting eosinophils to the site [48]. Several studies showed that PGE2, a prostaglandin derived from arachidonic acid through cyclooxygenase (COX-2) pathway, has association with IL-6 production [49,50].

Findings evidenced that PGE2 increases IL-6 expression [51], where COX-2 gene seems to regulate IL-6 expression [52].IL-1β also has a pro-inflammatory role, participating in important processes such as: activation of lymphocytes,

macrophage stimulation, adhesion of macrophages to endothelial cells [53,54]. Endo et al. (2014) showed that IL-1β contributed to PGE2 production in LPS-treated monocytes [55], indicating the association between IL-1β and prostaglandins. Studies in rats demonstrated high levels of COX-2 following administration of LPS [56]. Bacterial LPS are often of interest in medicine for their immunomodulatory properties [57], which involves COX-2 production [58] and releasing inflammatory cytokines [59]. In vitro model of LPS-induced PBMC, presented an increase in IL-6 and IL-1β production considerably [60]. We have performed PBMC stimuli with LPS and the C2PD peptide down-regulated IL-6 and IL-1β, which corroborates with Lu et al. (2018) where it was demonstrated the effect of an anti-inflammatory agent on LPS-stimulated PBMC by inhibiting the production of these inflammatory cytokines [61].

In addition to LPS, cytokine production can also be stimulated by PLA_2s from snake venom. Marangoni et al. (2013) [62] observed the increased in the levels of IL-1, IL-6, and TNF-α when Bleu TX-III, a PLA_2 from *Bothrops leucurus*, was administrated by intravenous route in mice. Nunes et al. (2011) [13] showed that *Bl*-PLA_2, also a PLA_2 from *Bothrops leucurus*, was able to induce an increase in IL-1β and IL-6 in human PBMC stimulated *in vitro*. Another PLA_2, BatroxPLA_2, isolated from *Bothrops atrox*, increased the IL-6 response in macrophages from C57BL/6 mice stimulated *in vitro* [63]. Several other studies have proven the role of different snake venom PLA_2s in the increasing production of various inflammatory mediators [64–66]. We suggest that the ability of the C2PD peptide to affect phospholipase activity is associated with the reduction of IL-6 and IL-1β production.

IL-10 is an anti-inflammatory cytokine with immunoregulatory functions capable of inhibiting the expression of pro-inflammatory enzymes and mediators such as TNF-α, IL-1β and prostaglandins [67]. This immunoregulation is in agreement with our findings since the C2PD peptide increased IL-10 production and reduced the IL-1β response, which may prevent excessive inflammation and tissue damage [68].

Inflammatory diseases are heterogeneous pathologies and, despite promising strategies have been elucidated, it is still necessary the development of appropriate therapeutical strategies [69]. Our findings suggest a potential use of the C2PD peptide in the inflammatory modulation, but further studies must be explored.

5. Conclusion

C2PD peptide was selected through PhD technology mimicking γCdcPLI, a PLA_2 inhibitor from *Crotalus durissus collilineatus,* it had its ability to reduce phospholipase activity confirmed and it did not present harmful effect on human PBMC. Besides, C2PD peptide displayed an anti-inflammatory response down-regulating the pro-inflammatory cytokines IL-6 and IL-1β and up-regulating IL-10, an essential anti-inflammatory cytokine. Furthermore, the synthetic peptide was able to modulate the immune response only in an inflammatory microenvironment, indicating that its use is safe. Although further investigations are needed, we propose that this synthetic peptide may be a potential model for development of therapies against inflammatory diseases.

Conflict of Interest

The authors declare that there is no conflict of interests.

Acknowledgments

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Capítulo III

Development of phospholipase A_2 binding peptide by Combining Phage Display-Derived Sequence Motifs and Docking Analysis with Potential Therapeutics

Development of phospholipase A_2 binding peptide by Combining Phage Display-Derived Sequence Motifs and Docking Analysis with Potential Therapeutics

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ABSTRACT: Phospholipase A_2 (PLA_2) is an important family of enzymes implicated in various biological functions. The secreted PLA_2s ($sPLA_2$s) are found in vertebrate and invertebrate secretions, such as snake venom, which is the most studied type due to its potential biotechnological application, since PLA_2 expression is increased in inflammatory diseases and some cancers. Based on phage display-derived peptides from a selection that mimics a PLA_2 inhibitor, we have attempted to optimize its inhibitory activity by modeling a novel peptide through bioinformatics using extensive 3D alignment and peptide docking to the $sPLA_2$ protein structure. *In silico* analysis generated the PepSeq9 peptide with the greatest coverage of the $sPLA_2$ active site. Interestingly, we have shown that PepSeq9 potentiated the enzyme activity rather than inhibiting it, thus demonstrating that bioinformatics may be a valuable tool for predicting structures, but function prediction is a complex process. In turn, the novel peptide was cytotoxic to MDA-MB-231 cell lines, thus suggesting a potential therapeutic role in breast cancer.

Keywords: PLA_2, PLA_2 inhibitor, bioinformatics, peptides, breast cancer.

1. Introduction

Phospholipase A_2 (PLA_2) constitutes a superfamily of enzymes widely distributed in nature [1]. PLA_2s are divided into six groups: secreted PLA_2 ($sPLA_2$); Cytosolic PLA_2 (cPLA2); Ca^{2+} independent PLA_2 (iPLA2); PLA_2 Platelet-Activating Factor Acetylhydrolase (PAF-AH); Lysosomal PLA_2 (LyPLA2) and adipose-specific PLA_2 (AdPLA2) [2]. The secreted PLA_2 ($sPLA_2$) is a lipolytic enzyme with a molecular weight of 14-18 kDa that uses a His/Asp dyad and requires calcium for catalytic activity [3]. Notably, $sPLA_2$s are found in vertebrate and invertebrate secretions and, structurally, they contain three long α-helices, two-stranded β-sheets referred to as β-wings, and a conserved Ca^{2+} binding loop [4].

Snake venom PLA_2 is a secreted type belonging to groups I (*Elapidae* / *Hydrophiidae*) and II (*Crotalidae* / *Viperidae*), depending on its primary structure and disulfide bond pattern [5]. Group II PLA_2s are classified into two subtypes: Asp49-PLA_2s, which are catalytically active, and Lys49-PLA_2s, which are catalytically inactive. During evolution, Lys49-PLA_2 lost the ability to hydrolyze phospholipids due to the substitution of the Aspartate residue by a Lysine residue at position 49 [6]. Although catalytically inactive, Lys49-PLA_2s demonstrate membrane toxicity through a calcium-independent mechanism [4].

PLA_2 specifically catalyzes the hydrolysis of the ester bond at the sn-2 position of phospholipid substrates, releasing free fatty acids as bioproducts, including the polyunsaturated arachidonic acid and lysophospholipids [2]. Arachidonic acid is a precursor of bioactive lipids, such as prostaglandins, leukotrienes and thromboxanes, which act on various biological functions, such as immune response, inflammation, and pain perception. Additionally, PLA_2s play a pivotal role in metabolism, reproduction, membrane remodeling for antimicrobial signaling and defense [3,4].

Numerous studies have shown that PLA_2 is known for its high expression in inflammatory diseases, for example, rheumatoid arthritis [7], adult respiratory distress syndrome, and inflammatory bowel disease [8]. Furthermore, studies correlate secreted PLA_2 ($sPLA_2$) with some cancers, such as breast cancer [9], prostate cancer [10], and colorectal tumor [11]. However, the role of $sPLA_2$ in cancer is controversial. Indeed, it is not established whether the enzyme is a tumor suppressor or tumor promoter. These enzymes can modulate tumorigenesis in a tissue- and context-dependent manner, which means that they can be pro-

tumorigenic in cancer at some tissue sites or anti-tumorigenic in tumors at other sites [12,13].

Importantly, Bioinformatics is a valuable tool for drug discovery through a series of biological and structural predictions by *in silico* analyses, using sequence similarities and alignments, domain identification, protein fold recognition and model validation, but function prediction remains elusive and inconsistent [14,15]. On the other hand, Phage Display has become one of the most successful technologies for selecting functional peptides directly against specific targets, but bacteriophage-fused peptides are not always useful when selected sequences are synthesized [16,17]. In this investigation, we used Bioinformatics and Phage Display to optimize peptides using consensus sequences and structures of both PLA_2 inhibitor and Asp49-PLA_2 as target molecules. Thus, this work aims to select the best mimetic peptide of PLA_2 inhibitor, change its sequence for better binding to PLA_2 and analyze its performance against breast tumor cells. The novel peptide was cytotoxic to MDA-MB-231 cell lineages, hence suggesting it can be explored on further investigations as a potential a therapeutic potential in breast cancer, which will be discussed herein.

2. Materials and Methods

2.1. *In silico* analysis

2.1.1. Peptides and PLA_2 inhibitor structure prediction

Mimetic peptides of PLA_2 inhibitor were previously selected by Phage Display and three peptide motifs (D10, E11 and F7) with specific inhibitory activities against BpPLA_2-TXI, a PLA_2 from *Bothrops pauloensis* [18] were identified. The native PLA_2 inhibitor (γCdcPLI) from *Crotalus durissus collilineatus* was used for competitive elution, and its sequence (UniProtKB – C0HJU4) and predicted structure were selected for docking analysis and peptide alignments.

Three-dimensional (3D) structural models of peptides and PLA_2 inhibitor were predicted by I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/).

We performed the validation of three-dimensional structure of the PLA_2 inhibitor using Ramachandran Server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php). The structure of BbPLA_2-

TXI is absent in Protein Data Bank (PDB), therefore, a PLA_2 from *Crotalus durissus terrificus* (also an Asp49) was used for bioinformatics analysis (PDB: 2QOG), since γCdcPLI is also from *Crotalus durissus* and it has an inhibitory effect against different PLA_2s from snake venoms [19]. Multiple sequence alignment of PLA_2s was executed by Clustal Omega program.

2.1.2. Molecular dockings

In order to analyze the interaction between peptides and PLA_2 and the interaction between PLA_2 and PLA_2 inhibitor, molecular dockings were performed using Patchdock program (http://bioinfo3d.cs.tau.ac.il/PatchDock/).

2.1.3. Interaction analysis

Three-dimensional alignments were performed to assess the similarity between mimetic peptides and PLA_2 inhibitor. The PLA_2 domain was analyzed to map calcium ions and catalytic site, both crucial for enzyme activity. All interaction analyses were visualized and performed by UCSF Chimera [20].

2.2. PepSeq9 peptide synthesis

PepSeq9 was chemically synthesized by Peptide 2.0 Inc. (Chantilly, VA, USA). The peptide was constructed with 39 residues (PILKEGGGSAETVESPPGGSGGPPSEVTEASGGGEKLIP). Part of the F7 peptide sequence (original and mirrored sequence) and p3 protein (AETVES) were included in PepSeq9 following the phage display instruction manual [21].

2.3. Affinity chromatography

PepSeq9 peptide was applied to the N-hydroxysuccinimide (NHS HiTrap) affinity column previously immobilized with BnSP-7, a PLA_2 from *Bothrops pauloensis*. The column coupled to the AKTA PRIME GE Healthcare system was initially equilibrated with 10 mM Tris-HCl / pH 7.5 buffer, and chromatography was started with this buffer. Then, the binding proteins were eluted with 100 mM Glycine/L pH 2 buffer. This fraction was neutralized with a neutralization buffer (1M Tris-HCl, pH 8).

2.4. Phospholipase A_2 activity

Enzymatic activity was assayed by potentiometric titration as described by De Haas [22]. Phospholipase activity was measured using egg yolk as a substrate

in the presence of 0.03 M sodium deoxycholate and 0.6 M $CaCl_2$. PepSeq9 peptide was previously incubated with the PLA_2 (*Bl*D-PLA_2) [23] at 37 °C for 30 minutes.

PLA_2 concentration was 5 µg and PepSeq9 peptide was 100 µg. Phospholipase activity was calculated per amount of microequivalents of NaOH consumed per minute, by mg of protein.

2.5. Cell viability by MTT assay

The viability of triple-negative breast cancer (TNBC) cell subtype (MDA-MB-231) and a monkey kidney cell line (Vero) was assessed by the MTT assay. The cells were seeded at 2 $\times 10^4$ cells/well in 96-well microplates and were allowed to attach for 24 h at 37 °C and 5% CO_2. MDA-MB-231 were incubated with the medium in the absence (control cells) or presence of different concentrations of PepSeq9 (200, 100, 50, 25, 12.5, 6.25 and 3.125 µg/mL). Vero cells were incubated with the medium in the absence (control cells) or presence of PepSeq9 at 1 µg, 10 µg, 50 µg and 100 µg/mL concentrations. After 24 h, cells were incubated with 20 µL/well of MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (5 mg/ml diluted in PBS) and maintained for 3 h at 37 °C. The formazan crystals were dissolved in 100 µL phosphate buffered saline (PBS) with 10% SDS and 0.01 N HCL for 18 h until complete dissolution of crystals; then the absorbance at 570 nm was determined (Multiskan GO Thermo Scientific, USA). Cell growth inhibition (%) = [1 − (OD Treated/OD Control)] × 100.

2.6. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 9.0 (GraphPad Software, USA). Statistical significance was estimated by ANOVA followed by Tukey's post-hoc test. Differences with p-values less than 5% were considered significant ($P < 0.05$).

3. Results and Discussion

The present investigation developed a novel synthetic peptide with potential antitumor activity, based on phage display-derived peptide sequences that mimicked a PLA_2 inhibitor coupled with 3D alignments and peptide docking to the $sPLA_2$ protein structure. However, the improved peptide action did not show inhibitory activity against $sPLA_2$. Interestingly, *in silico* analyzes showed that the PepSeq9 peptide had a good interaction and coverage of the PLA_2 catalytic site, but

surprisingly, the enzyme activity was potentiated rather than inhibited. The findings evidenced that bioinformatics tools still need further improvements to incorporate functions' prediction, which are currently obtained only by functional assays. Even with computational approaches, molecules must be synthesized and experimentally tested to confirm their predicted effects against targets [24].

3.1. Bioinformatics analysis

Peptides were selected by Phage Display with action similar to the PLA_2 inhibitor. The sequences are depicted in Table 1.

Table 1. Sequences of the selected mimetic peptides to PLA_2 inhibitor by Phage Display.

Peptide	Sequence
D10	ACGLAPSHTCGGGSAETVES
E11	ACHSHNVKSCGGGSAETVES
F7	ACNPILKEACGGGSAETVES

3.1.1. Three-dimensional structure prediction of the PLA_2 inhibitor and mimetic peptides: PLA_2 inhibitor models 1 and 4 showed 74.9% of residues in favored region

The probable models obtained for PLA_2 inhibitor and the three-dimensional structures of PLA_2 inhibitor mimetic peptides are illustrated in Fig. 1 (A-E). The findings indicated the presence of β-sheets in all models. β-sheet is an element of protein secondary structure, formed when several β-strands self-assemble [25]. Thereby, there will be the formation of extended amphipathic sheets in which hydrophobic side chains point in one direction and polar side chains in the other [26]. This type of structure is stabilized by hydrogen bonding [25].

Validation of each model was performed to select the best predicted PLA_2 inhibitor structure. Ramachandran graph defines the residues that are in the most favorable and unfavorable regions and guides the evaluation of the quality of theoretical or experimental models of proteins [27]. Models 1 and 4 (Fig. 1 A; D) had

a higher percentage of residues in the favored region (74.9%), thus indicating that both structures are more likely to be correct. For this reason, models 1 and 4 were chosen for further investigations.

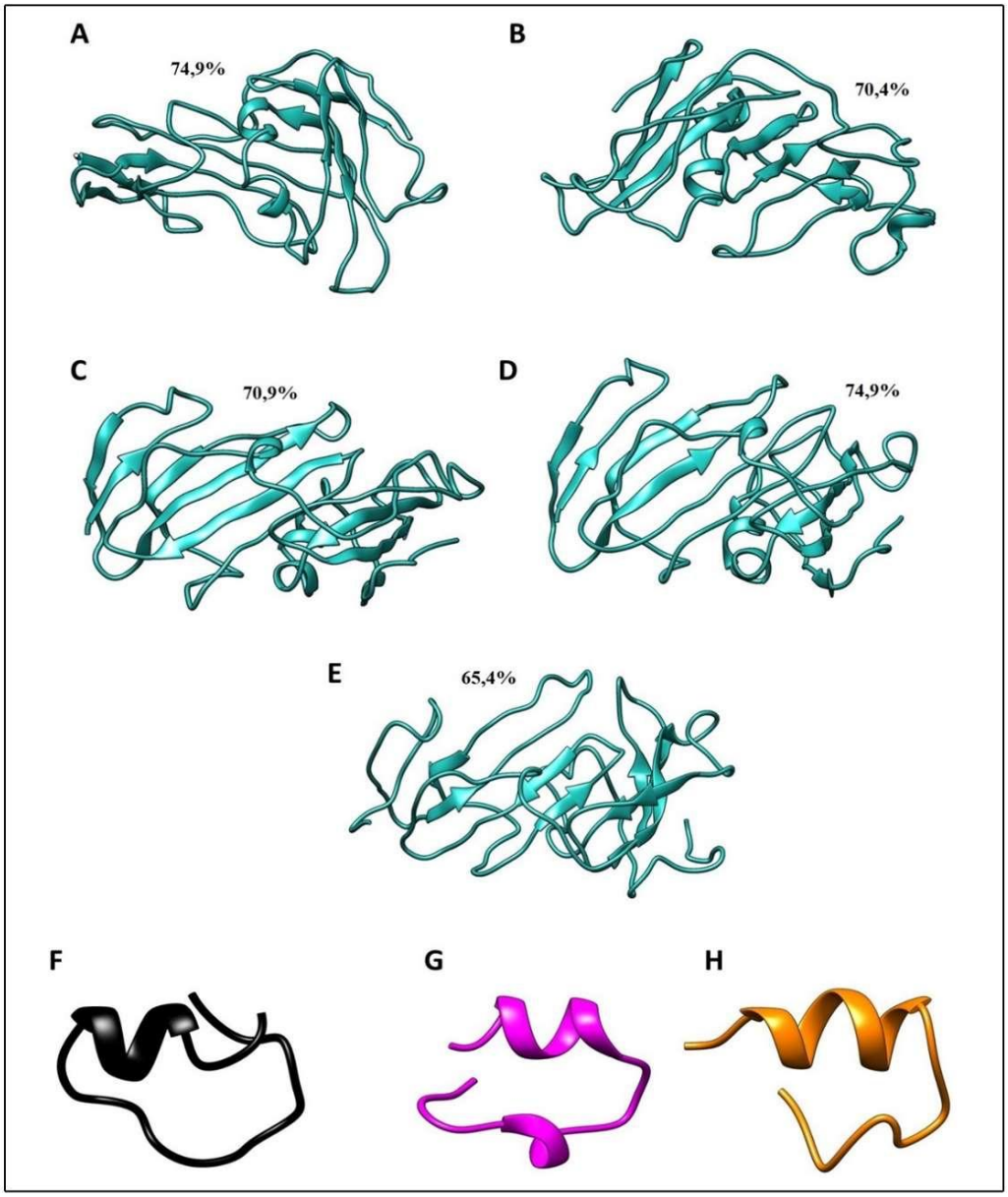

Fig. 1. PLA_2 inhibitor models and peptide structure prediction. (A) PLA_2 inhibitor model 1 showed 74.9% of residues in the favored region; (B) PLA_2 inhibitor model 2 showed 70.4% of residues in the favored region; (C) PLA_2 inhibitor model 3 showed 70.9% of residues in the favored region; (D) PLA_2 inhibitor model 4 showed 74.9% of residues in the favored region; (E) PLA_2 inhibitor model 5 showed 65.4% of residues in the favored region; (F) D10 peptide is shown in black; (G) E11 peptide is shown in magenta; (H) F7 peptide is shown in orange.

3.1.2. Three-dimensional alignments between PLA_2 inhibitor models and mimetic peptides: F7 peptide showed higher similarity with both models

Three-dimensional alignments were executed between the mimetic peptides and PLA_2 inhibitor models (Fig. 2) to select the peptide structure most similar to the inhibitor structure. In the alignment executed by Chimera tool, it was possible to visualize the amino acids in the structure. The results showed a higher similarity between F7 peptide and PLA_2 inhibitor model 4 (Fig. 2 F) when compared to D10 and E11 peptides. F7 peptide was able to align with most part of the inhibitor, unlike the others.

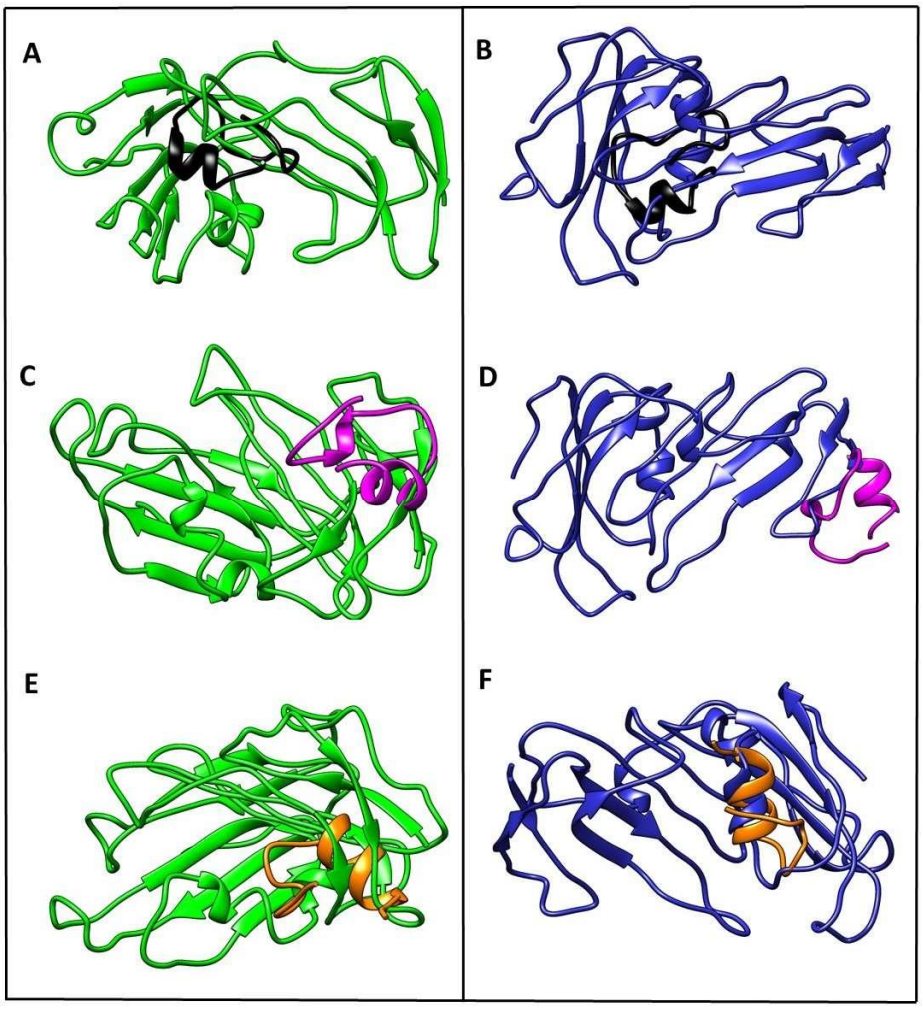

Fig. 2. Three-dimensional alignments between PLA_2 inhibitor models and peptides. (A) D10 peptide is shown in black and PLA_2 inhibitor model 1 is shown in green; (B) D10 peptide is shown in black and PLA_2 inhibitor model 4 is shown in blue; (C) E11 peptide is shown in magenta and PLA_2 inhibitor model 1 is shown in green; (D) E11 peptide is shown in magenta and PLA_2 inhibitor model 4 is shown in blue; (E) F7 peptide is shown in orange

and PLA_2 inhibitor model 1 is shown in green; (F) F7 peptide is shown in orange and PLA_2 inhibitor model 4 is shown in blue.

3.1.3. Multiple alignment of PLA_2s from snake venom showing their conserved domain (calcium binding and catalytic sites)

It is known that most $sPLA_2$s contain a highly conserved Ca^{2+} binding loop (XCGXGG) and a catalytic site (DXCCXXHD) [4]. Ca^{2+} is an essential cofactor for typical PLA_2s activity in which Tyr28, Gly30, Gly32 and Asp49 are involved in ion coordination [28].

In this study, several PLA_2s were investigated, and their alignments were performed. Important amino acids for catalysis previously mentioned are present in most sequences (Fig. 3). BID-PLA_2 and 2QOG are the enzymes used in the work for phospholipase A_2 activity and for bioinformatics analysis, respectively.

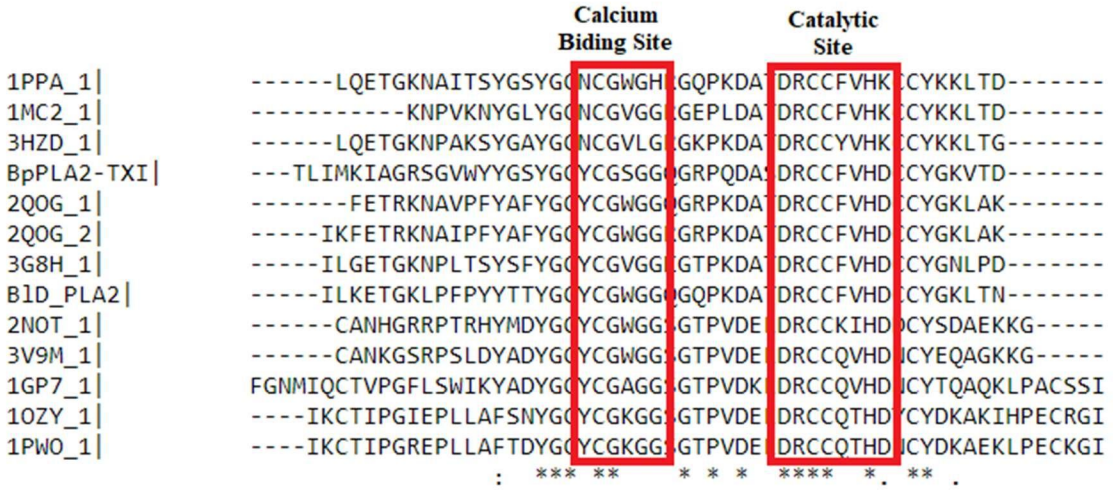

Fig. 3. Multiple alignment of PLA_2 from snake venom and its conserved domain.
(*) indicates the same amino acids; **(.)** indicates amino acids with low similarity; **(-)** indicates no similarity between amino acids.
1PPA: PLA_2 from *Agkistrodon piscivorus piscivorus*; 1MC2: PLA_2 from *Deinagkistrodon acutus*; 3HZD: PLA_2 from *Bothrops jararacussu;* BpPLA2-TXI: PLA_2 from *Bothrops pauloensis*; 2QOG: PLA_2 from *Crotalus durissus terrificus;* 3G8H: PLA_2 from *vipera ammodytes ammodytes; BID-PLA2: PLA*$_2$ from *Botrhops leucurus;* 2NOT: PLA_2 from *Notechis scutatus scutatus*; 3V9M: PLA_2 from *Pseudechis australis;* 1GP7: PLA_2 from *Ophiophagus hannah*; 1OZY: PLA_2 from *Micropechis ikaheka;* 1PW0: PLA_2 from *Micropechis ikaheka.*

3.1.4. Molecular docking between PLA_2 and mimetic peptides: D10 and F7 peptides bind into the PLA_2 catalytic site

Molecular docking is an established method based on a computer simulation structure, which explores the behavior of molecules in the binding site of a target

protein [29]. Molecular docking was executed herein to verify the interaction sites between mimetic peptides and PLA_2.

The three-dimensional structure of PLA_2 is illustrated in Fig. 4-A. Green, yellow and red colors represent catalytic site, calcium binding site and Ca^{2+}, respectively. As can be noticed, D10 and F7 peptides bind to the catalytic and calcium binding sites (Fig. 4-B; D), which is the most important region of the enzyme. For this reason, we chose both for further investigations.

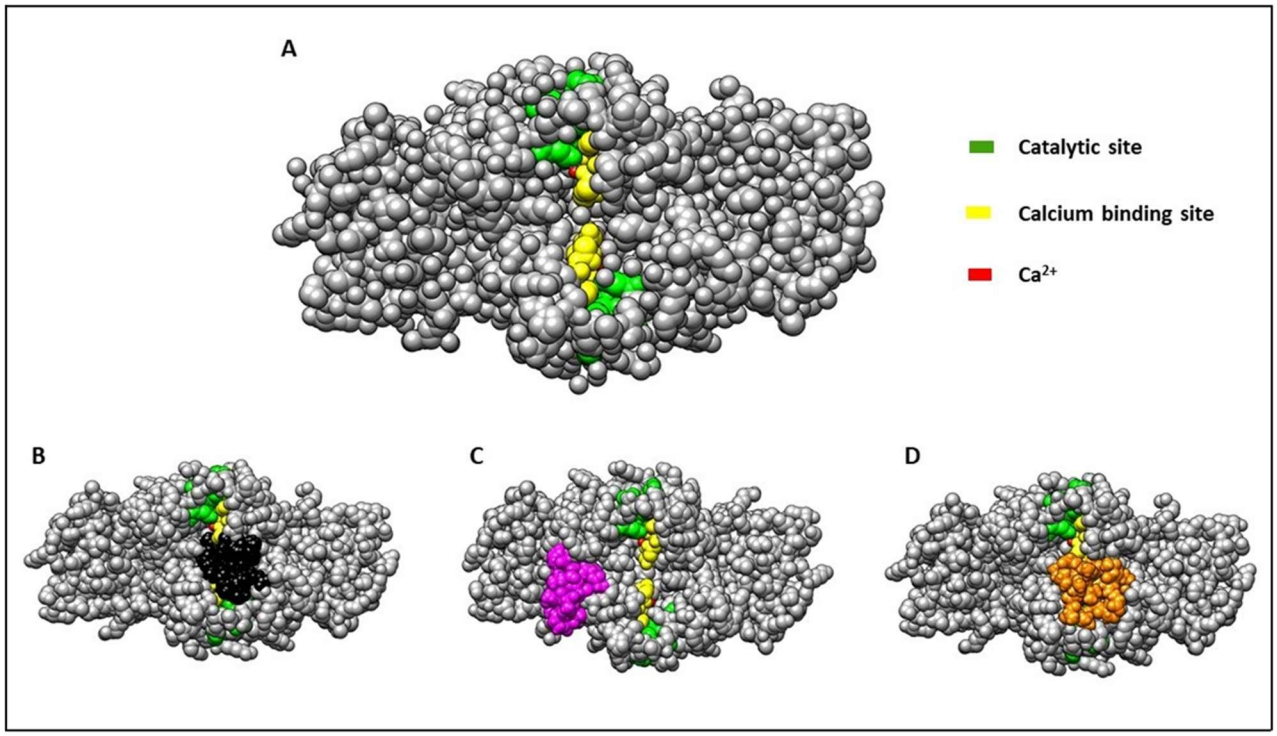

Fig. 4. Molecular docking between PLA_2 and mimetic peptides. (A) $sPLA_2$ catalytic and calcium binding sites in 3D model. Green, yellow and red colors represent catalytic site, calcium binding site and Ca^{2+}, respectively; (B) D10 peptide is shown in black and PLA_2 is shown in gray; (C) E11 peptide is shown in magenta and PLA_2 is shown in gray; (D) F7 peptide is shown in orange and PLA_2 is shown in gray.

3.1.5. F7 peptide had more similarity with the PLA_2 inhibitor than D10 peptide

We performed molecular docking studies to investigate which amino acids in PLA_2 interact with the inhibitor molecule. The amino acids of PLA_2 (in red) that interact with PLA_2 inhibitor and mimetic peptides (D10 and F7) are listed in Table 2. D10 had 16.6% of the same amino acids that interacted with the inhibitor, while F7 had 69.44%, making it more similar to the inhibitor molecule. Therefore, F7 peptide was chosen for molecular modeling.

Table 2. Interaction region between PLA_2-PLA_2 inhibitor and PLA_2-mimetic peptides

Binding molecule	PLA_2 subunit
PLA_2 inhibitor	HLLQFNKMIKFETRKNAIPFYAFYGCYCGWGGRGRPKDATDRC CFVHDCCYGKLAKCNTKWDIYPYSLKSGYITCGKGTWCEEQIC ECDRVAAECLRRSLSTYKYGYMFYPDSRCRGPSETC
D10 peptide	HLLQFNKMIKFETRKNAIPFYAFYGCYCGWGGRGRPKDATDRC CFVHDCCYGKLAKCNTKWDIYPYSLKSGYITCGKGTWCEEQIC ECDRVAAECLRRSLSTYKYGYMFYPDSRCRGPSETC
F7 peptide	HLLQFNKMIKFETRKNAIPFYAFYGCYCGWGGRGRPKDATDRC CFVHDCCYGKLAKCNTKWDIYPYSLKSGYITCGKGTWCEEQIC ECDRVAAECLRRSLSTYKYGYMFYPDSRCRGPSETC

3.1.6. F7 peptide modeling: Seq9 had the best coverage of the catalytic domain of PLA_2

After analyzing the molecular docking between PLA_2 and its inhibitor (Fig. 5-C), we performed molecular modeling with more than 40 sequences (not all shown) and chose the one that most covered the catalytic region of PLA_2. The three-dimensional structure of PLA_2 inhibitor (model 4) and the catalytic domain of PLA_2 are shown in Figures 5A and 5B.

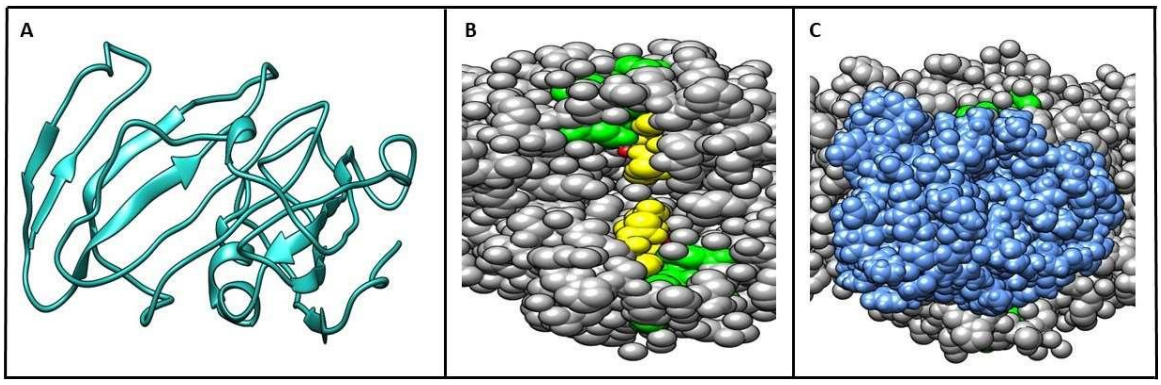

Fig. 5. Molecular docking analysis. (A) Three-dimensional structure of PLA_2 inhibitor; (B) Catalytic domain of PLA_2; (C) Molecular docking between PLA_2 and PLA_2 inhibitor. PLA_2 is shown in gray, and its catalytic site, calcium binding site and Ca^{2+} are represented by green, yellow and red colors, respectively.

Part of the F7 peptide sequence, including proline and glycine residues, was used to develop novel peptides. Proline-containing sequences have more rigidity, providing a locus for protein folding and assembly [30,31], and proline acts as a flexible element that could mediate rigid body motions of helical segments [32].

Furthermore, proline is usually found with glycine in peptide channels as well as within the putative transmembrane domains of several membrane ion channels [33].

The sequence, three-dimensional structure and docking into PLA_2 of each developed peptide are illustrated in Figures 6A, 6B and 6C, respectively. The sequence represented by the number 5 in Figure 6 (PILKEGGGSAETVESPPGGSGGPPSEVTEASGGGEKLIP) was able to cover the entire catalytic domain of PLA_2. For this reason, we chose it for synthesis and called it PepSeq9.

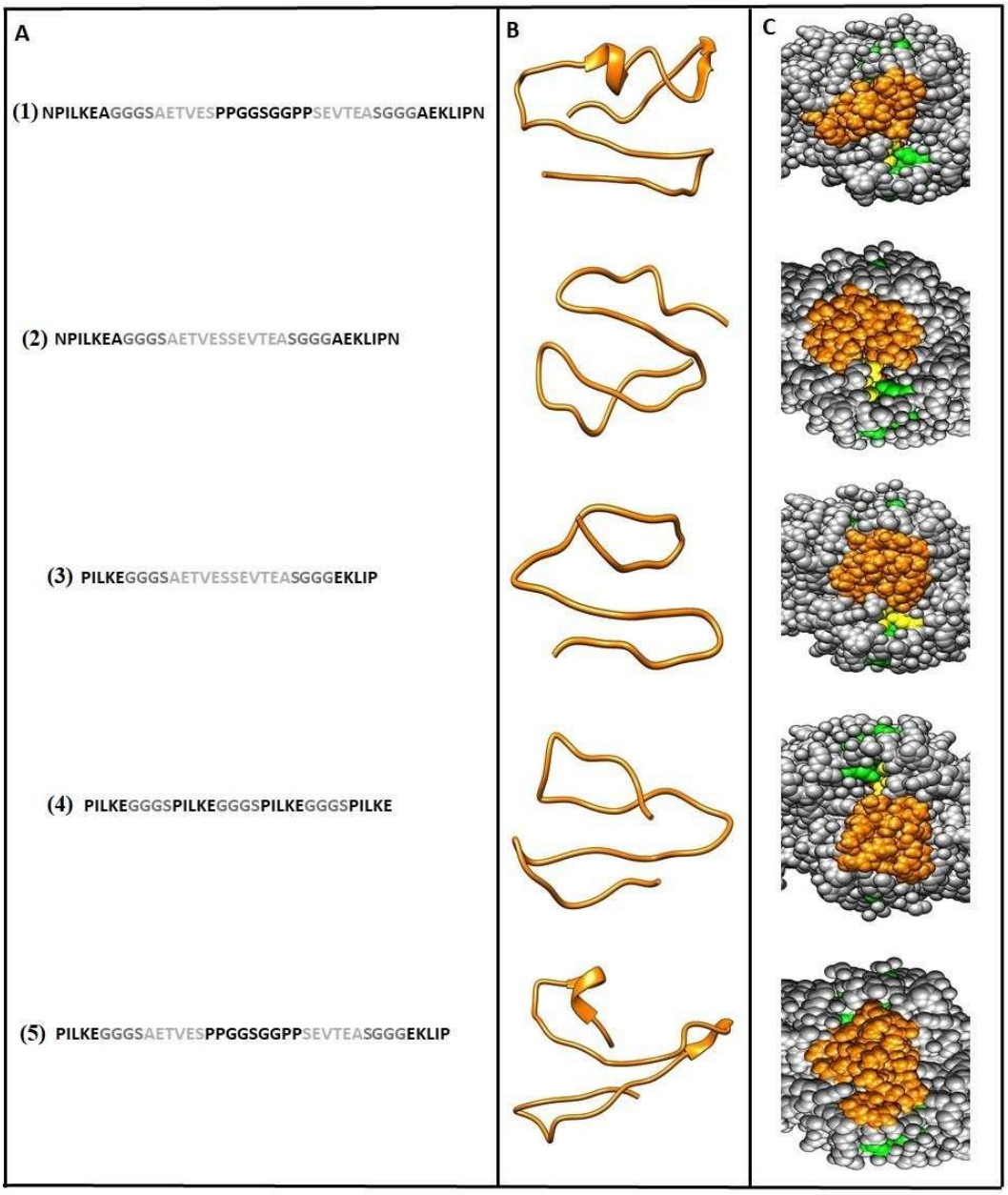

Fig. 6. F7 peptide modeling. Column A. Peptide sequences; Column B. Three-dimensional structure of peptide sequences; Column 3. Docking between peptides and PLA_2. Peptides

are shown in orange, PLA_2 is shown in gray, and its catalytic site, calcium binding site and Ca^{2+} are represented by green, yellow and red colors, respectively.

3.1.7. Amino acid interactions between PepSeq9 and PLA_2

The interaction between PepSeq9 peptide and PLA_2 is displayed in Figure 7. PepSeq9 interacts with amino acids that are important to the PLA_2 catalysis, such as His48 and Asp49, and also interacts with calcium binding region (GLY32), which is pivotal for enzyme activity [3,34,35]. For this reason, PepSeq9 peptide, theoretically, would have a major impact on enzymatic catalysis.

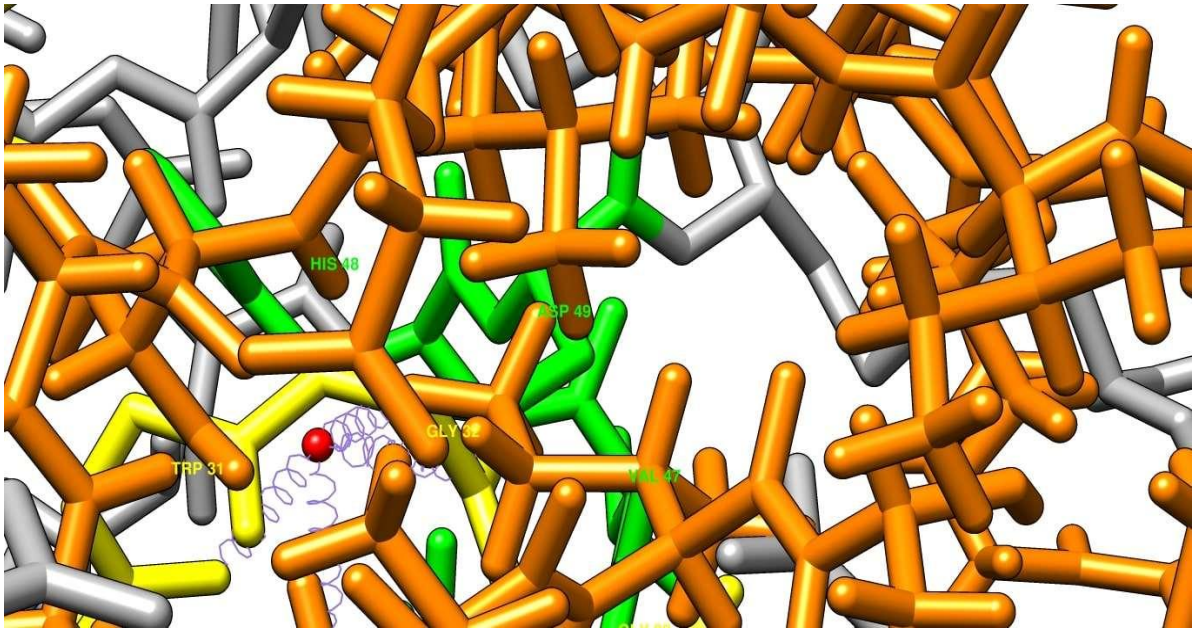

Fig. 7. Interaction between PepSeq9 peptide and PLA_2. The PLA_2 catalytic site, calcium binding site and PepSeq9 peptide are shown in green, yellow and orange, respectively.

3.2. Affinity chromatography showed that PepSeq9 molecule is a PLA_2 ligand

In order to confirm that PepSeq9 molecule is ligand of PLA_2, an affinity chromatography was carried out (Fig. 8). The second peak (highlighted in green) represents PepSeq9 that was bound to the column. Thus, it is possible to affirm that PepSeq9 is a PLA_2 ligand, even though it has been redesigned by bioinformatics. This result is expected, since the PepSeq9 sequence came from a peptide modeling that was selected to have the same inhibitory action. In fact, it is known that PLA_2 inhibitor must be a PLA_2 ligand to perform its function.

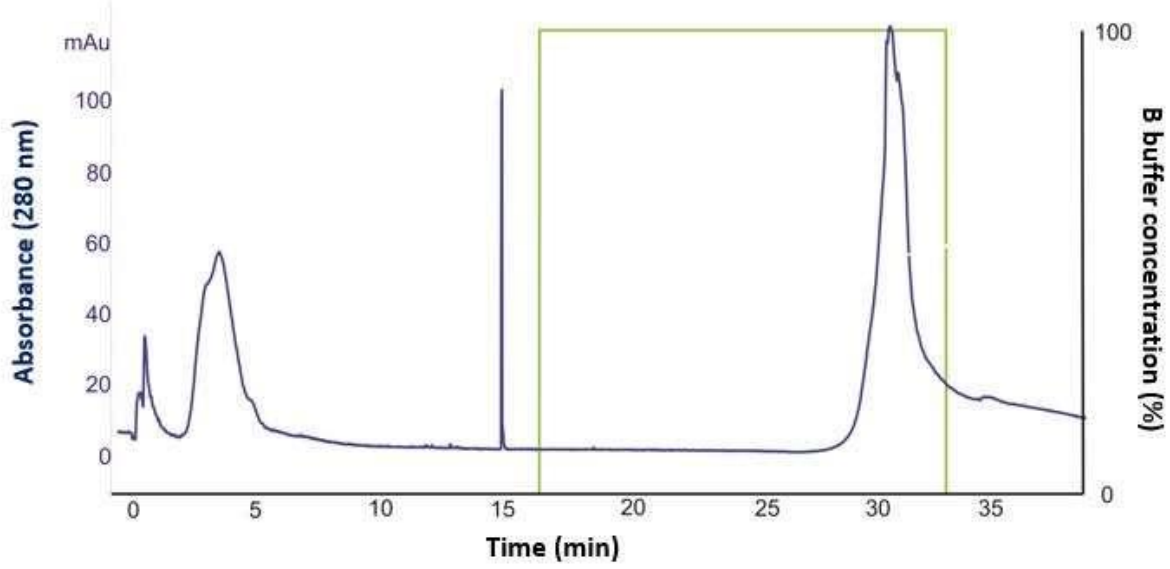

Fig. 8. Chromatographic profile of PepSEQ9 peptide. NHS HiTrap column previously immobilized with PLA_2 BnSP-7, equilibrated with 10mM/L Tris-HCl pH 7.5 buffer and eluted with 100mM/L Glycine pH 2.5 buffer, under constant flow 1.0 mL/min.

3.3. Phospholipase A_2 activity

Regarding phospholipase activity, Figure 9 exhibits that PepSeq9 peptide added to PLA_2 was able to increase the enzymatic catalysis when compared to control (only PLA_2). The concentrations used were 5 µg and 100 µg of PLA_2 and PepSeq9, respectively.

This result is curious since PepSeq9 peptide came from a sequence selected by Phage Display with similar structure to the PLA_2 inhibitor. Moreover, through bioinformatics analysis, PepSeq9 peptide interacted directly into the catalytic site. Thus, the expected result would be an inhibition by the peptide. Intriguingly, it was not an inhibition, but a potentiation of enzymatic activity.

It is noteworthy that several factors are included in a peptide-protein interaction and its inhibition mechanisms. An interesting work discussed the enzymatic inhibition mechanisms and obtained notable results. The researchers studied multi-conformational enzyme structure and intrinsic randomness and concluded that stochastic fluctuations at the single-enzyme level could make inhibitors to act as activators. These findings may explain why certain molecules inhibit enzymatic activity when substrate concentrations are high and elicit a non-monotonic dose response when substrate concentrations are low [36].

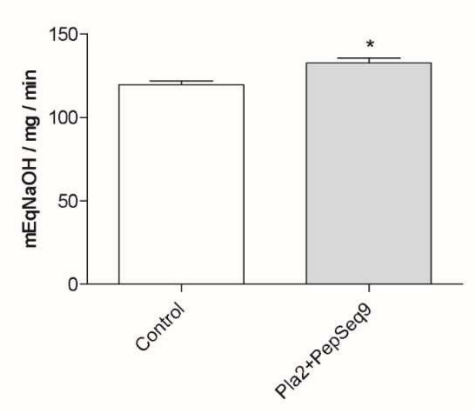

Fig. 9. Phospholipase A_2 activity. PepSeq9 peptide added to PLA_2 significantly increased the phospholipase activity when compared to control ($P < 0.05$).

The activation and inhibition mechanisms of PLA_2 represent another important issue that have not yet been fully elucidated. It is known that $sPLA_2$ enzymes do not hydrolyze the sn-1 position of the lysophospholipid products while some of the intracellular enzymes do. Therefore, these enzymes display an increase in activity when substrate forms aggregates rather than monomers, a phenomenon known as interfacial activation. The interfacial activation mechanism of PLA_2 is an interesting topic in membrane protein enzymology [37]. There are aromatic residues on the interfacial binding surface. Studies using hydrogen-deuterium exchange mass spectrometry (HDX-MS) show that the surface hydrophobic residues penetrate the lipid membrane to allow PLA_2 to access the substrate from the lipid aggregates [38]. Remarkably, tryptophan is an aromatic amino acid considered to be the most potent contributor to the interfacial binding [39]. Bezzine et al. revealed with a human $sPLA_2$ a higher activity of the enzyme on dioleoylphosphocholine (DOPC) membranes when a surface valine residue was mutated to tryptophan [40]. Therefore, perhaps even PepSeq9 peptide had interacted with the catalytic region (as shown in *in silico* tests), it was unable to prevent these aromatic residues from encountering the lipid surface and thus preventing catalysis. On the other hand, it could have assisted in this process in some way. By analyzing the interactions between PepSeq9 and PLA_2 (Fig. 7), it is possible to identify aromatic residues with which the peptide could be interacting, such as tryptophan.

Furthermore, the residues Tyr28, Gly30, Gly32 and Asp49 are involved in the coordination of the calcium ion, an indispensable cofactor for phospholipase activity

[28]. Cofactors are crucial constituents capable of endowing proteins with a range of functions that may be difficult or even impossible to achieve using a polypeptide sequence alone [41]. Asp49-PLA_2s have a highly conserved aspartic acid at position 49, which is completely involved in the binding of the essential cofactor Ca^{2+} ion [35,42]. Asp49-PLA_2s use the catalytic site His and Asp to polarize a bound H_2O to attack the carbonyl group. Therefore, calcium ion stabilizes the transition state by coordinating the carbonyl group and the negative charge from the phosphate oxygen [4]. Thus, we propose that PepSeq9 interacted with Ca^{2+} to potentiate the hydrolysis performed by this ion, which would explain the increase in the activity performed.

3.4. Viability assay in VERO cell line

Vero cells are used for biomaterial cytotoxicity assessment, toxin detection, function testing, media testing and mycoplasma [43]. To demonstrate that the PepSeq9 peptide has no cytotoxic action on non-tumorigenic cells, we performed MTT assays using Vero cell line. The investigation revealed that the PepSeq9 peptide tested at 1 µg, 10 µg, 50 µg and 100 µg concentrations did not affect cell viability and had no significant differences from control group (Fig. 10). This result suggests that PepSeq9 peptide can be proposed as a therapy, since it has no harmful effect on normal cells.

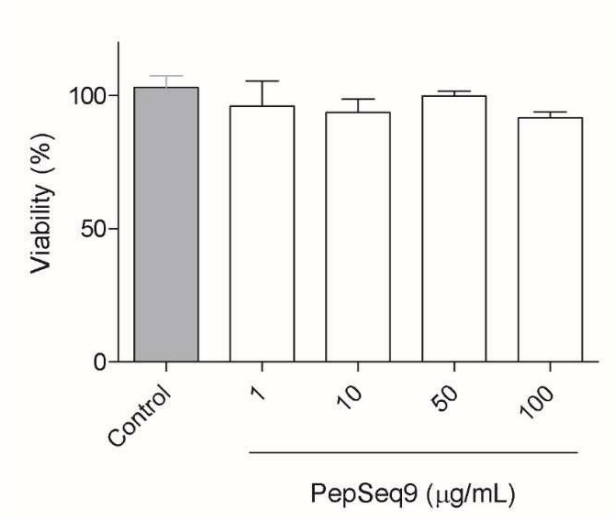

Fig. 10. Viability analysis of the synthetic peptide in Vero cell line. PepSeq9 peptide showed no statistical difference when compared to control (Vero cells without treatment), indicating that the concentrations tested had no cytotoxic effect.

3.5. PepSeq9 induces cell death in MDA-MB-231 cells

PepSeq9 peptide significantly reduced viability MDA-MB-231 cells at all concentrations used when compared to control (Fig. 11). Azevedo et al. (2019) reported that BthTX-II, a PLA_2 from *Bothrops jararacussu* venom, had antitumor and antimetastatic effects on MDA-MB-231 [44]. Since PepSeq9 peptide increased phospholipase A_2 activity and was toxic to breast cancer cells, we suggest further tests to verify wether PepSeq9 incubated with a antitumoral PLA_2 could improve that action.

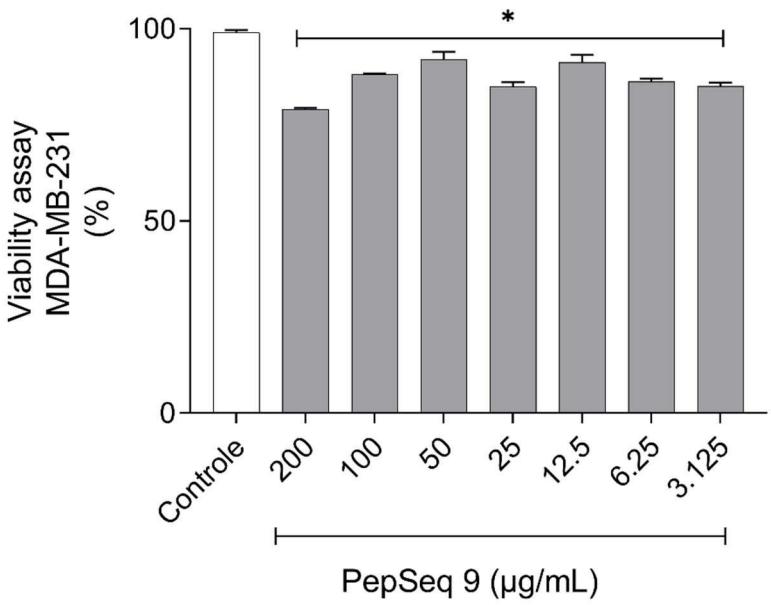

Fig. 11. Viability analysis of the synthetic peptide in MDA-MB-231 cells. PepSeq9 presented a significant decrease of MDA-MB-231 viability when compared to control ($P < 0.05$).

Prior studies have proposed that the cytotoxic effect of PLA_2 in tumor lines may be associated with the induction of apoptosis [45,46]. Panini et al. (2001) found that the activity of this enzyme accelerates the phospholipid turnover, influencing changes in the membrane, and inducing the apoptosis process [47]. Although previous works have demonstrated that different PLA_2s from several snake venoms induced death in cancer cells [48–51], further in-depth studies are warranted to explore the effects of PLA_2s.

Our findings are intriguing, and more studies are needed to better understand the results here found. Nevertheless, PepSeq9 peptide aroused interest because

its functional analysis was the opposite of the predicted bioinformatics analysis. Interestingly, this work showed that the PepSeq9 peptide was cytotoxic to MDA-MB-231, a triple-negative breast cancer cell line, and revealed a non-cytotoxic effect in Vero cells, a monkey kidney cell line, thereby indicating that PepSeq9 peptide may have specific antitumor effect. Besides, PepSeq9 conjugated with PLA_2 may be a future strategy that can be explored in further investigations.

4. Conclusion

Molecular modeling and docking were performed with sequences of three peptides selected by through PhD technology mimicking γCdcPLI, a PLA_2 inhibitor from *Crotalus durissus collilineatus*. PepSeq9 showed similarity with PLA_2 inhibitor, good interaction with catalytic site of PLA_2 and was toxic to MDA-MB-231 cells. Although initial studies were presented and further investigations are still required, these findings suggest that PepSeq9 is a PLA_2 ligand, and its action should be explored as a potential therapeutic in breast cancer.

Conflict of Interest

The authors declare that there is no conflict of interests.

Acknowledgments

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