UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE – UFCSPA
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Bruno de Souza Mayer

Peptídeos derivados da cistatina RHcyst-1 do carrapato *Rhipicephalus haemaphysaloides* como potenciais inibidores de catepsinas humanas envolvidas no câncer

Porto Alegre
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Dissertação submetida ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Mestre

Orientadora: Profª Dra. Adriana Seixas
Coorientador: Prof. Dr. Rodrigo Ligabue-Braun

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De acordo com o estabelecido previamente pela Comissão Coordenadora do Curso de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre, realizou-se aos vinte e oito dias do mês de abril de 2022, às quatorze horas, na modalidade remota, via Google Meet, a defesa da Dissertação de Mestrado do aluno **Bruno de Souza Mayer** orientado pela professora Adriana Seixas no Programa de Pós-graduação em Ciências da Saúde da UFCSPA. O trabalho defendido intitula-se "**Peptídeos derivados da cistatina RHcyst-1 do carrapato Rhipicephalus haemaphysaloides como potenciais inibidores de catepsinas humanas envolvidas no câncer**". A Banca Examinadora foi composta pelos professores Eliane Dallegrave (UFCSPA), Lucas Tirloni (Texas A&M University Estados Unidos) e Abid Ali (Abdul Wali Khan University Mardan). Após a abertura da sessão o aluno dispôs de 40 minutos para expor seu trabalho. Ao término da apresentação, foi realizada a arguição do aluno pelos membros da banca examinadora. Cada examinador teve até 20 minutos para sua arguição, tendo o aluno igual tempo para resposta. Ao término da Sessão foi anunciada a aprovação do aluno que, após a homologação da Dissertação de Mestrado, receberá o título de Mestre em Ciências da Saúde: Epidemiologia e Métodos Diagnósticos. Nada mais havendo a tratar, foi encerrada a sessão e lavrada a presente ata, que será assinada pela orientadora do aluno e pela Coordenação do Programa.

Porto Alegre, 28 de abril de 2022.

Prof. Pedro Roosevelt Torres Romão
Coordenador

Profa. Adriana Seixas
Orientadora

DEDICATÓRIA

Dedico este estudo à minha família, em especial,
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EPÍGRAFE

“Pobre é o discípulo que não excede seu mestre”

- Leonardo da Vinci

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RESUMO

A atividade das catepsinas é frequentemente desregulada no contexto da transformação neoplásica. O aumento da atividade e a localização desordenada de proteases no microambiente tumoral têm um papel fundamental na condução da progressão, proliferação, invasão e metastização do câncer. As catepsinas cisteínicas têm sua atividade proteolítica naturalmente controlada por inibidores proteicos denominados cistatinas. Esses inibidores podem ser obtidos a partir de parasitas hematófagos e já demonstraram atividades antitumorais. A proteína foco deste estudo, a RHcyst-1, já teve seu potencial anticâncer comprovado, tendo inibido significativamente a proliferação, migração e invasão de quatro tipos de células tumorais. Além disso, inibe o crescimento de tumores e aumenta a sobrevivência de animais *in vivo*. No estudo realizado, foram desenhados e sintetizados peptídeos baseados na RHcyst-1, e avaliada sua capacidade inibitória em estudos *in silico* e *in vitro*. Os estudos *in silico* demonstraram que os peptídeos Rhcyst-1pep e Rhcyst-1pep2 podem inibir a atividade das catepsinas B e L humanas com potencial similar a proteína recombinante. Esse potencial foi confirmado pelos ensaios *in vitro* sobre substrato sintético. Adicionalmente, as análises de sequência dos aminoácidos sugerem que os peptídeos propostos possuem menor capacidade de gerar resposta imune em relação a proteína RHcyst-1. Nossos resultados sugerem que os peptídeos propostos neste trabalho possam apresentar atividade semelhante a RHcyst-1 sendo novos candidatos no estudo de farmácos antitumorais ou antimetastáticos.

Palavras-chave: Cistatina; metástases, antitumorais, catepsinas, inibidores de protease, câncer.

ABSTRACT

Cathepsin activity is often dysregulated in the context of neoplastic transformation. Increased activity and disordered localization of proteases in the tumor microenvironment play a potent role in driving cancer progression, proliferation, invasion and metastasis. Cysteine cathepsins have their protease activity naturally controlled by protein inhibitors called cystatins. The inhibitors can be obtained from haematophagous ectoparasites and have already demonstrated antitumor activities. The protein focus of this study, RHcyst-1, had its antimural potential proven, having significantly inhibited the proliferation, migration and invasion of four types of tumor cells. In addition, it inhibied the growth of tumors and increases the survival of animals *in vivo*. In this study, two peptides based on RHcyst-1 were designed and synthesized, and the inhibitory capacity were evaluated *in silico* and *in vitro* studies. *In silico* studies showed that Rhcyst-1pep and Rhcyst-1pep2 can inhibit the activity of human cathepsins B and L as the recombinant protein. This potential was confirmed by *in vitro* assays on using synthetic substrate. Additionally, *in silico* studies showed that the proposed peptides have a lower capacity to generate an immune response in relation to the RHcyst-1 protein. Our results suggest that the peptides proposed in this work may have antitumor activity as well as RHcyst-1 being considered new candidates for studies on the development of antitumor or antimetastatic drugs.

Key words: Cystatin; metastases, antitumor, cathepsins, protease inhibitors, cancer.

1 INTRODUÇÃO

Segundo a Organização Mundial da Saúde (OMS), o câncer é uma das principais causas de morte no mundo, sendo responsável por quase 10 milhões de mortes em 2020. Neste mesmo ano os cânceres mais diagnosticados em todo o mundo foram câncer de mama feminino (2,26 milhões de casos), câncer de pulmão (2,21 milhões de casos) e câncer de próstata (1,41 milhões de casos); as causas mais comuns de morte por câncer foram pulmão (1,79 milhões de mortes), fígado (830.000) e câncer de estômago (769.000) (FERLAY et al., 2021).

O câncer é caracterizado como um grande grupo de doenças ocasionadas pelo crescimento descontrolado de células alteradas, podendo começar em qualquer órgão ou tecido do corpo com possibilidade de invadir partes adjacentes e/ou se espalhar para outros órgãos (LÚCIA DE ALMEIDA et al., 2005). Esse processo chamado de metástase é a principal causa de morte por câncer (FERLAY et al., 2021). A metástase é um processo complexo de várias etapas, que envolve alterações genéticas e epigenéticas nas células tumorais. Além disso, o câncer se desenvolve dentro de um microambiente tecidual complexo, que regula a invasão, migração e metástase de células tumorais (ZHANG et al., 2019). O microambiente da frente de invasão tumoral pode permitir que as células cancerosas obtenham uma capacidade de motilidade anormalmente alta e penetrem no estroma circundante. A frente de invasão do tumor é particularmente rica em uma variedade de proteases, que facilitam a invasão e as metástases do câncer através da remodelação da matriz extracelular promovendo a migração celular (YANG et al., 2017).

As catepsinas são proteases que estão envolvidas em diversos tipos de câncer, exibindo características e funções variadas (WEI et al., 2018). Em humanos, as catepsinas cisteínicas têm 11 membros: catepsina B (Cat B), C (Cat C), F (Cat F), H (Cat H), K (Cat K), L (Cat L), O (Cat O), S (Cat S), V (Cat V, também conhecida como L2), W (Cat W), e Z (Cat Z), também denominada X) (ROSSI et al., 2004). Essas catepsinas têm sido associadas à proliferação, sobrevivência e metástase contribuindo, assim, para a progressão tumoral em vários tipos de câncer através de diversos mecanismos (OLSON; JOYCE, 2015). Nas células normais apenas uma pequena quantidade de catepsinas são secretadas (TANDON et al., 1990), e sob condições fisiológicas normais estas são sequestradas pelos lisossomos. No entanto, nas células tumorais, já é bem compreendido que alterações nos níveis de expressão

e nas vias de tráfego resultam na secreção destas proteinases para o meio extracelular (DENHARDT et al., 1987). Inibidores que bloqueiam a atividade das catepsinas ou diminuem seus níveis de atividade proteolítica no ambiente periférico, têm sido investigados pois podem impedir a progressão do tumor e ocorrência de metástases (GOCHEVA; JOYCE, 2007). As cistatinas constituem uma família evolutivamente relacionada de inibidores reversíveis e de forte ligação, que atuam sobre as catepsinas lisossomais pertencentes à família C1 (JAKOŠ et al., 2019). O papel principal das cistatinas é limitar a atividade excessiva de cisteíno endopeptidases. Dessa forma, a combinação de inibidores de catepsina com quimioterapia e radiação para tratamento de câncer tem sido proposto (LI et al., 2021; SUDHAN; SIEMANN, 2015a).

A busca por moléculas e compostos com atividade antitumoral é constante devido à importância dessa doença. As cistatinas estão entre algumas das moléculas que vêm sendo estudadas com este propósito. Além da presença em vertebrados, essas moléculas estão presentes em diversas espécies de artrópodes, dentre elas os carrapatos. Os carrapatos são ectoparasitos hematófagos obrigatórios de animais selvagens e domésticos, bem como de seres humanos. Eles perdem apenas para os mosquitos como vetores globais de doenças humanas (ZHANG et al., 2018). Devido ao longo e complexo processo evolutivo gerado pelo parasitismo, estes hematófagos representam uma importante fonte de novas moléculas bioativas.

Diversos compostos bioativos provenientes de animais hematófagos vem sendo estudados e inúmeras aplicações biotecnológicas têm sido reportadas, como por exemplo: substâncias anti-hemostáticas (RIBEIRO et al., 1985), inibidores da coagulação sanguínea (DAVIE; FUJIKAWA; KISIEL, 1991), inibidores da agregação plaquetária (CHAMPAGNE; NUSSENZVEIG; RIBEIRO, 1995; MANS et al., 1998; RIBEIRO; GARCIA, 1981; VALENZUELA et al., 1998, 2001), vasodilatadores (CHAMPAGNE; RIBEIRO, 1994; LERNER et al., 1991; LERNER; SHOEMAKER, 1992), imunossupressores (WEI et al., 2019) e antitumorais (WEI et al., 2018). Algumas espécies de carrapatos apresentam mais de uma cistatina, como os carrapatos da espécie *Rhipicephalus microplus, no qual foram identificadas* diferentes cistatinas. Dados de transcriptoma e proteoma mostram que a presença de mais de uma cistatina não seria exclusiva de *Rhipicephalus microplus*. (CARDOSO et al., 2017; LIMA; SASAKI; TANAKA, 2006; LU et al., 2020a; PARIZI et al., 2013).

A atividade antitumoral de uma cistatina obtida de carrapato já foi comprovada. A RHcyst-1, é uma cistatina obtida da espécie *Rhipicephalus haemaphysaloides*, que apresentou significativa inibição da proliferação, da migração e da invasão de células tumorais (WEI et al., 2018). *In vitro,* RHcyst-1 inibiu significativamente a proliferação, migração e invasão das linhagens celulares de câncer cervical humano (HeLa), pulmão (A549), ovário (SKOV-3) e melanoma murino (B16-F10). Além disso, quando testada *in vivo*, em modelo murino, inibiu o crescimento do tumor e melhorou a sobrevivência dos animais. A RHcyst-1 obtida do carrapato *Rhipicephalus. hemaphisaloides* possui atividade antitumoral potencial, que pode estar associada a alterações na expressão da catepsina, podendo ser considerada passível de aplicação no tratamento do câncer (WEI et al., 2018).

Neste trabalho nós estudamos a estrutura terciária da RHcyst-1 e a interação inibidor-enzima. A partir desses resultados projetamos e testamos (*in silico* e *in vitro*) o potencial inibitório de dois peptídeos derivados das regiões inibitórias da RHcyst-1 como inibidores de catepsinas L e B.

2 REVISÃO BIBLIOGRÁFICA

2.1 Câncer

Câncer é o nome dado a um conjunto de mais de 100 doenças que têm em comum o crescimento desordenado de células, que invadem tecidos e órgãos. Estas células tendem a ser muito agressivas e incontroláveis, pois têm capacidade de se dividir de forma acelerada, levando a formação de tumores, que podem espalhar-se para outras regiões do corpo (INCA, 2019).

O câncer surge a partir de mutações genéticas, alterações no DNA das células que podem acontecer em qualquer parte do corpo. No entanto, alguns órgãos são mais afetados do que outros; e cada órgão, por sua vez, pode ser acometido por tipos diferenciados de tumor, mais ou menos agressivos. Sendo assim, são evidenciados variados tipos de câncer, que correspondem aos diversos tipos de células do corpo. Alterações reportadas em tecidos epiteliais, como pele ou mucosas, são denominados carcinomas. Quando os alvos são os tecidos conjuntivos, como ossos, músculos e cartilagens, trata-se de sarcomas. A velocidade de multiplicação das células e a

capacidade de invadir tecidos e órgãos vizinhos ou distantes, também diferenciam os tipos de câncer (INCA, 2019).

O processo geralmente lento de formação do câncer é chamado de carcinogênese ou oncogênese, podendo levar vários anos para que uma célula cancerosa se prolifere e origine um tumor visível. Esse processo possui três estágios principais, sendo o primeiro o estágio de iniciação. Nesta etapa as células sofrem modificações em seus genes, causadas por agentes cancerosos, no entanto essas modificações não são clinicamente detectáveis. Na segunda etapa, denominada estágio de promoção, a célula geneticamente modificada se torna maligna, em um processo geralmente lento, gradual e de contato continuado com o agente promotor, podendo este agente ser desde um componente da alimentação, ou até mesmo hormônios. Dessa forma, as células cancerosas podem progredir para a terceira etapa, conhecida como estágio de progressão, que é caracterizado pela multiplicação acelerada, desordenada e irreversível das células alteradas. Nesse estágio, o câncer evolui até o surgimento de manifestações clínicas no paciente (INCA, 2019).

2.1.1 Metástase

A possibilidade do câncer invadir células adjacentes e/ou se espalhar para outros órgãos leva ao processo de metástase. Esse processo ocorre pela capacidade de disseminação de células tumorais através dos vasos sanguíneos ou linfáticos culminando na colonização de órgãos específicos (FONT-CLOS; ZAPPERI; LA PORTA, 2020). Em meados de 1889, Paget afirmou que uma metástase não ocorre por acaso, mas sim em decorrência da adaptação de células tumorais a um microambiente permissivo de um determinado órgão (PAGET; STEPHEN, 1889). Ao se espalhar pelo corpo e formar um novo foco do tumor em outro órgão, longe do sítio primário ou local de origem da doença, esse novo tumor é chamado de metastático (INCA, 2019). As enzimas proteolíticas implicadas na progressão tumoral e metástase pertencem a quatro principais classes catalíticas de proteases: metaloproteinases, serino-proteases, cinteíno-proteases e aspartil-proteases (VASILJEVA et al., 2006).

2.2 Catepsinas

O nome catepsina é derivado do grego “kathepsein” que significa digerir, esse nome foi proposto para as proteases que são ativadas em ambiente levemente ácido (WILLSTÄTTER; BAMANN, 1929). As catepsinas são enzimas sintetizadas como pré-pro-catepsinas e ativadas no lisossomo. Incluem-se as catepsinas D, E (aspartil-proteases), A, G (serino-proteases) e B, C, F, H, L, K, O, S, V, W e X (cisteíno-proteases). Todas são importantes proteases com funções que vão além da proteólise, estando envolvidas em importantes processos fisiológicos (SAFTIG et al., 1998).

2.2.1 Catepsinas cisteínicas e seus inibidores naturais

A maioria das catepsinas cisteínicas são localizadas e expressas ubiquamente em tecidos humanos, como a catepsina B, H, L, C, X, F, O e V. Seu perfil de expressão indica que essas enzimas estão envolvidas na degradação e renovação natural das proteínas celulares. Em contraste, as catepsinas K, W e S apresentam uma distribuição restrita específica nas células ou tecidos, indicando seus papéis mais específicos, que podem ser observados na Tabela 1 (TURK et al., 2012).

Em geral, as catepsinas cisteínicas são pequenas proteínas monoméricas com uma massa média de aproximadamente 30 kDa, sua estrutura consiste em dois domínios (esquerdo e direito) com uma fenda de sítio ativo em forma de V localizada ao longo da interface do domínio (TURK, 2000). Nas catepsinas cisteínicas esse local contém dois resíduos ativos formando uma díade catalítica, um de cisteína (Cys-25) localizado no domínio esquerdo e um de histidina (His 159) localizado no domínio direito que, juntos, formam um par de íons tiolato-imidazól estável necessário para a atividade da enzima (FALGUEYRET et al., 2001). Atualmente são conhecidas 11 catepsinas cisteínicas humanas, sendo elas: catepsina B, C, F, H, K, L, O, S, V, X e W, informação que foi confirmada por análises de bioinformática da sequência do genoma humano (ROSSI et al., 2004).

Tabela 1 - Tecidos de expressão e funções das catepsinas cisteínicas humanas.

Catepsina	Tecido de expressão	Função
B	Cérebro, tecido endócrino, sistema respiratório, trato digestivo proximal, trato gastrointestinal, fígado, vesícula biliar, pâncreas, rim, bexiga, aparelho reprodutor masculino, aparelho reprodutor feminino, tecido muscular, pele, medula óssea e tecidos linfoides	Catabolismo de proteínas, processamento de ativação de hormônios, antígenos e renovação óssea
C	Cérebro, tecido endócrino, sistema respiratório, trato digestivo proximal, trato gastrointestinal, fígado, vesícula biliar, pâncreas, rim, bexiga, aparelho reprodutor masculino, aparelho reprodutor feminino, tecido muscular, pele, medula óssea, tecidos linfoides, tecido adiposo e tecido mole	Hidrólise de ésteres de dipeptideos, amidas, anilidas e beta-naftilamidas
F	Cérebro, tecido endócrino, sistema respiratório, trato digestivo proximal, trato gastrointestinal, fígado, vesícula biliar, pâncreas, rim, bexiga, aparelho reprodutor masculino, aparelho reprodutor feminino, tecido muscular, pele, tecido adiposo e tecido mole	Apresentação de antígeno
H	Tecido endócrino, sistema respiratório, trato digestivo proximal, trato gastrointestinal, fígado, vesícula biliar, pâncreas, rim, bexiga, aparelho reprodutor masculino, aparelho reprodutor feminino, pele, medula óssea e tecidos linfoides	Atividade de endopeptidase
L	Cérebro, tecido endócrino, sistema respiratório, trato digestivo proximal, trato gastrointestinal, fígado, vesícula	Diferenciação de queratinócitos, renovação proteica, apresentação de antígenos

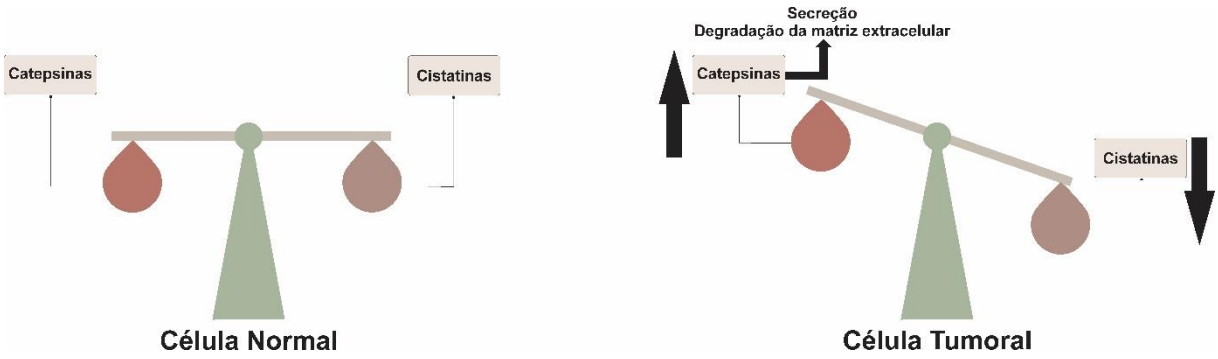
	biliar, pâncreas, rim, bexiga, aparelho reprodutor masculino, aparelho reprodutor feminino, tecido muscular, medula óssea e tecidos linfoides	
K	Pouco claro	Remodelação óssea
O	Cérebro, tecido endócrino, sistema respiratório, trato digestivo proximal, trato gastrointestinal, fígado, vesícula biliar, pâncreas, rim, bexiga, aparelho reprodutor masculino, aparelho reprodutor feminino, tecido muscular e pele	Degradação e rotatividade de proteínas
S	Cérebro, tecido endócrino, sistema respiratório, trato digestivo proximal, trato gastrointestinal, fígado, vesícula biliar, pâncreas, rim, bexiga, aparelho reprodutor masculino, aparelho reprodutor feminino, pele, medula óssea e tecidos linfoides	Proteólise da cadeia Ii nos linfócitos B
V	Cérebro, tecido endócrino, sistema respiratório, trato digestivo proximal, trato gastrointestinal, fígado, vesícula biliar, pâncreas, rim, bexiga, aparelho reprodutor masculino, aparelho reprodutor feminino, tecido muscular, pele, medula óssea, tecidos linfoides, tecido adiposo e tecido mole	Produção de encefalina e neuropeptídeo Y
W	Sistema respiratório, trato digestivo proximal, fígado, vesícula biliar, pâncreas, rim, bexiga, aparelho reprodutor masculino, pele, medula óssea e tecidos linfoides	Citotoxicidade mediada por células
X	Pouco Claro	Fagocitose, regulação das respostas imunes

Fonte: *The Human Protein Atlas*

Nos últimos anos as catepsinas cisteínicas se tornaram um alvo importante para terapias contra o câncer, pois sabe-se que o aumento da atividade proteolítica destas facilita a progressão maligna e tem sido associada a papéis promotores de tumores (JAKOŠ et al., 2019). Contudo as catepsinas podem ser reguladas por diversos inibidores proteicos descritos na literatura, conforme a Tabela 2 (LECAILLE; KALETA; BRÖMME, 2002). Esses inibidores podem interagir com o sítio ativo de forma reversível ou irreversível (LEUNG; ABBENANTE; FAIRLIE, 2000), sendo classificados em quatro grandes famílias conforme sua estrutura primária, sendo elas as estefinas (família I), cistatinas (família II), cininogênios (família III) e fitocistatinas (família IV) (DICKINSON, 2002). Além de inibidores proteicos, as catepsinas podem ser inibidas por compostos sintéticos que vem sendo descritos desde 1977 (LEARY; SHAW, 1977).

O desequilíbrio entre os níveis de catepsinas e seus inibidores é relatado como parte do processo de invasão e metástase tumoral, a super expressão e secreção das catepsinas auxiliam na degradação da matriz extracelular facilitando assim, o processo de metástase tumoral (NOMURA; KATUNUMA, 2005), conforme ilustrado na Figura 1 (FRIEDRICH et al., 1999). Níveis reduzidos de inibidores de proteases foram observados em diversos tipos de malignidades incluindo câncer de próstata (COLELLA; GOODWYN; GOPAL, 2002) e de mama (ZAJC et al., 2002) sendo que esse desequilíbrio contribui para desenvolvimento e progressão das células malignas (KOS; LAH, 1998).

Figura 1 - Equilíbrio entre catepsinas e inibidores de protease.



Fonte: Adaptado de NOMURA; KATUNUMA, (2005)

Tabela 2 - Inibidores proteicos de catepsinas cisteínicas.

(continua)

Catepsina	Inibidor	Referência
Cat B	alpha-2-macroglobulin	(BARRETT et al., 1982)
	leupeptina	(BAICI; GYGER-MARAZZI, 1982)
	cistatina A	(BARRETT, 1987)
	cistatina B	(BARRETT, 1987)
	cistatina C	(BARRETT, 1987)
	ovocystatin	(BARRETT, 1987)
	kininogen	(BARRETT, 1987)
	snake venom cystatin (*Bitis sp.*)	(EVANS; BARRETT, 1987)
	cistatina SN	(ABRAHAMSON, 1994)
	cistatina D	(ABRAHAMSON, 1994)
	cistatina E	(NI et al., 1997)
	T-kininogen	(GUTMAN et al., 1988)
	equistatin	(LENARCIC et al., 1998)
	clitocypin	(BRZIN et al., 2000)
	onchocystatin	(NEWLANDS et al., 2001)
	serpin SPI2	(PHILLIPS et al., 2004)
	phytocystatin (*Actinidia sp.*)	(RASSAM; LAING, 2004)
	amoebiasin-2	(ŠARIĆ et al., 2006)
	crammer peptidase inhibitor	(DESHAPRIYA et al., 2007)
	caneCPI cystatin (*Saccharum sp.*)	(GIANOTTI et al., 2008)
	toxostatin-1 (*Toxoplasma gondii*)	(HUANG et al., 2009)
	macrocypin 4	(RENKO et al., 2010)
	macrocypin 1	(RENKO et al., 2010)
	cystatin 2b (*R. microplus*)	(PARIZI et al., 2015)
	cystatin 2a (*Ixodes ovatus*)	(PARIZI et al., 2015)
	RHcyst-1 (*R. haemaphysaloides*)	(WANG et al., 2015)
	DARPin	(KRAMER et al., 2017)
	cistatina S	(SOOND et al., 2019)
	CP14019 (*Giardia intestinalis*)	(LIU; SVÄRD; KLOTZ, 2019)
	CP16160 (*Giardia intestinalis*)	(LIU; SVÄRD; KLOTZ, 2019)
	CP16779 (*Giardia intestinalis*)	(LIU; SVÄRD; KLOTZ, 2019)
	TK-cystatin (*Thelohanellus kitauei*)	(ZHANG et al., 2020)
Cat C	cistatina A	(BARRETT, 1987)
	cistatina B	(BARRETT, 1987)
	cistatina C	(BARRETT, 1987)
	ovocystatin	(BARRETT, 1987)
	snake venom cystatin (*Bitis sp.*)	(EVANS; BARRETT, 1987)
	cistatina SN	(TSENG et al., 2000)

Tabela 2 - Inibidores proteicos de catepsinas cisteínicas.

(continua)

Catepsina	Inibidor	Referência
	cystatin 2b (*R. microplus*)	(PARIZI et al., 2015)
	cystatin 2a (*Ixodes ovatus*)	(PARIZI et al., 2015)
	cystatin 2c (*R. microplus*)	(PARIZI et al., 2015)
	RHcyst-1 (*R. haemaphysaloides*)	(WANG et al., 2015)
Cat F	cruzipain propeptide	(REIS et al., 2007)
Cat H	alpha-2-macroglobulin	(BARRETT et al., 1982)
	cistatina A	(BARRETT, 1987)
	cistatina B	(BARRETT, 1987)
	cistatina C	(BARRETT, 1987)
	kininogen	(BARRETT, 1987)
	ovocystatin	(BARRETT, 1987)
	T-kininogen	(GUTMAN et al., 1988)
	Oryzacystatin II	(KONDO et al., 1990)
	cytotoxic T-lymphocyte antigen-2 alpha	(DELARIA et al., 1994)
	cytotoxic T-lymphocyte antigen-2 beta	(DELARIA et al., 1994)
	cistatina D	(ABRAHAMSON, 1994)
	MHC II invariant chain p41 form	(BEVEC et al., 1996)
	onchocystatin	(NEWLANDS et al., 2001)
Cat L	leupeptin	(KIRSCHKE et al., 1976)
	alpha-2-macroglobulin	(BARRETT et al., 1982)
	Ovocystatin	(BARRETT, 1987)
	cistatina A	(BARRETT, 1987)
	cistatina B	(BARRETT, 1987)
	kininogen inhibitor unit 2	(BARRETT, 1987)
	kininogen inhibitor unit 3	(BARRETT, 1987)
	kininogen	(BARRETT, 1987)
	T-kininogen inhibitor unit 2	(GUTMAN et al., 1988)
	T-kininogen inhibitor unit 3	(GUTMAN et al., 1988)
	T-kininogen	(GUTMAN et al., 1988)
	cistatina C	(ABRAHAMSON, 1994)
	cistatina D	(ABRAHAMSON, 1994)

Tabela 2 - Inibidores proteicos de catepsinas cisteínicas.

(continua)

Catepsina	Inibidor	Referência
	cytotoxic T-lymphocyte antigen-2 alpha	(DELARIA et al., 1994)
	cytotoxic T-lymphocyte antigen-2 beta	(DELARIA et al., 1994)
	SSCA	(TAKEDA et al., 1995)
	MHC II invariant chain p41 form	(BEVEC et al., 1996)
	histidine-rich glycoprotein inhibitor unit 1	(MARKS et al., 1988)
	cistatina F	(NI et al., 1998)
	equistatin	(LENARCIC et al., 1998)
	cathepsin L propeptide	(GUAY et al., 2000)
	cathepsin K propeptide	(GUAY et al., 2000)
	cathepsin S propeptide	(GUAY et al., 2000)
	clitocypin	(BRZIN et al., 2000)
	Cruzipain inhibitor (*Bauhinia bauhinioides*)	(OLIVEIRA et al., 2001)
	Bombyx-type cysteine peptidase inhibitor	(KURATA et al., 2001)
	Colligin 2	(SIAVASH et al., 2002)
	Serpin B13	(JAYAKUMAR et al., 2003)
	testican-1	(BOCOCK et al., 2003)
	Serpin srp-2 (*Caenorhabditis elegans*)	(PAK et al., 2004)
	Serpinb3b (*Mus musculus*)	(SAKATA et al., 2004)
	phytocystatin (*Actinidia sp.*)	(RASSAM; LAING, 2004)
	Endopin 2C	(HWANG et al., 2005)
	sialostatin L (*Ixodes scapularis*)	(KOTSYFAKIS et al., 2007)
	cistatina M/E	(CHENG et al., 2006)
	Falstatin	(PANDEY et al., 2006)
	Serpin srp-6 (*Caenorhabditis elegans*)	(LUKE et al., 2007)
	crammer peptidase inhibitor	(DESHAPRIYA et al., 2007)
	cathepsin V propeptide	(BURDEN et al., 2007)
	caneCPI cystatin (*Saccharum sp.*)	(GIANOTTI et al., 2008)
	toxostatin-1 (*Toxoplasma gondii*)	(HUANG et al., 2009)
	Macrocypin 1	(RENKO et al., 2010)
	Macrocypin 3	(RENKO et al., 2010)
	Macrocypin 4	(RENKO et al., 2010)
	Protein C inhibitor	(FORTENBERRY et al., 2010)
	cystatin 2b (*R. microplus*)	(PARIZI et al., 2015)
	cystatin 2a (*Ixodes ovatus*)	(PARIZI et al., 2015)
	cystatin 2c (*R. microplus*)	(PARIZI et al., 2015)
	RHcyst-1 (*R. haemaphysaloides*)	(WANG et al., 2015)

Tabela 2 - Inibidores proteicos de catepsinas cisteínicas.

(continua)

Catepsina	Inibidor	Referência
	FhKT1 (*Fasciola hepatica*)	(SMITH et al., 2016)
	cistatina E	(SOOND et al., 2019)
	cistatina M	(SOOND et al., 2019)
	Galinamida A	(BOUDREAU et al., 2019)
	TK-cystatin (*Thelohanellus kitauei*)	(ZHANG et al., 2020)
	rAacystatin	(OLIVEIRA et al., 2020)
	rRmcystatin-1b	(LU et al., 2020)
Cat K	cathepsin K propeptide	(GUAY et al., 2000)
	SCCA1	(STENMAN et al., 2001)
	Serpin B13	(JAYAKUMAR et al., 2003)
	Serpin srp-2 (*Caenorhabditis elegans*)	(PAK et al., 2004)
	Falstatin	(PANDEY et al., 2006)
	cathepsin V propeptide	(BURDEN et al., 2007)
	Serpin srp-6 (*Caenorhabditis elegans*)	(LUKE et al., 2007)
	Clitocypin	(RENKO et al., 2010)
Cat O	Pouco Claro	
Cat S	cistatina A	(ABRAHAMSON, 1994)
	cistatina B	(ABRAHAMSON, 1994)
	cistatina C	(ABRAHAMSON, 1994)
	cistatina D	(ABRAHAMSON, 1994)
	cathepsin L propeptide	(GUAY et al., 2000)
	cathepsin S propeptide	(GUAY et al., 2000)
	cathepsin K propeptide	(GUAY et al., 2000)
	squamous cell carcinoma antigen 1	(SAKATA et al., 2004)
	Serpin srp-2 (*Caenorhabditis elegans*)	(PAK et al., 2004)
	cathepsin V propeptide	(BURDEN et al., 2007)
	Macrocypin 1	(RENKO et al., 2010)
	Macrocypin 3	(RENKO et al., 2010)
	Macrocypin 4	(RENKO et al., 2010)
	clitocypin	(RENKO et al., 2010)
	RHcyst-1 (*R. haemaphysaloides*)	(WANG et al., 2015)
Cat V	Serpin srp-2 (*Caenorhabditis elegans*)	(PAK et al., 2004)
	cistatina M/E	(CHENG et al., 2006)
	Serpin srp-6 (*Caenorhabditis elegans*)	(LUKE et al., 2007)
	cathepsin V propeptide	(BURDEN et al., 2007)

Tabela 2 - Inibidores proteicos de catepsinas cisteínicas.

(conclusão)

Catepsina	Inibidor	Referência
	Clitocypin	(RENKO et al., 2010)
	Macrocypin 1	(RENKO et al., 2010)
	Macrocypin 3	(RENKO et al., 2010)
	Macrocypin 4	(RENKO et al., 2010)
Cat W	Pouco claro	
Cat X	monoclonal antibody 2F12	(OBERMAJER et al., 2006)

Fonte: Elaborado pelo autor (2022) a partir do banco de dados MEROPS acessado em março de 2022.

Embora a maioria das cisteíno-peptidases sejam endopeptidases (catepsinas L, V, F, S, K e W), algumas, como as catepsinas X e C são exopeptidases. As catepsinas B e H possuem atividade de exopeptidases e endopeptidases (TURK, 2000).

2.3 Características funcionais e estruturais das catepsinas cisteínicas e envolvimento em patologias

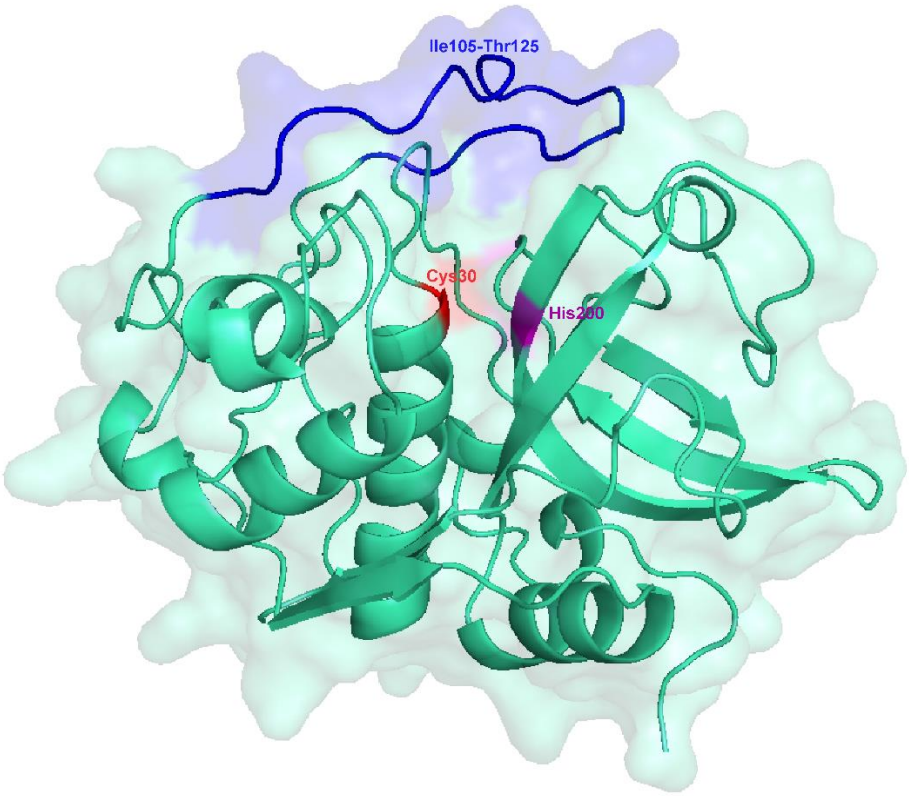
2.3.1 Catepsina B

A Cat B (EC 3.4.22.1) têm diferentes localizações subcelulares que podem determinar suas funções distintas e mecanismos de regulação independentes (BAICI et al., 2006; BESTVATER; DALLNER; SPIESS, 2005; YAN; SLOANE, 2003). Ela é única entre as catepsinas porque tem duas conformações, exopeptidase (carboxidipeptidase) e endopeptidase, devido à presença de uma inserção de 20 aminoácidos (Ile105 - Thr125) denominada alça de oclusão, que fornece a Cat B uma dupla atividade pois interfere no acesso dos substratos ao sítio ativo (ILLY et al., 1997). Em condições fisiológicas normais, a regulação da Cat B é bem coordenada e rígidamente controlada em vários níveis. Em condições patológicas, a regulação pode ser alterada em mais de um nível, resultando na superprodução, redistribuição e secreção de Cat B (MIJANOVIĆ et al., 2019).

Nos cânceres, a Cat B é encontrada em lisossomos que são perinucleares, bem como em vesículas por todo o citoplasma e na periferia da célula, sua forma ativa é secretada pelas células tumorais (KLOSE et al., 2006; RECKLIES et al., 1980; SAMENI et al., 1995; SLOANE et al., 1994). A secreção de Cat B pode ocorrer através da liberação de vesículas de membrana, microvesículas ou exossomos (CAVANAUGH et al., 1983; GIUSTI et al., 2008). No microambiente tumoral a Cat B está envolvida na proliferação, disseminação, degradação da membrana extracelular, aumento da motilidade, invasão e mediação da angiogênese de variados tipos de cânceres como os carcinomas de mama, melanoma, câncer gástrico, câncer de pulmão, câncer de cólon, câncer de ovário, câncer de colo do útero, câncer de pâncreas, carcinoma de glioblastoma e tireoide, colangiocarcinomas, carcinomas hepatocelulares e câncer de bexiga (TAN et al., 2013).

A estrutura terciária da catepsina B (verde) com destaque para o aminoácido Cys30 (vermelho), His200 (rosa) que juntamente com a asparagina (Asn220) formam o sítio ativo da proteína. Pode ser visualizada também a alça de oclusão Ile105-Thr125 (azul) na Figura 2. A estrutura proteica foi obtida através do RCSB PDB (BERMAN, 2000) código de identificação 1GMY (GREENSPAN et al., 2001).

Figura 2 - Estrutura terciária da Catepsina B humana.



Fonte: Elaborado pelo autor (2022)

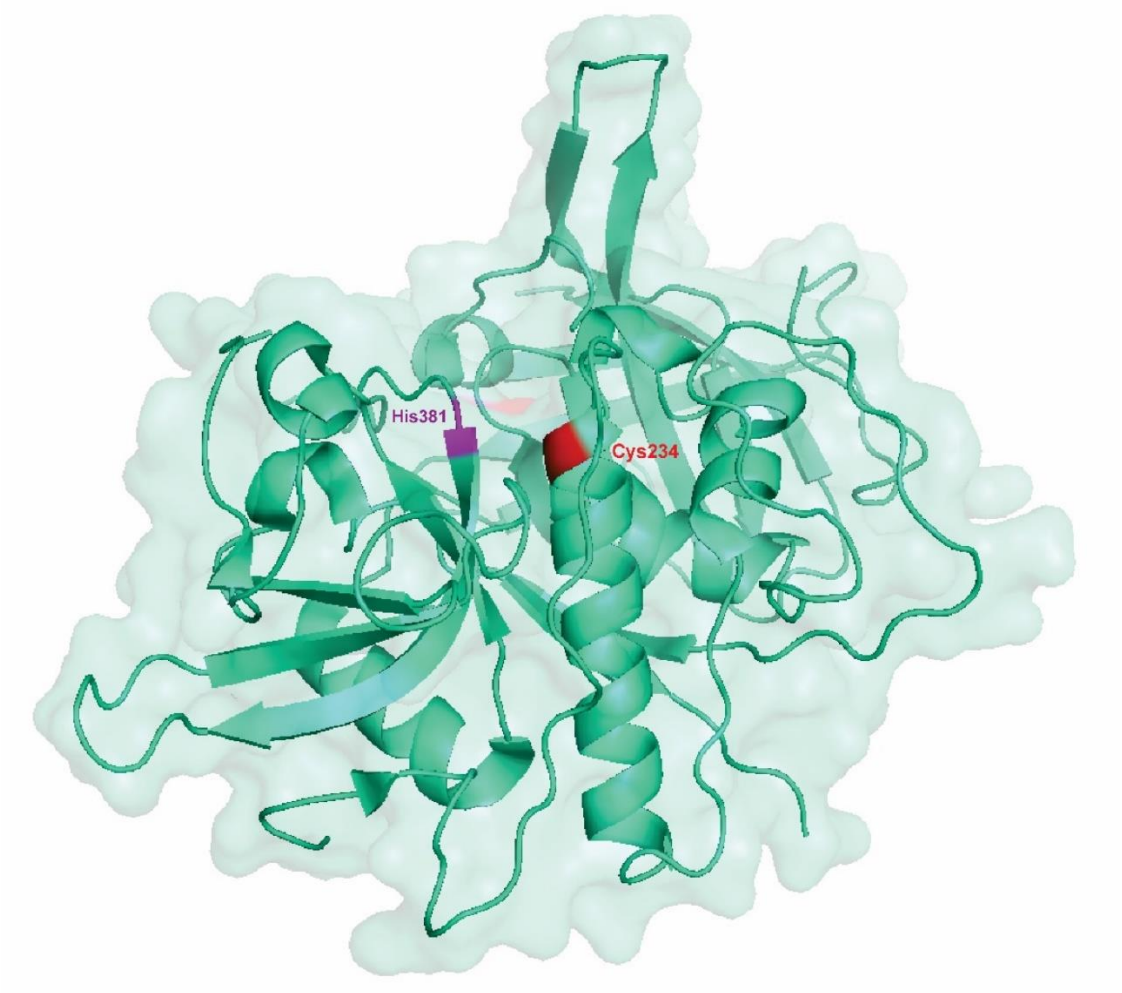
2.3.2 Catepsina C

Cat C (EC 3.4.14.1) também chamada de dipeptidil peptidase I, é sintetizada como um zimogênio proteoliticamente inativo, em seguida, processada em uma série de clivagens proteolíticas para produzir a forma madura da enzima (YANG et al., 2011). Assim como outros membros da família das catepsinas ela é responsável pela degradação de proteínas não específicas no meio ligeiramente ácido do lisossoma. No entanto, experimentos com ratos *knock-out* e estudos genéticos também identificaram papéis específicos para a Cat C nas células do sistema imunológico (TURK et al., 2012). Foi demonstrado que a Cat C ativa serino proteases efetoras no sistema imunológico e sua atividade excessiva tem sido associada a diferentes doenças inflamatórias, como doença pulmonar obstrutiva crônica e fibrose cística (METHOT et al., 2008; PHAM; LEY, 1999). Por outro lado, a atividade insuficiente da Cat C, leva a doenças autoimunes como a síndrome de Papillon-Lefèvre e Haim-Munk, caracterizada por ceratodermia palmoplantar e periodontite severa (HART et al., 2000; NAKANO et al., 2001).

O papel da Cat C no câncer não é claro, embora existam relatos de aumento significativo da sua expressão em adenocarcinomas, carcinogênese pancreática, mamária e escamosa (ALTORJAY et al., 2005; JOYCE; HANAHAN, 2004; RUFFELL et al., 2013). Foi revelado que a carcinogênese escamosa é funcionalmente dependente da expressão de Cat C (RUFFELL et al., 2013).

A estrutura terciária da Catepsina C (verde) com destaque para os aminoácidos da díade catalítica Cys234 (vermelho) e Hys381 (rosa) pode ser visualizada na Figura 3. A estrutura proteica foi obtida através do RCSB PDB (BERMAN, 2000) código de identificação 1K3B (TURK, 2001).

Figura 3 - Estrutura terciária da Catepsina C humana.



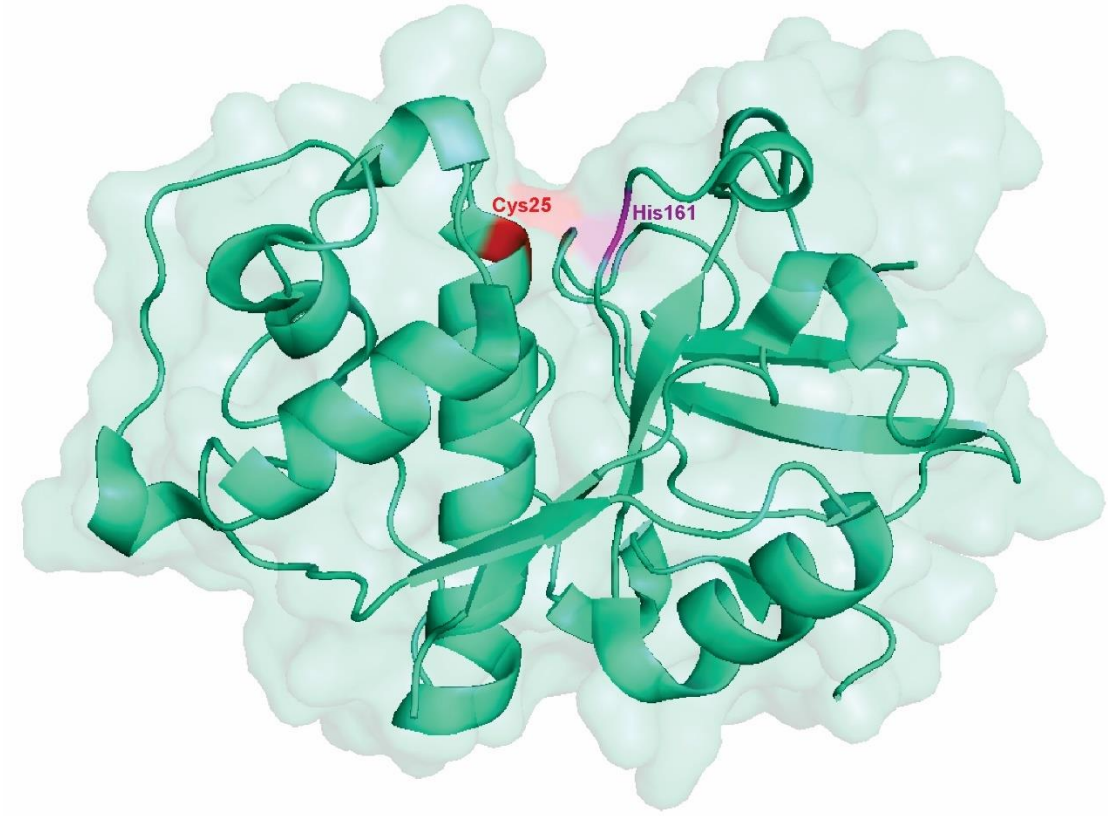
Fonte: Elaborado pelo autor (2022)

2.3.3 Catepsina F

Análises realizadas com *Northern blot* demonstraram que a Cat F (EC 3.4.22.41) é expressa de forma ubíqua em vários tecidos com uma expressão mais elevada no músculo esquelético, testículo, cérebro e fígado. Os transcritos da Cat F também foram encontrados em várias linhagens de células cancerosas, sugerindo que esta enzima poderia estar envolvida em processos degradativos durante a progressão do tumor (SANTAMARÍA et al., 1999). Posteriormente foi descrito que em carcinoma cervical e câncer gástrico a Cat F participa do processo de tumorigênese celular (TAN et al., 2013).

A estrutura terciária da catepsina F (verde) com destaque para os aminoácidos da díade catalítica Cys25 (vermelho) e His161 (rosa) pode ser visualizada na Figura 4. A estrutura proteica foi obtida através do RCSB PDB (BERMAN, 2000) código de identificação 1M6D (SOMOZA; PALMER; HO, 2002).

Figura 4 - Estrutura terciária da Catepsina F humana.



Fonte: Elaborado pelo autor (2022)

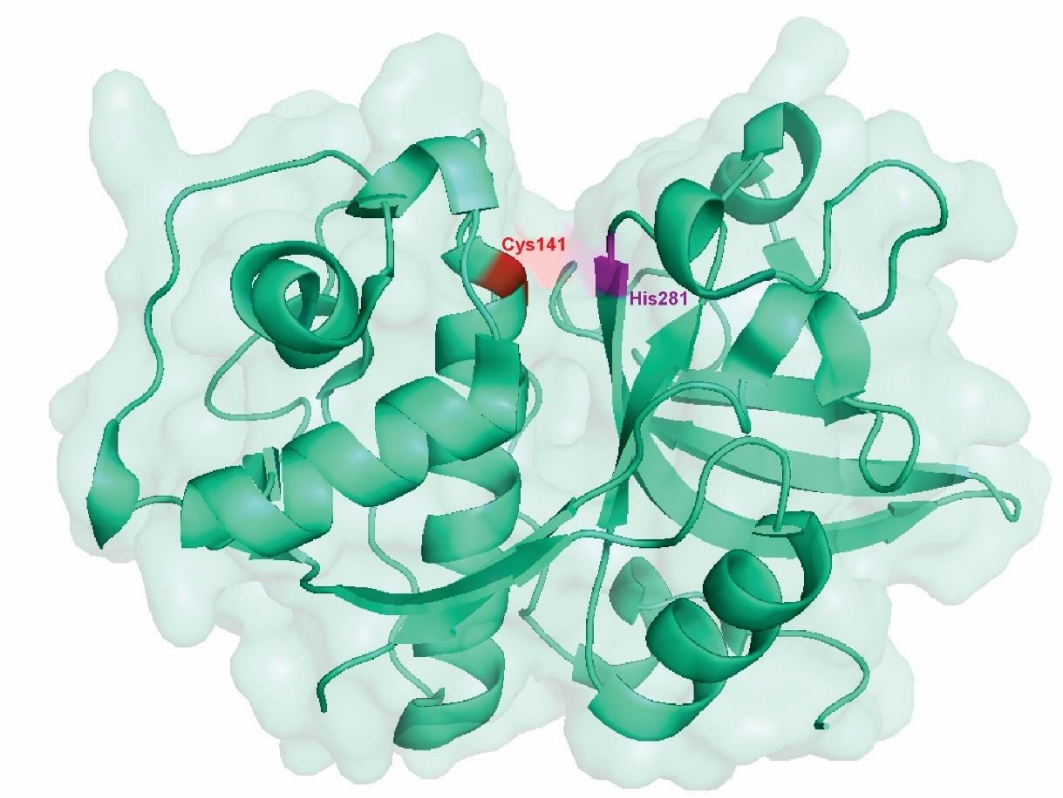
2.3.4 Catepsina H

Conhecida por ser expressa no pulmão adulto, a Cat H (EC 3.4.22.16) processa a proteína surfactante pulmonar (UENO et al., 2004), sendo também expressa ubiquamente em outros tecidos (TURK et al., 2012).

A Cat H está associada a várias condições patológicas, sua capacidade de elevar a motilidade, invasão, proliferação e participar da mediação da angiogênese é fator importante no desenvolvimento de carcinoma de mama, câncer colorretal, melanoma, carcinoma de cabeça e pescoço, glioma e câncer de próstata (TAN et al., 2013). Em pacientes com melanoma a Cat H também demonstrou ser um bom marcador prognóstico. Pacientes com altos níveis séricos de Cat H tem sobrevida global significativamente menor do que os pacientes com baixos níveis dessa proteína (KOS; LAH, 1998).

A estrutura terciária da catepsina H (verde) com destaque para os aminoácidos da díade catalítica Cys141 (vermelho) e His281 (rosa) pode ser visualizada na Figura 5. A estrutura proteica foi obtida através do RCSB PDB (BERMAN, 2000) código de identificação 6CZS (HAO et al., 2018).

Figura 5 - Estrutura terciária da Catepsina H humana.



Fonte: Elaborado pelo autor (2022)

2.3.5 Catepsina K

A Cat K (EC 3.4.22.38) é uma cisteíno protease altamente expressa nos osteoclastos, na maioria das células epiteliais e nos fibroblastos sinoviais das articulações na artrite reumatoide. Entre as enzimas que degradam a matriz, a Cat K é a única enzima para a qual um papel essencial na reabsorção óssea foi documentado em camundongos e seres humanos (TURK et al., 2012). A Cat K ativada é capaz de degradar vários componentes da matriz óssea, incluindo o colágeno tipo I (responsável por aproximadamente 90% da matriz óssea), osteopontina e osteonectina (STOCH; WAGNER, 2008).

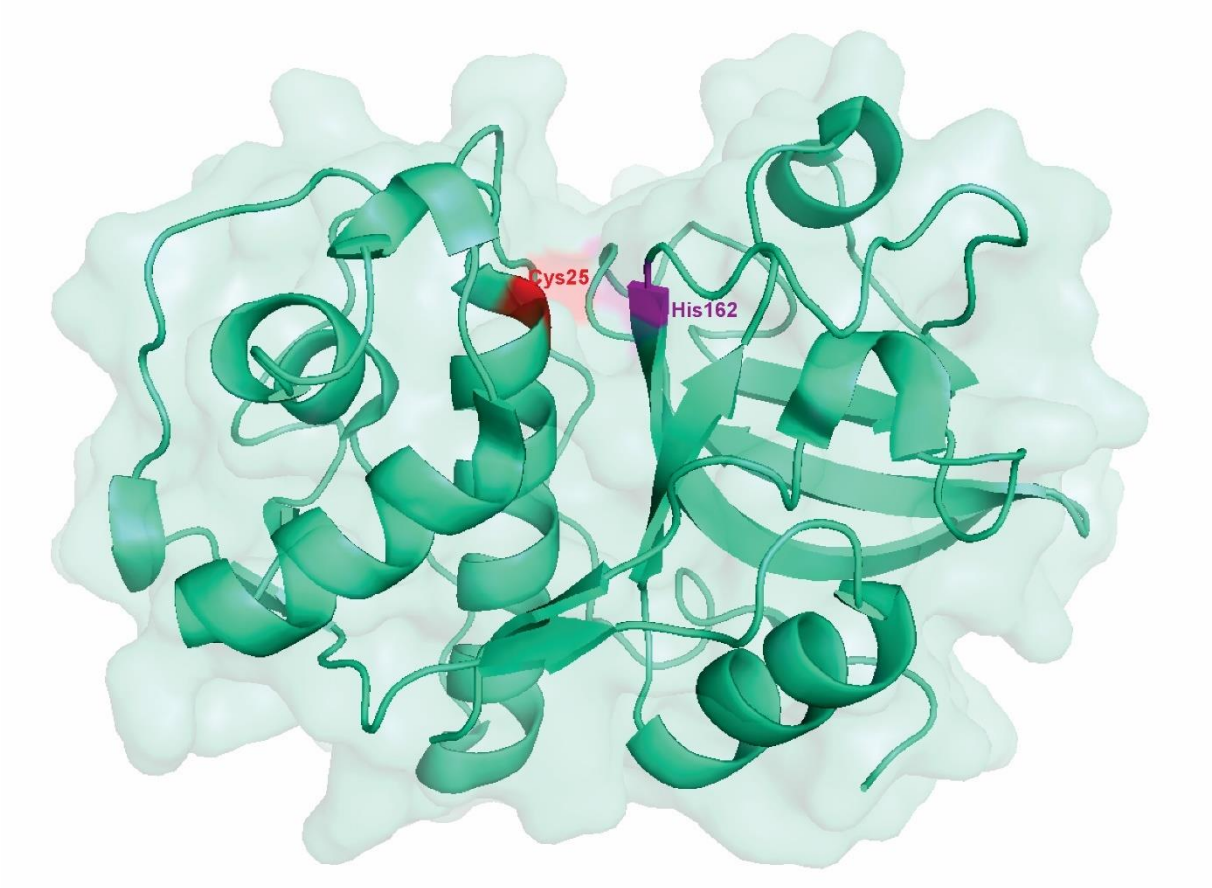
Mutações raras no gene codificador da Cat K levam a picnodisostose, uma doença que causa aumento da massa óssea (CHAVASSIEUX et al., 2008), em contrapartida uma baixa massa óssea juntamente com fragilidade esquelética causa osteoporose, que ocorre devido a um desequilíbrio entre a reabsorção óssea (influenciada pelos osteoclastos) e a formação óssea (influenciada pelos osteoblastos) resultando em perda óssea (GARNERO et al., 1998; PECK, 1993).

Nas últimas décadas, depois da confirmação da relação entre Cat K e doenças relacionadas aos ossos, como a osteoporose, muitos esforços têm sido dedicados ao desenvolvimento de inibidores de Cat K (LU et al., 2018). Enquanto permitem a desmineralização, os inibidores da Cat K suprimem a degradação do colágeno tipo I (a principal matriz orgânica do osso) aumentando a formação óssea (NEGRI, 2008).

A capacidade de degradação da membrana extracelular pela Cat K é fator importante na progressão do câncer gástrico, carcinoma de células escamosas, carcinoma basocelular, tumor de mama, câncer de pulmão, melanomas, tumores de próstata e tumor renal (TAN et al., 2013). Alguns inibidores de Cat K foram aprovados em estudos pré-clínicos e atualmente estão em ensaios clínicos em diferentes estágios de desenvolvimento (NEGRI, 2008).

A estrutura terciária da Catepsina K (verde) com destaque para os aminoácidos da díade catalítica Cys25 (vermelho) e His162 (rosa) pode ser visualizada na Figura 6. A estrutura proteica foi obtida através do RCSB PDB (BERMAN, 2000) código de identificação 2ATO (WEIDAUER et al., 2007).

Figura 6 - Estrutura terciária da Catepsina K humana.



Fonte: Elaborado pelo autor (2022)

2.3.6 Catepsina L

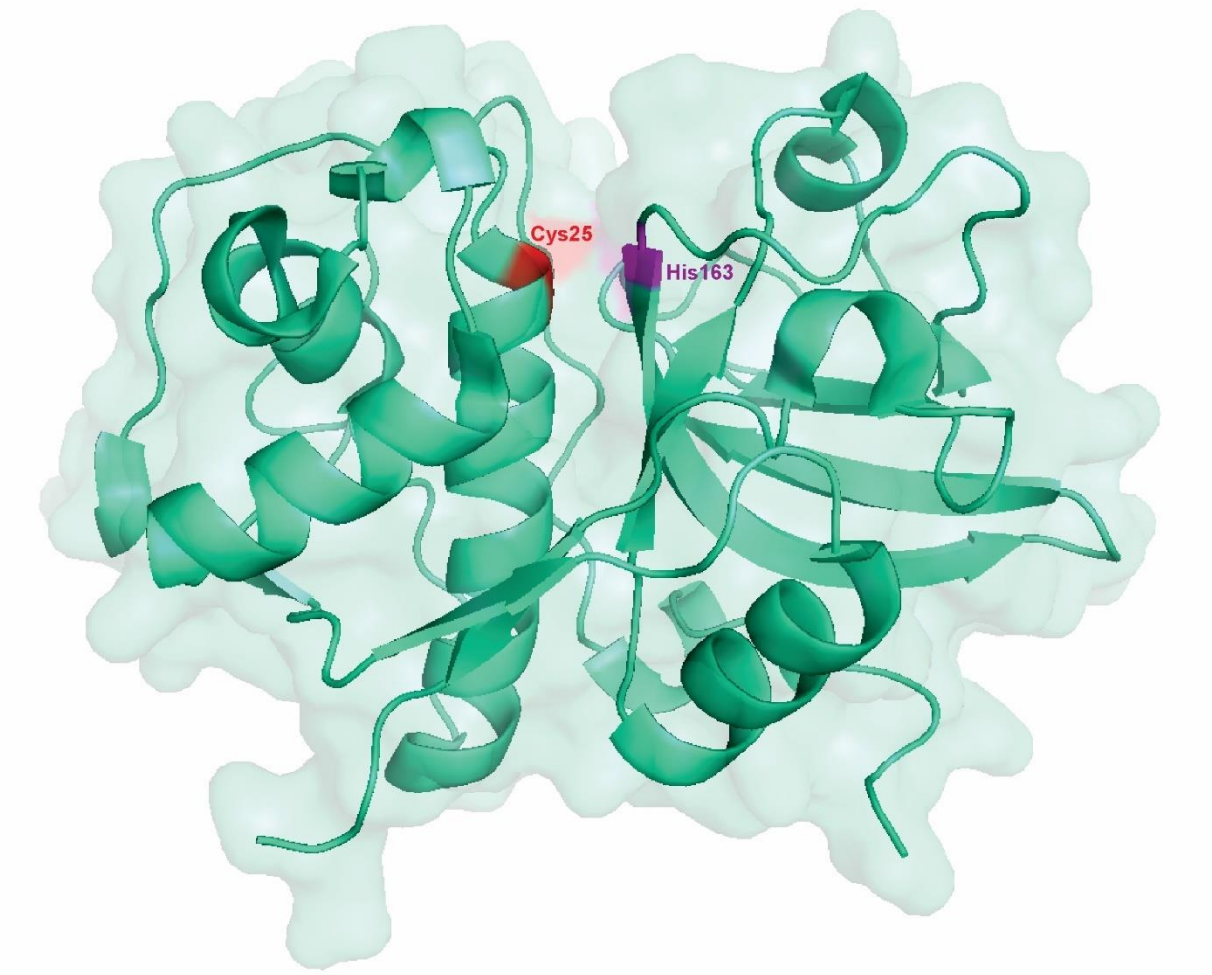
A Cat L (EC 3.4.22.15) é a proteína mais conhecida e estudada dentre as cisteíno proteinases. A Cat L como outras proteinases da família C1, é sintetizada em uma forma inativa que contêm seu pró-peptídeo, posteriormente a remoção do pró-peptídeo a catepsina L pode ser encontrada em sua forma ativa (KIRSCHKE et al., 1981).

Estudos realizados em 2003 por MACABEO-ONG e colaboradores demonstraram que a catepsina L está super expressa nos carcinomas orais e alguns parâmetros como tamanho do tumor e estágio da doença foram correlacionados com a expressão da Cat L (MACABEO-ONG et al., 2003). O envolvimento da Cat L em tumores ósseos como osteosarcoma, mieloma múltiplo e condrossarcoma encontra-se bem fundamentado (LETO et al., 2010). A habilidade de degradação da membrana extracelular é fator importante para o aumento da motilidade e invasão tumoral em variados tipos de cânceres como: câncer de mama, câncer de pulmão, câncer

gástrico, câncer de cólon, carcinomas de cabeça e pescoço, melanomas, gliomas, câncer de ovário, câncer de pâncreas (TAN et al., 2013).

A estrutura terciária da catepsina L (verde) com destaque para os aminoácidos da díade catalítica Cys25 (vermelho) e His163 (rosa) pode ser visualizada na Figura 7. A estrutura proteica foi obtida através do RCSB PDB (BERMAN, 2000) código de identificação 1ICF (GUNČAR et al., 1999).

Figura 7 - Estrutura terciária da catepsina L humana.



Fonte: Elaborado pelo autor (2022)

2.3.7 Catepsina O

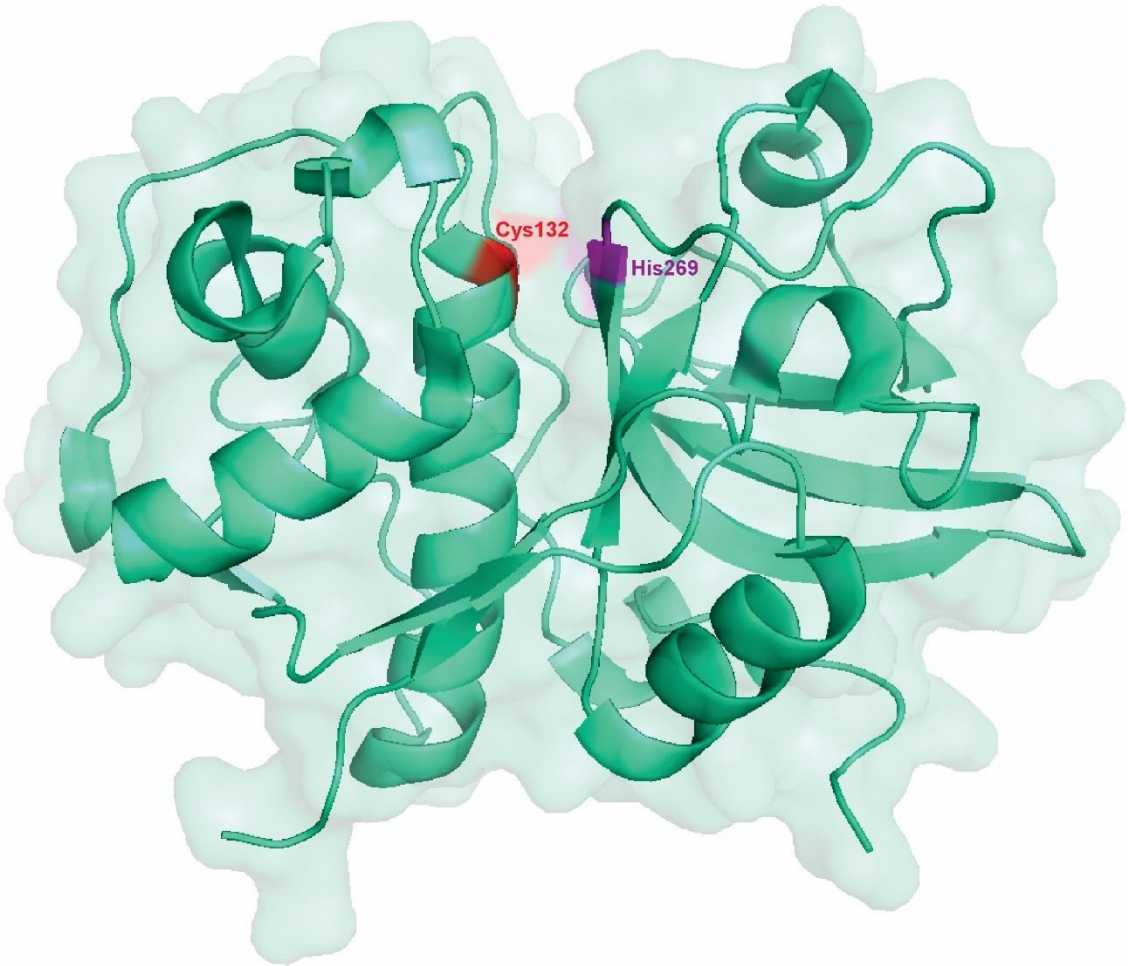
Existem poucos estudos sobre a Cat O (EC 3.4.22.42), esta proteína foi relatada pela primeira vez quando descrita a partir de uma biblioteca de cDNA de câncer de mama humano (VELASCO et al., 1994). Posteriormente foi demonstrado que a Cat O tem atividade de endoprotease contra o fibrinogênio em pH ácido,

indicando que pode desempenhar um papel na degradação da matriz extracelular (SHI et al., 1995).

O papel da Cat O no câncer é pouco claro, estudos apresentaram evidências de que a Cat O possui alguns mecanismos subjacentes que podem dificultar a atuação do medicamento tamoxifeno, utilizado para o tratamento do câncer de mama (CAIRNS et al., 2017).

A estrutura terciária da catepsina O (verde) com destaque para alguns dos aminoácidos da tríade catalítica Cys132 (vermelho) e His269 (rosa) pode ser visualizada na Figura 8. A Cat O ainda apresenta no seu sítio ativo um resíduo de asparagina Asn289 formando assim a tríade catalítica. A estrutura molecular de Cat O foi elaborada com previsão de estrutura baseada em *deep learning* utilizando AlphaFold2 (JUMPER et al., 2021), o resultado apresentado foi considerado de alta confidencialidade.

Figura 8 - Estrutura terciária da Catepsina O humana.



Fonte: Elaborado pelo autor (2022)

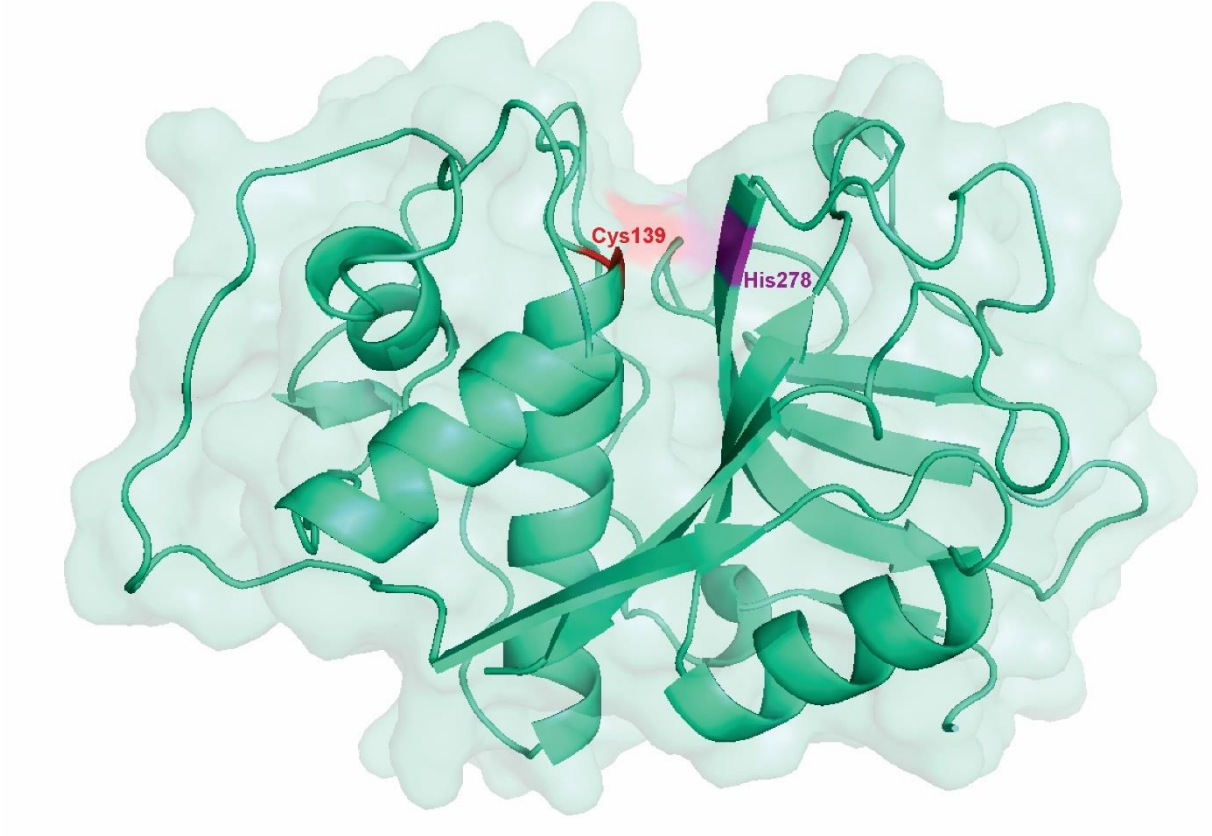
2.3.8 Catepsina S

A catepsina S (EC 3.4.22.27) é uma cisteíno-peptidase lisossomal caracterizada como endopeptidase e sem nenhuma atividade exopeptidase detectada (FUCHS et al., 2020). Esta enzima está envolvida na remodelação e degradação do tecido conjuntivo e da membrana basal. A expressão ou atividade desregulada de Cat S tem sido associada a uma variedade de doenças, incluindo artrite, câncer, doenças cardiovasculares e pulmonares. Alguns fatores podem influenciar na desregulação da expressão e atividade da Cat S como o tabagismo que por sua vez impulsiona a regulação positiva da Cat S nos tecidos pulmonares, elevando a possibilidade de desenvolvimento tumoral (ANDRAULT et al., 2019).

Com relação ao ambiente tumoral, a Cat S é relacionada com o aumento da motilidade, invasão e mediação da angiogênese. Sendo descrito seu papel em carcinoma de células gliais ou astrocitomas, câncer gástrico, carcinoma hepatocelular, glioblastomas, melanoma, câncer de células das ilhotas pancreáticas, carcinoma basocelular, tumor de mama, câncer de pulmão, melanomas, tumores de próstata e tumor renal (TAN et al., 2013).

A estrutura terciária da catepsina S (verde) com destaque para alguns dos aminoácidos da tríade catalítica Cys139 (vermelho) e His278 (rosa) pode ser visualizada na Figura 9. A Cat S ainda apresenta no seu sítio ativo um resíduo de asparagina Asn298 formando assim a tríade catalítica. A estrutura molecular de Cat S foi elaborada com previsão de estrutura baseada em *deep learning* utilizando AlphaFold2 (JUMPER et al., 2021), o resultado apresentado foi considerado de alta confidencialidade.

Figura 9 - Estrutura terciária da Catepsina S humana.



Fonte: Elaborado pelo autor (2022)

2.3.9 Catepsina V

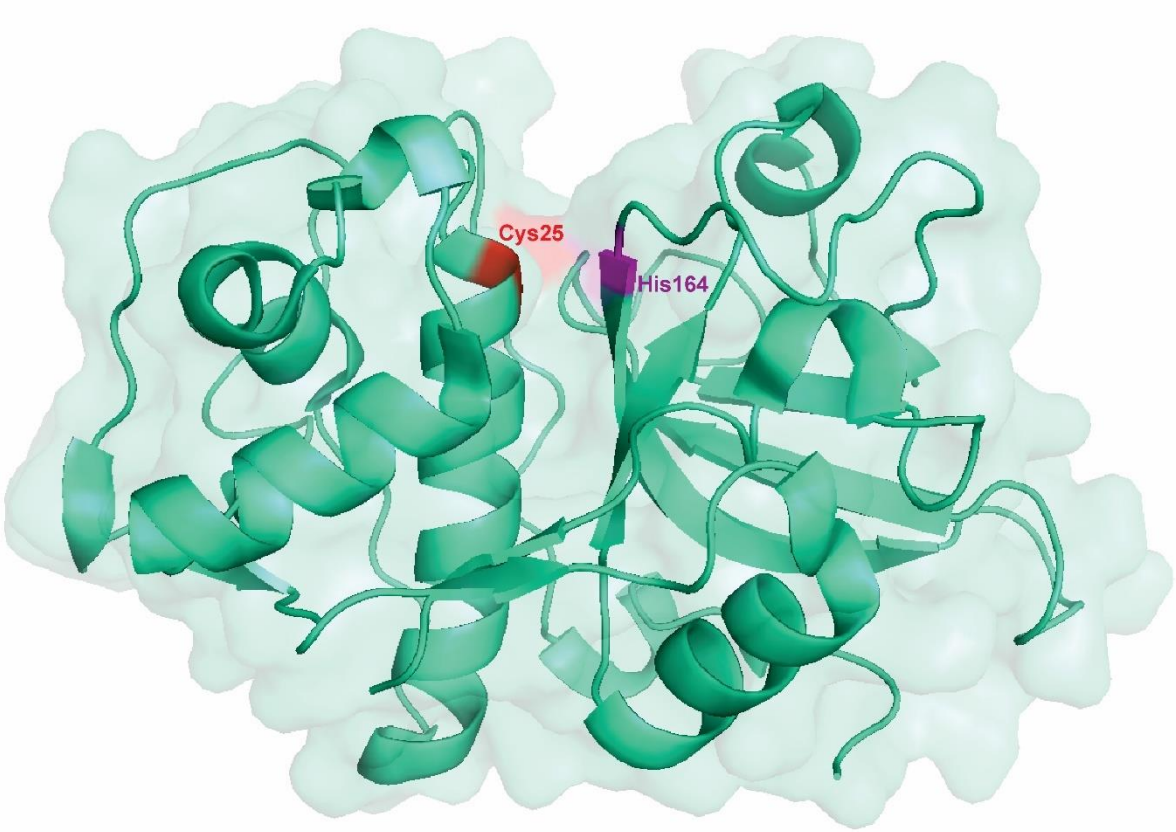
A catepsina V também chamada de catepsina L2 (EC 3.4.22.43) é uma cisteíno protease que foi clonada a partir de bibliotecas de cDNA derivadas do epitélio da córnea, timo e testículo, por três grupos de pesquisa independentes (ADACHI et al., 1998; BRÖMME et al., 1999; SANTAMARÍA et al., 1998).

A elevação dos níveis de expressão de Cat V está fortemente correlacionada com diversas malignidades como o câncer de cólon (GORETZKI et al., 1992; KOBAYASHI et al., 1991) adenocarcinoma renal, carcinoma de ovário e endométrio (SANTAMARÍA et al., 1998) carcinoma hepatocelular e câncer de mama (JING et al., 2018; SANTAMARÍA et al., 1998; SUN et al., 2016; TOSS et al., 2020) câncer de pulmão, tireoide e timo (TEDELIND et al., 2010; TOLOSA et al., 2003; WILDER et al., 2011) alguns estudos apontam ainda um papel da Cat V na desregulação da apoptose celular (FEHRENBACHER et al., 2008). Baseado na literatura a Cat V pode ser um

biomarcador relevante para cânceres específicos, sendo uma promissora proteína a ser investigada (LECAILLE; KALETA; BRÖMME, 2002).

A estrutura terciária da catepsina V (verde) com destaque para alguns dos aminoácidos da tríade catalítica Cys25 (vermelho) e His164 (rosa) pode ser visualizada na Figura 10. A Cat V ainda apresenta no seu sítio ativo um resíduo de asparagina Asn188 formando assim a tríade catalítica. A estrutura proteica foi obtida através do RCSB PDB (BERMAN, 2000) código de identificação 1FH0 (SOMOZA et al., 2000).

Figura 10 - Estrutura terciária da Catepsina V humana.



Fonte: Elaborado pelo autor (2022)

2.3.10 Catepsina X

A catepsina X (EC 3.4.18.1), também conhecida como catepsina Z, é uma cisteino-carboxipeptidase ativada por endopeptidades lisossomais (KOS et al., 2015). Cat X é expressa em células do sistema imune, bem como linfócitos T, monócitos, macrófagos e até mesmo células dentríticas, justamente por regular a maturação destas células no início da imunidade adaptativa. Além disso, apresenta função de fagocitose, maturação, proliferação, migração, adesão e transdução de sinal das células imune (DOLENC et al., 2021; KOS et al., 2015).

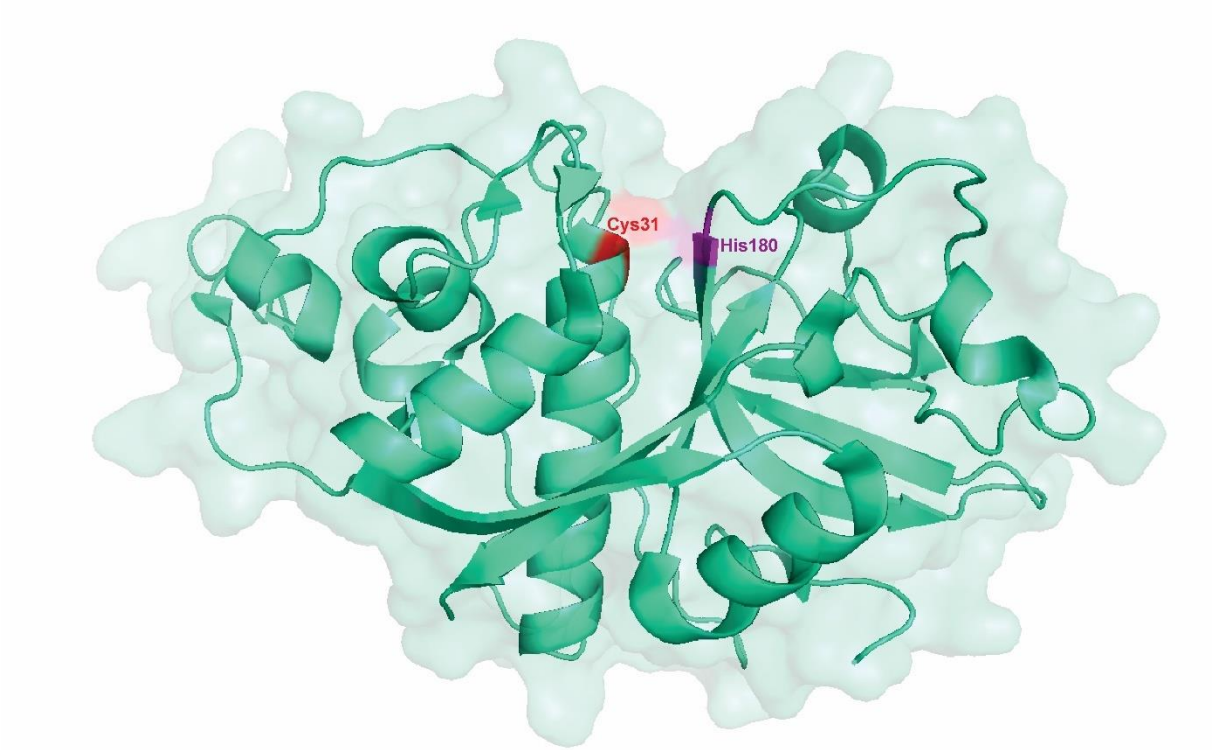
Estudos mostraram que em células tumorais deficientes em catepsina B a catepsina X substitui a cat B na superfície das células. Sondas baseadas em atividade de catepsinas cisteínicas marcaram a catepsina B na superfície destas células tumorais, e foram capazes de mostrar tal processo (VASILJEVA et al., 2006).

A catepsina X tem papel na proliferação e mediação da transição epitélio-mesenquimal em células tumorais, tendo níveis de expressão elevada em melanomas malignos, tumores de pulmão, câncer de mama, câncer colorretal, especialmente no câncer de próstata (TAN et al., 2013). No entanto, também são encontrados níveis expressos em doenças inflamatórias e distúrbios neurodegenerativos relacionados à inflamação (DOLENC et al., 2021; KOS et al., 2015).

Cat X possui baixo referêncial teórico a respeito do câncer e seu papel em processos malignos ainda não é claro. Entretanto, quando se trata de câncer colorretal, Cat X pode se apresentar como excelente indicador de prognóstico para determinar a sobrevida do paciente (VIZIN et al., 2012).

A estrutura terciária da catepsina X (verde) com destaque para alguns aminoácidos da tríade catalítica Cys31 (vermelho) e His180 (rosa) pode ser visualizada na Figura 11. A Cat X ainda apresenta no seu sítio ativo um resíduo de asparagina Asn200 formando assim a tríade catalítica. A estrutura proteica foi obtida através do RCSB PDB (BERMAN, 2000) código de identificação 1EF7 (GUNČAR et al., 2000).

Figura 11 - Estrutura terciária da Catepsina X humana.



Fonte: Elaborado pelo autor (2022)

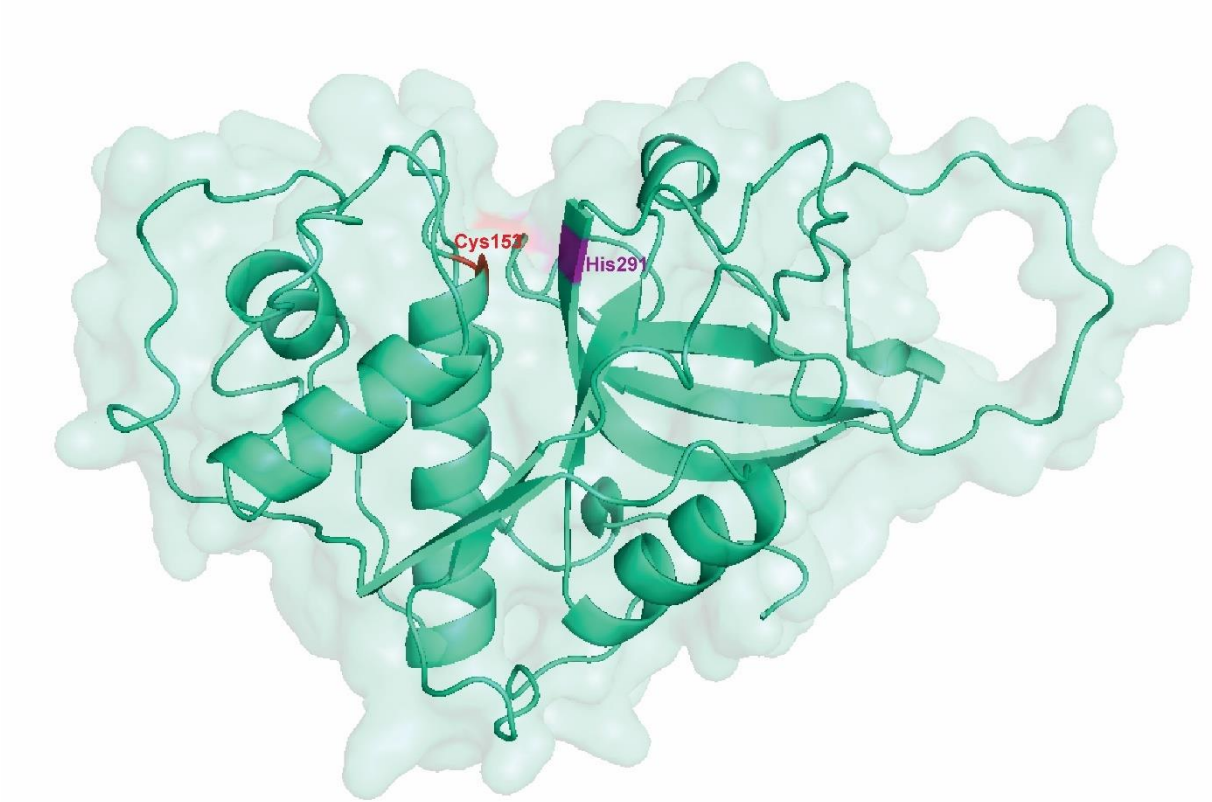
2.3.11 Catepsina W

A catepsina W (EC 3.4.22.1) também denominada linfopaína, foi identificada por Linnevers e colaboradores, em 1997 (LINNEVERS; SMEEKENS; BROMME, 1997) e foi classificada como membro da família da papaína. Cat W é expressa nas células exterminadoras naturais ou células NK (do inglês *Natural Killer*) e em células T citotóxicas (CTL), mais especificamente nos linfócitos T CD8+ (ONDR; PHAM, 2004; VIVIER et al., 2008; WEX et al., 2003).

Cat W tem suas funções e propriedades pouco estudadas, porém sugere-se que ela possui papel no processo citotóxico produzido pelas células NK e CTL (WEX et al., 2001, 2003). A estrutura terciária da Catepsina W (verde) com destaque para para alguns aminoácidos da tríade catalítica Cys153 (vermelho) e His291 (rosa) pode ser visualizada na Figura 12. A Cat X ainda apresenta no seu sítio ativo um resíduo de asparagina Asn331 formando assim a tríade catalítica. A estrutura molecular de

Cat W foi elaborada com previsão de estrutura baseada em *deep learning* utilizando AlphaFold2 (JUMPER et al., 2021), o resultado apresentado foi considerado de alta confidencialidade.

Figura 12 - Estrutura terciária da Catepsina W humana



Fonte: Elaborado pelo autor (2022)

2.4 Cistatinas

A regulação da atividade das catepsinas ocorre por meio de inibidores de proteases, um grupo específico de moléculas que têm a propriedade de reduzir a atividade catalítica destas enzimas proteolíticas. As cistatinas são proteínas que inibem especificamente a atividade da papaína e das proteases cisteínicas relacionadas. Cistatinas são inibidores competitivos que interagindo com o sítio ativo da enzima-alvo, bloqueiam o acesso ao substrato. A afinidade com as proteases-alvo é extremamente alta, tornando difícil a reversão da inibição (BARRETT, 1987). Descrita pela primeira vez em clara de ovo e posteriormente denominada como cistatina de clara de ovo de galinha, as cistatinas foram caracterizadas também em microrganismos, espécies animais e vegetais (COLELLA et al., 1989).

Verificou-se que as cistatinas possuem homólogos em diferentes animais pluricelulares, dessa maneira foi denominada a “superfamília das cistatinas" (BARRETT, 1987). A classificação nas famílias é baseada em similaridades de sequência primária, massas moleculares, número de ligações dissulfeto e localização subcelular (ABRAHAMSON; ALVAREZ-FERNANDEZ; NATHANSON, 2003; BENCHABANE et al., 2010; TURK; TURK, 2008).

A família 1 (estefinas) é composta por dois membros (estefina A e B) os quais são encontrados no citosol, possuem uma massa molecular de aproximadamente 11 kDa, com apenas um domínio inibitório e sem pontes dissulfeto. Promovem ação inibitória nas catepsinas liberadas pelos lisossomos (ABRAHAMSON; ALVAREZ-FERNANDEZ; NATHANSON, 2003; BENCHABANE et al., 2010; TURK; TURK, 2008).

A família 2 cistatinas propriamente ditas, também apresentam um domínio inibitório único e sua atuação é extracelular e/ou transcelular, possuem massa molecular de ~15 kDa com quatro resíduos cisteínicos formando pontes dissulfeto. Nesta família está a mais bem estudada cistatina, a cistatina C humana descrita pela primeira vez em 1961 como constituinte da urina de pacientes com falhas renais e que, atualmente, seus níveis são utilizados como marcador endógeno da função renal (ODDEN et al., 2010; TURK; TURK, 2008).

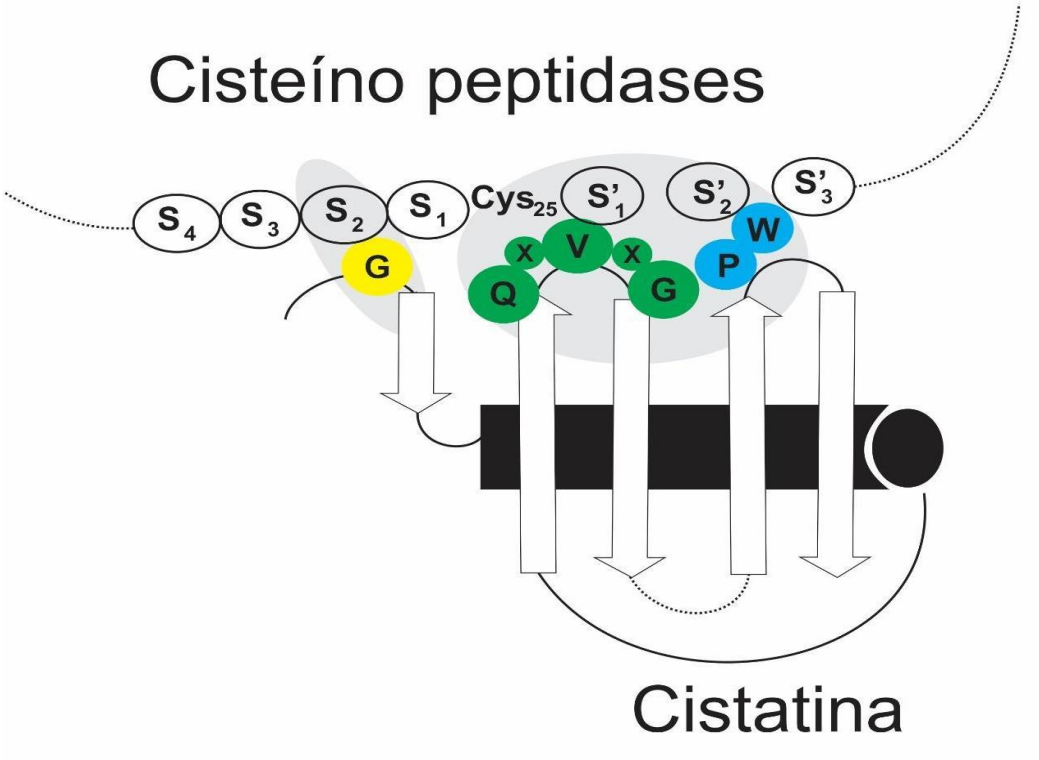
A família 3 (cininogênios) pode também ser denominada de cistatinas intravasculares visto que seus representantes são encontrados no plasma sanguíneo e em secreções de mamíferos. Esta família mostra uma organização estrutural mais complexa, possuem domínios inibitórios múltiplos, possivelmente resultantes de eventos de duplicação gênica, que originam proteínas de altas massas moleculares (60-120kDa). Esse grupo atua como fator de conversão e/ou ativação nos processos de coagulação sanguínea (ABRAHAMSON; ALVAREZ-FERNANDEZ; NATHANSON, 2003; BENCHABANE et al., 2010; TURK; TURK, 2008).

A família 4 (fitocistatinas) se caracteriza por apresentar uma sequência conservada Leu-Ala-Arg-Ala-Val (LARFAV). A presença da sequência LARFAV é exclusiva das cistatinas de plantas e é usada para identificar fitocistatinas em particular (VALUEVA; MOSOLOV, 2004). As fitocistatinas possuem uma atividade inibitória bifuncional contra papaína e legumaínas. Fitocistatinas têm sido identificadas em diversas espécies vegetais, tanto em monocotiledôneas quanto em dicotiledôneas e constituem uma família formada por mais de 80 membros oriundos de um mesmo ramo evolutivo (HABIB; FAZILI, 2007).

O mecanismo de interação entre as cistatinas e suas proteases alvo, é caracterizado especificamente pela presença de domínios altamente conservados: (1) um resíduo de glicina (G) na região amino-terminal; (2) o motivo QxVxG; e (3) um triptofano (W) na região carboxi-terminal, sendo que, estes dois últimos motivos estruturais localizam-se no primeiro e segundo *loops* entre as folhas-β da estrutura terciária. Estes motivos proteicos são os principais responsáveis pela atividade inibitória do sítio ativo das enzimas-alvo, as proteases cisteínicas. Sendo assim, a interação desses inibidores com a protease alvo deve seguir o modelo proposto para a já citada anteriormente cistatina do ovo de galinha (ABE et al., 1988; BODE et al., 1988)

A interação entre cistatina e cisteíno peptidase alvo pode ser visualizada na Figura 13, em destaque as regiões envolvidas na inibição: em amarelo o segmento N-terminal, contendo o resíduo de glicina conservado interagindo com a peptidase no subsítio S2; em verde a alça central, formada pelo motivo Q-X-V-X-G interagindo com no subsítio S'1 e em azul o domínio C-Terminal, contendo o resíduo conservado de triptofano interagindo com a peptidase no subsítio S'2.

Figura 13 - Demonstração esquemática do mecanismo de interação entre uma cistatina e sua cisteíno peptidase alvo.



Fonte: Adaptado de ALBAREZ-FERNANDEZ; ABRAHAMSON, M., (2006)

2.5 Estudos com as cistatinas de carrapatos

Os carrapatos têm sucesso na sua atividade de parasitismo pois conseguiram se adaptar ao longo do tempo, sendo capazes de realizar a modulação do sistema imune do hospedeiro, capacidade essa adquirida durante a co-evolução parasita-hospedeiro (RECH, 2007). O entendimento sobre as funções e mecanismos de ação dos diferentes tipos de cistatinas tem sido possível, em grande parte, graças aos experimentos de clonagem e expressão heteróloga destes inibidores de proteases provenientes de diferentes espécies. Diversos trabalhos foram realizados ao redor do mundo com foco na clonagem e caracterização de cistatinas de carrapatos, obtendo as proteínas recombinantes purificadas e funcionais.

Algumas espécies de carrapatos apresentaram mais de uma cistatina como os carrapatos da espécie *Rhipicephalus microplus,* no qual já foram identificadas diferentes cistatinas: BrBmcys2a, BrBmcys2b, BrBmcys2c, BrBmcys2d, BrBmcys2e (PARIZI et al., 2013), Bmcystatin (LIMA; SASAKI; TANAKA, 2006), Rmcystatin-4 (CARDOSO et al., 2017) e Rmcystatin-1b (LU et al., 2020). A cistatina alvo desse estudo é proveniente da espécie *Rhipicephalus haemaphysaloides, e* foi descrita e caracterizada por um grupo chines, o qual nomeou essa cistatina como RHcyst-1 e a classificou como membro do grupo 1 das cistatinas (WANG et al., 2015). Outras espécies de carrapatos também já tiveram suas cistatinas estudadas, conforme descrito na Tabela 3.

Tabela 3 - Cistatinas de carrapatos descritas na literatura

Espécie	Referência
Ixodes scapularis	(KOTSYFAKIS et al., 2007; VALENZUELA et al., 2002)
Amblyomma variegatum	(NENE et al., 2002; RIBEIRO et al., 2011)
Rhipicephalus appendiculatus	(NENE et al., 2004; PARIZI et al., 2020)
Ixodes pacificus	(FRANCISCHETTI et al., 2005)
Dermacentor andersoni	(ALARCON-CHAIDEZ; SUN; WIKEL, 2007)
Ixodes ricinus	(CHMELAŘ et al., 2008; COTTÉ et al., 2014; LIU et al., 2014; SCHWARZ et al., 2013)
Ornithodorus parkeri	(FRANCISCHETTI et al., 2008)
Ornithodoros coriaceus	(FRANCISCHETTI et al., 2008)
Argas monolakensis	(MANS et al., 2008)

Amblyomma cajennense	(BATISTA et al., 2008)
Amblyomma americanum	(ALJAMALI et al., 2009; RADULOVIĆ et al., 2014)
Rhipicephalus sanguineus	(ANATRIELLO et al., 2010; OLIVEIRA et al., 2013)
Ornithodoros moubata	(SALÁT et al., 2010)
Amblyomma maculatum	(KARIM; SINGH; RIBEIRO, 2011)
Hyaloma marginatum rufipes	(FRANCISCHETTI et al., 2011)
Ornithodoros moubata	(DÍAZ-MARTÍN et al., 2013)
Dermacentor andersoni	(MUDENDA et al., 2014)
Amblyomma triste	(GARCIA et al., 2014)
Amblyomma parvum	(GARCIA et al., 2014)
Amblyomma cajennense	(GARCIA et al., 2014)
Haemaphysalis longicornis	(TIRLONI et al., 2015)
Rhipicephalus pulchellus	(TAN et al., 2015)
Haemaphysalis flava	(XU et al., 2015)
Amblyomma sculptum	(ESTEVES et al., 2017)
Rhipicephalus zambeziensis	(DE CASTRO et al., 2017)
Hyalomma excavatum	(RIBEIRO; SLOVÁK; FRANCISCHETTI, 2017)
Rhipicephalus bursa	(ANTUNES et al., 2018)
Ixodes holocyclus	(RODRIGUEZ-VALLE et al., 2018)
Hyalomma dromedarii	(BENSAOUD et al., 2018)
Amblyomma aureolatum	(MARTINS et al., 2019)
Ornithodoros rostratus	(ARAUJO et al., 2019)
Ixodes ricinus	(KOTÁL et al., 2021)

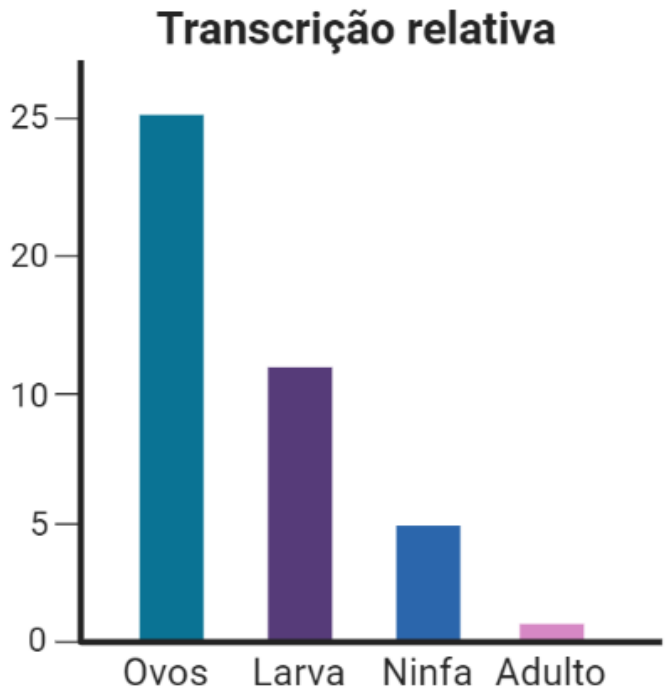
Fonte: Elaborado pelo autor (2022)

Estudos envolvendo variadas espécies de carrapatos mostraram que estes inibidores de protease estão envolvidos em processos fisiológicos naturais destes parasitas, por exemplo, no sistema imunológico dos artrópodes através da mediação da coagulação e produção de peptídeos antimicrobianos (BLISNICK; FOULON; BONNET, 2017).

2.6 RHcyst-1

Rhipicephalus haemaphysaloides é uma espécie de carrapato encontrada na China e no sul da Ásia, é responsável pela transmissão de algumas doenças como a babesiose e doença da floresta de Kyasanur (ZHOU et al., 2006). RHcyst-1 é uma proteína caracterizada e classificada como uma cistatina do tipo I, a qual foi identificada a partir de fêmeas dessa espécie. Acredita-se que este inibidor tenha papel no desenvolvimento embrionário dos carrapatos por ser mais expresso nos ovos destes parasitas Figura 14 (WANG et al., 2015). RHcyst-1 exibe atividade inibitória significante contra as cisteíno proteases incluindo a Cat L e Cat B e possui atividade antitumoral comprovada *in vitro* e em modelos murinos *in vivo* (WEI et al., 2018). Verificou-se que RHcyst-1 pode inibir significativamente a proliferação, migração e invasão das linhagens celulares de câncer cervical humano (HeLa), pulmão (A549), ovariano (SKOV-3) e melanoma murino (B16-F10) *in vitro,* além disso, inibiu o crescimento do tumor de células B16-F10 e melhorou a sobrevivência *in vivo* (WEI et al., 2018).

Figura 14 - Expressão gênica da RHcyst-1 em diferentes estágios do desenvolvimento de *R. haemaphysaloides.*



Fonte: Adaptado de WANG et al., (2015).

Estudos como os realizados com a Rhcyst-1 indicam que moléculas bioativas derivadas de carrapatos têm potencial aplicação na terapia do câncer, além disso podem ter baixa toxicidade e imunogenicidade em humanos (SIMONS et al., 2011). Nosso interesse no potencial bioativo da Rhcyst-1 é na sua capacidade (já comprovada), mais especificamente de peptídeos derivados dela, de inibir as catepsinas B e L que são liberadas pelas células cancerosas no ambiente tumoral e atuam na degradação da matriz extracelular contribuindo para a evolução de metastases. O papel das cistatinas no câncer e como imunossupressor pode ser bem mais complexo do que isso e vem sendo estudado (WEI et al., 2019).

Neste trabalho nós primeiramente modelamos e apresentamos a estrutura tridimensional da RHcyst-1 utilizando AlphaFold2, e realizamos estudos *in silico* da interação entre RHCyst-1 com duas das principais catepsinas humanas envolvidas no processo tumoral e metástase (Cat B e Cat L). Posteriormente, desenhamos e sintetizamos peptídeos baseados nas regiões inibitórias da RHcyst-1, esses peptídeos foram caracterizados como inibidores de catepsinas L e B. Inibidores com tamanho reduzido têm como vantagem a capacidade de síntese química, e menor potencial de gerar uma resposta imune.

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4 OBJETIVOS

5.1 Objetivo Geral

Projetar peptídeos a partir da estrutura terciária da cistatina Rhcyst-1 e avaliar o potencial inibitório de catepsinas humanas envolvidas em progressão tumoral e metástase.

5.2 Objetivos Específicos

- Modelar tridimensionalmente a estrutura da cistatina Rhcyst-1.
- Realizar estudos *in silico* de interação entre a cistatina Rhcyst-1 e catepsinas humanas envolvidas no ambiente tumoral e na metástase.
- Propor a sequência e modelar tridimensionalmente a estrutura de um peptídeo inibidor de catepsinas derivado da cistatina Rhcyst-1.
- Avaliar *in silico* a interação e o potencial inibitório de catepsinas do peptídeo derivado da RHcyst-1.
- Realizar uma confirmação *in vitro* dos resultados obtidos *in silico*.

5 ARTIGO CIENTÍFICO

Periódico: Process Biochemistry

Título: **Design of a new peptide derived from the *Rhipicephalus haemaphysaloides* Cystatin (RHcyst-1) with inhibitory potential against human cathepsins L and B involved in cancer invasion**

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Projeto desenvolvido é derivado do projeto derivado de um projeto maior, intitulado “Ectoparasitos como fonte de novas moléculas com potencial farmacológico: agentes anticoagulantes e antitumorais” e registrado na compesq sob o número 042/2017.

Declaramos a não existência de conflitos de interesse.

Design of a new peptide derived from the *Rhipicephalus haemaphysaloides* Cystatin (RHcyst-1) with inhibitory potential against human cathepsins L and B involved in cancer invasion

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Abstract

Cysteine cathepsins are enzymes involved in several physiological processes and its role in various diseases including cancers. These enzymes have their protein activity naturally controlled by protein inhibitors called cystatins. These inhibitors can be obtained from vegetal and animal sources and have already demonstrated antitumor activity. In this way, considering that cathepsins are proteases involved in several diseases, exhibiting varied characteristics and functions, our purpose is to study the cathepsins as therapeutic targets for the development of new drugs, based on natural inhibitors such as cystatins. So, in this study we investigate the *Rhipicephalus haemaphysaloides* tick inhibitor RHcyst-1, which was previously characterized and classified as a type I cystatin. RHcyst-1 exhibits *in vitro* inhibitory activity against cysteine proteases including cathepsin L, cathepsin B, cathepsin C, and cathepsin S. The peptides were designed and chemically synthesized, and its inhibitory activity against cathepsin L and B was identified by fluorogenic substrate analysis. *In silico* analyzes were performed to predict the ability to generate immune and allergic responses. The present research demonstrates that the peptides (Rhcyst-1pep and Rhcyst-1pep2) resulting from the RHcyst-1 inhibitory regions are effective inhibitors of cathepsin L and cathepsin B enzymes. We hypothesized that the peptides, even used in higher concentration, could play a role like RHcys-1 in the tumor microenvironment and exhibit potential antitumor and antimetastatic efficacy. In future studies we will investigate the potential of these peptides as antitumoral drugs.

Key words: Cystatin; metastases, antitumor, cathepsins, protease inhibitors, cancer.

1. Introduction

According to the World Health Organization (WHO), cancer is a leading cause of death worldwide, accounting for nearly 10 million deaths in 2020 (FERLAY et al., 2021). Cancer is characterized as a large group of diseases caused by the uncontrolled growth of transformed cells, which can start in any organ or tissue of the body with the possibility of invading adjacent parts and / or spreading to other organs (LÚCIA DE ALMEIDA et al., 2005). This process is called metastasis and is a major cause of cancer-caused death (FERLAY et al., 2021). Metastasis is a complex, multi-step process that involves genetic and epigenetic changes in tumor cells. In addition, cancer develops within a complex tissue microenvironment, which also regulates the invasion, migration, and metastasis of tumor cells (ZHANG et al., 2019). The microenvironment of the tumor invasion can allow cancer cells to reach an abnormally high motility capacity and penetrate the surrounding stroma. It is noteworthy that the tumor invasion front is particularly rich in a variety of proteases, which facilitates cancer invasion and metastasis through remodeling of the extracellular matrix and promote cell migration (YANG et al., 2017).

Cathepsins are proteases that are involved in several types of cancer, exhibiting varied characteristics and functions (WEI et al., 2018). In humans, cysteine cathepsins, a lysosomal protease group, comprise 11 members: Cathepsin B (CtsB), C (CtsC), F (CtsF), H (CtsH), K (CtsK), L (CtsL), O (CtsO), S (CtsS), V (CtsV, also known as L2), W (CtsW), and Z (CtsZ, also called X) (ROSSI et al., 2004). These cathepsins have been associated with cancer proliferation, survival, and metastasis, thus contributing to tumor progression in various types of cancer through different mechanisms (OLSON; JOYCE, 2015). In normal cells, only a small amount of cathepsins is secreted (TANDON et al., 1990) and under normal physiological conditions, these are sequestered in lysosomes. However, in tumor cells, it is already well known that changes in expression levels and trafficking pathways result in the secretion of these proteinases in the extracellular environment (DENHARDT et al., 1987). Inhibitors that block the activity of cathepsins or decrease their levels in the peripheral environment, called cystatins, have been investigated once they can prevent the progression of the tumor and metastasis. Cystatins are an evolutionary related family of reversible and tightly bind inhibitors of lysosomal cathepsins belonging to the C1 family (JAKOŠ et al., 2019). The main role of cystatins is to limit the excessive activity of cysteine endopeptidases. Thus, the combination of cathepsin inhibitors with chemotherapy and radiation for cancer treatment has been reported

(NALLA et al., 2010; SUDHAN; SIEMANN, 2015b). Recently, researchers have reported that selective CatK inhibitor can prevent the establishment and progression of bone-invading metastatic prostate cancer, thus becoming a new therapeutic approach for advanced prostate cancer (LIANG et al., 2019).

Some species of ticks presented one or more isoforms of cystatins such as ticks of the species *Rhipicephalus microplus*, source of at least five different cystatins (PARIZI et al., 2013). Ticks are obligatory blood-sucking ectoparasites of wild and domestic animals, as well as humans. They are second only to mosquitoes as global vectors of human diseases (ZHANG et al., 2018). Due to the long and complex evolutionary process generated by parasitism, these hematophagous represent an important source of new bioactive molecules, such as cystatins. Such molecules have been explored for several applications such as anticoagulant, immunosuppressive, antiangiogenic, and antitumor potential (PARIZI et al., 2013; WEI et al., 2018).

The antitumor activity of some cystatins obtained from ticks has already been proven. RHcyst-1, for example, is a cystatin obtained from *Rhipicephalus haemaphysaloides* ticks, which showed significant inhibition of tumor cell proliferation, migration, and invasion (WEI et al., 2018). *In vitro*, RHcyst-1 was shown to have positive inhibition results in human cervical cancer (HeLa), lung (A549), ovarian (SKOV-3), and melanoma cells. Furthermore, when tested *in vivo*, in a murine model, it inhibited tumor growth and improved the animals' survival. The RHcyst-1 has potential antitumor activity that may be associated with changes in cathepsin expression, which can be considered a potential application in the treatment of cancer (WEI et al., 2018). The aim of this study was to design and evaluate a synthetic peptide derived from the inhibitory regions of RHcyst-1 considering the possibility to use this molecule as a biopharmaceutical.

2. Materials & Methods

2.1 Molecular modeling of the RHcyst-1

The molecular structure of RHcyst-1 was elaborated with deep learning-based structure prediction with AlphaFold2 (JUMPER et al., 2021). The selected model was evaluated with dedicated tools provided by the software, as well as visually, in order to detect possible structural changes, using the PyMOL 2.4 software (SCHRÖDINGER LLC, 2015). The RHcyst-1 sequence was obtained from MEROPS (MERNUM MER0595513) (RAWLINGS et al., 2018).

2.2 Design of the three-dimensional structure of peptides based on RHcyst-1

The sequence of the peptides based on the RHcyst-1 inhibitor, named RHcyst1-peps, was determined based on the results of RHcyst-1 molecular modeling. With the model of the built RHcyst-1, the cystatin was docked with the human cathepsin L structure obtained through the RCSB PDB (BERMAN, 2000) under the identification code 1ICF (GUNČAR et al., 1999). The structure was subjected to a preparation step, with the removal of water molecules and any existing binders. At the end, the missing hydrogen atoms were added using the AutoDock version 4.2 program (MORRIS et al., 2009). After the structural determination of RHcyst-1, its docking with cathepsin L was performed using the ClusPro software (KOZAKOV et al., 2017). Docking was carried out blindly, allowing the platform to assess the most suitable place for the interaction between the two input molecules occur. The selected docking pose was visualized in the PyMOL software (SCHRÖDINGER LLC, 2015). To observe the interactions between amino acids at the catalytic site and the nature of the bonds between the ligand and the protein, the PDBsum software was used (LASKOWSKI et al., 2018). With the results of the interactions between RHcyst-1 and cathepsin L, two peptides were designed to contain all the amino acids that participate in such interaction. Later, we used the AlphaFold2 Software to perform the three-dimensional modeling of the peptides. The first peptide containing 24 amino acids was named Rhcyst-1pep and the amino acid sequence of Rhcyst-1pep is as follows: LCGGLSQLVNGINYAHKAFQSEHF. The second peptide, being a shortly version of the first, contains 14 amino acids and is called Rhcyst-1pep2, the amino acid sequence of Rhcyst-1pep2 is as follows: QLVNGINYAHKAFQ.

2.3 Allergenicity and toxicity assessment *in silico*

The web-based AllerTOP v.2.0 (DIMITROV et al., 2014) was used to check the allergenicity of the peptides and of the complete inhibitor sequence. To classify allergens and nonallergens, AllerTOP v. 2.0 has been established on the basis of k-nearest neighbors (kNN) method. The web server ToxinPred was used to predict toxicity of the peptides (GUPTA et al., 2013). This strategy was developed on the basis of machine learning technique and quantitative matrix utilizing distinctive properties of peptides. For the mapping of IgE epitopes and mapping of motifs by Multiple Em for Motif Elicitation (MEME) / Motif Alignment and Search Tool (MAST) we use AlgPred tools (SAHA; RAGHAVA, 2006a).

2.4 B-cell and T-cell epitopes prediction

Linear B-cell epitopes of the RHcyst1-pep sequences were predicted by ABCpred (SAHA; RAGHAVA, 2006b). The length of peptide fragments was not set to any specific value to find all potential linear epitopes. Predicted peptides with scores above the threshold of 0.50 were selected.

NetMHCIIpan 4.0 servers were used for the likelihood of peptide binding to Major Histocompatibility Complex (MHC) class II molecules for T-cell epitope prediction (REYNISSON et al., 2020).

2.5 Peptide synthesis

An automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system from Shimadzu) was used for the solid-phase synthesis of the peptides by the Fmoc-procedure (HIRATA et al., 1995; KORKMAZ et al., 2008). The final peptides were deprotected in TFA and purified by semipreparative HPLC using an Econosil C-18 column (10 µ, 22.5 x 250 mm) and a two-solvent system: (A) trifluoroacetic acid (TFA)/H2O (1:1000) and (B) TFA/acetonitrile (ACN)/H2O (1:900:100). The column was eluted at a flow rate of 8 ml/min with a 10 (or 30) - 50 (or 60)% gradient of solvent B over 30 or 45 min. Analytical HPLC was performed using a binary HPLC system from Shimadzu with a SPD-10AV Shimadzu uv-vis detector, coupled to an Ultrasphere C-18 column (5 µ, 4.6 x 150 mm) which was eluted with solvent systems A1 (H3PO4/H2O, 1:1000) and B1 (ACN/ H2O/H3PO4, 900:100:1) at a flow rate of 1.0 ml/min and a 10-80% gradient of B1 over 10 min. The HPLC column eluates were monitored by their absorbance at 220 nm. The molecular weight, the purity of the synthesized peptides and the sequence of the peptides were verified by MALDI-TOF mass spectrometry (Bruker Daltons), electrospray LC / MS-2010 (Shimadzu) and nano-LC/MS-MS-Impact-II (Bruker Daltons).

2.6 Proteinase inhibitor assays

To verify the inhibitory activity of the RHcyst1-pep and RHcyst1-pep2 in vitro, the IC_{50} of peptides was measured. The peptides were pre-incubated with each enzyme (0.15 µM) in a reaction buffer for 30 min. Then, 0.25 mM of the protease-specific substrates was added to each well, and residual enzyme activity was measured at 370/460 nm, using a microplate

spectrophotometer (Spectramax Microplate Reader, Molecular Devices Corporation) (WANG et al., 2015; YAMAJI et al., 2009). The following enzymes were used: cathepsin B (from bovine spleen), and cathepsin L (from the human liver). These enzymes were all purchased from Sigma Co. (Sigma, USA). The reaction buffer consisted of 100 mM sodium acetate, 100 mM NaCl, 1 mM EDTA, 1 mg / ml cysteine and 0.005% Triton X-100, pH 5.5 in a reaction final volume of 100µL at 37°C (WANG et al., 2015; YAMAJI et al., 2009). The substrate used (Sigma, USA) was as follows: Z-Phe-Arg-AMC·HCl in a final concentration of 0.25 mM.

3. Results

3.1 Molecular modeling of RHcyst-1

The three-dimensional structure of RHcyst-1 from the tick *Rhipicephalus haemaphysaloides* is shown in Fig. 1. Five models were generated, and the one with the best energy score was selected. Visual inspection using PyMOL showed an expected structure, with an alpha helix and four beta strands forming a sheet. The quality of the models performed by AlphaFold2 can be seen in Supplementary Fig 1. The quality of the five models was considered high, being suitable for application of the model for molecular docking.

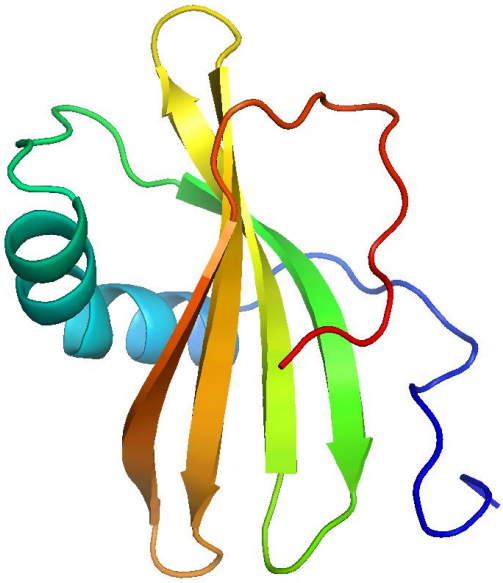

Fig. 1. The three-dimensional structure of RHcyst-1 from ticks *Rhipicephalus haemaphysaloides* that was modeled by AlphaFold2. Presented by the PyMOL program, in cartoon mode, colored from N-terminal in blue to C-terminal in red.

3.2 Design of the three-dimensional structure of Rhcyst-1pep1 and Rhcyst-1pep2

The results of the peptide structure project can be seen in Fig. 2. The peptide Rhcyst-1pep1 was projected to contain in a linear sequence all the amino acids that participate in the interaction between RHcyst-1 and the cathepsin L. The second peptide Rhcyst-1pep2 is a shorter version of the pep 1.

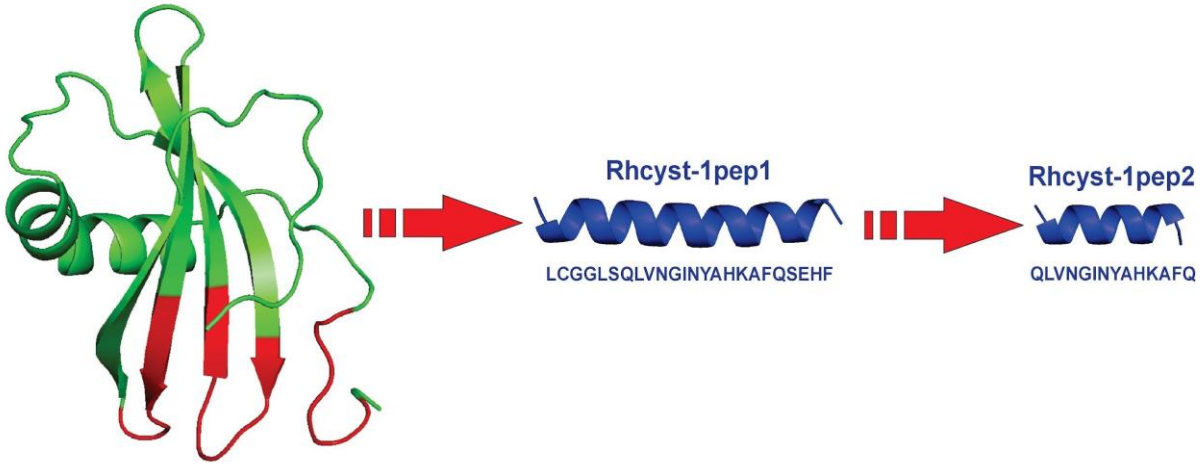

Fig. 2. The top of the figure, in green, shows the protein with regions in red showing the amino acids that participate in the interaction with CTS L. Highlighted in red are the regions containing the amino acids used for the modeling of the peptide Rhcyst-1pep (in blue). Figure also shows theRhcyst-1pep2 generated from Rhcyst-1pep1. Both peptides assumed an alfa helix conformation in our prediction.

3.3 ClusPro analysis

The results of peptide-protein anchoring using the ClusPro web server are presented in Table 1. Docking of inhibitors with cathepsins resulted in several clusters, most of which have been shown to interact with cathepsins across multiple regions. Inhibitor/Cathepsin complexes were ranked based on the lowest binding energy and binding to the active site of proteins. The highest ranked cluster was selected for further analysis. The results of these analyzes indicated that the peptides, having lower binding energy, would have a greater potential to inhibit the target cathepsins.

Table 1 - Blind coupling of RHcyst-1, RHcyst1-pep and RHcyst1-pep2 with cathepsins L and B using the ClusPro server.

Protein	Inhibitor	ClusPro energy docking			
		Cluster	Members	Central energy	Lowest energy
Cathepsin L	RHcyst-1 Protein	0	190	-729.8	-812.9
Cathepsin L	RHcyst1-pep	0	272	-676.1	-816.5
Cathepsin L	RHcyst1-pep2	0	296	-617.6	-716.2
Cathepsin B	RHcyst-1 Protein	0	186	-670.7	-732.0
Cathepsin B	RHcyst1-pep	1	140	-642.9	-872.8
Cathepsin B	RHcyst1-pep2	1	231	-664.2	-879.4

In the current investigation, ClusPro software was used to understand the interaction of RHcyst-1 and peptides with Cathepsins L and B. The computational molecular docking in which the inhibitors were docked with cathepsins served to determine favorite binding sites. The best conformation of the binding mode between inhibitors and cathepsins are shown in Fig. 3.

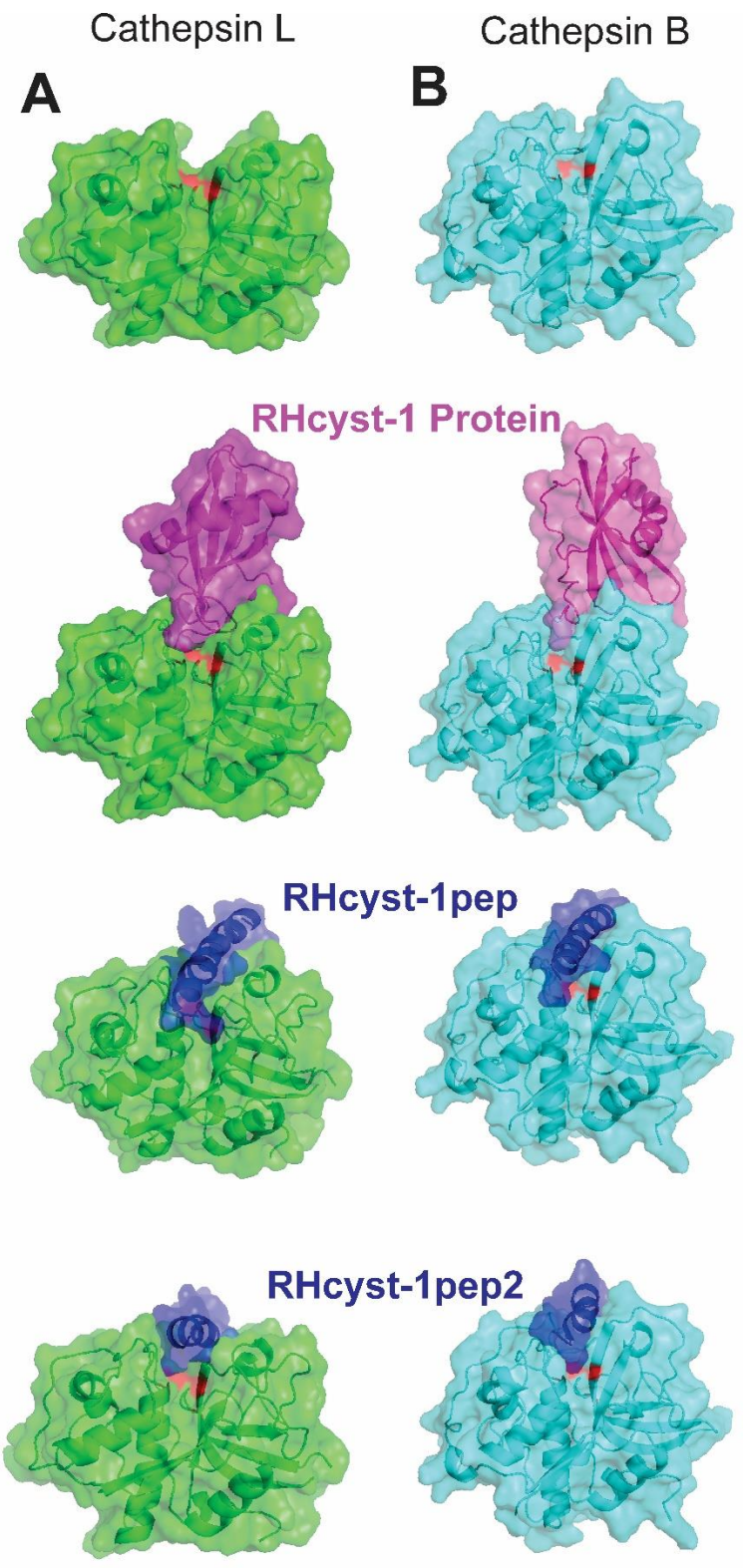

Fig. 3. Docked Cathepsin L and B with RHcyst-1, RHcyst-1pep and RHcyst-1pep2. The proteins are presented in surface mode with 50% transparency, making it possible to visualize the structure in cartoon mode. Cathepsin L is shown in green (A) and cathepsin B is shown in light blue (B) both with the active site highlighted in red. RHcyst-1 is shown in purple and peptides are shown in dark blue.

3.4 Allergenicity and toxicity assessment *in silico*

The AlgPred server attests the non-allergenic behavior of the RHcyst-1 protein and its derived peptides. All molecules were assessed as non-allergenic in nature. The RHcyst-1 protein and peptide sequences were predicted for their allergenicity using the AlgPred tool. Both protein sequences do not contain experimentally proven IgE epitopes. Identification of non-conserved MEME/MAST motifs considered all molecules as non-allergenic.

Toxicity prediction was undertaken by ToxinPred, all molecules were considered "Non-Toxin" as shown in table 2.

Table 2 - Toxicity prediction by ToxinPred

	ToxinPred output	
Molecule	Number of occurrences	Toxicity prediction
RHcyst-1 protein	89	Non-Toxin
RHcyst1-pep	1	Non-Toxin
RHcyst1-pep2	1	Non-Toxin

3.5 B-cell epitopes prediction

Using ABCpred, linear B-cell epitopes of the RHcyst-1 protein and the peptide RHcyst1-pep were predicted. Potential epitopes of RHcyst-1 and the potential epitopes of RHcyst1-pep are shown in Table 3. The RHcyst1-pep2 did not present any predicted epitope for B cells.

Table 3 - Predicted linear B-cell epitopes based on the RHcyst-1 protein and RHcyst1-pep sequences using ABCpred server.

Molecule	Potential linear B-cell epitope	Start position	Score
RHcyst-1 protein	CFPEFTPLKYRTQLVN	36	0.91
	SEEVKDADDTVREICE	8	0.82
	HIHVRAHKAFQGEISF	66	0.79
	MPLCGGLSEEVKDADD	1	0.79
	CEKVRAEVETKLGKCF	22	0.78
	DTVREICEKVRAEVET	16	0.74
	AVQEEKTLEDPLEHFQ	83	0.71
	GEISFSAVQEEKTLED	77	0.71
	HVGNNQHIHVRAHKAF	60	0.70
	VNGINYFVKVHVGNNQ	50	0.70
	PLKYRTQLVNGINYFV	42	0.65
RHcyst1-pep	QLVNGINYAHKAFQSE	7	0.67

3.6 T-cell epitopes prediction

Net-MHCIIpan-4.0 was used to predict binding between inhibitors and MHC-II. Cystatin RHcyst-1 showed 6 possible weak ligands as shown in Table 4. The peptides, on the other hand, had this number of ligands reduced to zero, no strong or weak ligands were present.

Table 4 - Location of the T-cell epitopes of the protein RHcyst-1 predicted by Net-MHCIIpan-4.0

Pos	Peptide	Of	Core	Core_Rel	Score_EL	%Rank_EL	BindLevel
41	TPLKYRTQ LVNGINY	4	YRTQLVNG	0.993	0.291731	4.62	WB
42	PLKYRTQL VNGINYF	3	YRTQLVNG	1.000	0.282884	4.75	WB
70	RAHKAFQG EISFSAV	5	FQGEISFSA	0.980	0.276952	4.84	WB
71	AHKAFQGE ISFSAVQ	4	FQGEISFSA	1.000	0.626440	1.73	WB
72	HKAFQGEI SFSAVQE	3	FQGEISFSA	1.000	0.763609	1.05	WB
73	KAFQGEISF SAVQEE	2	FQGEISFSA	1.000	0.383438	3.57	WB

Number of strong binders (SB): 0 Number of weak binders (WB): 6

3.7 In vitro Proteinase inhibition assays

The results showed that RHcyst1-pep and RHcyst1-pep2 can inhibit the enzymatic activities of cathepsin B and cathepsin L in vitro (Fig. 4) and (Fig. 5) respectively. The IC50s of RHcyst1-pep1 targeting cathepsin B and cathepsin L, 504.05 µM, and 534.72 µM, respectively. The IC50 values for the RHcyst1-pep2 targeting cathepsins B and L were 658.99 µM and 494.52 µM, respectively. The IC50 values were calculated based on the log values using GraphPad Prism 8 Software.

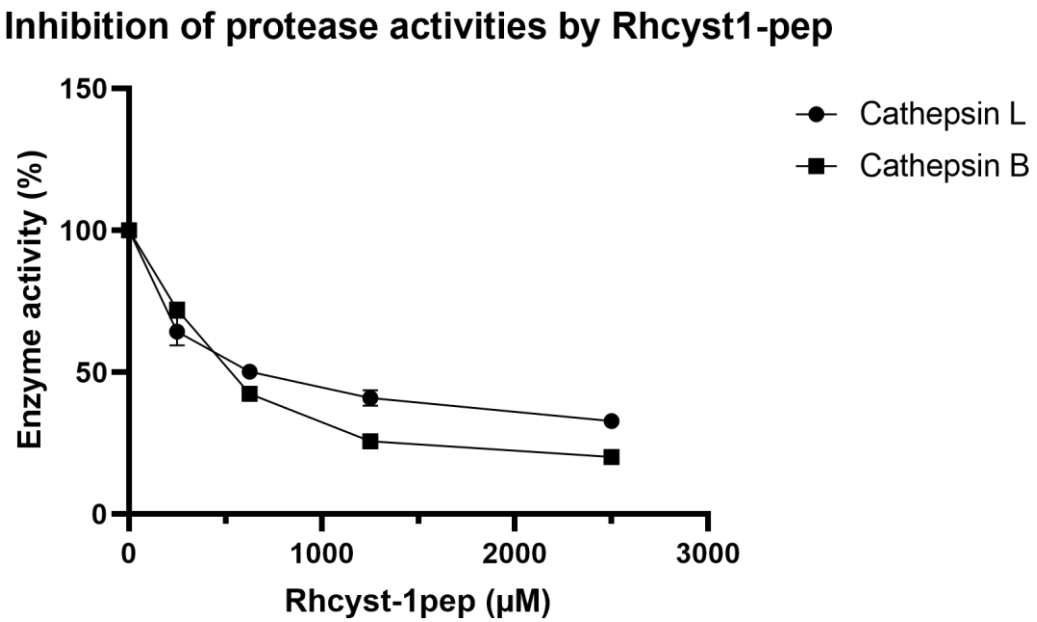

Fig. 4. Inhibition of protease activities by Rhcyst-1pep. Cathepsins B and L were incubated with the substrate in the presence of different concentrations of Rhcyst-1pep. Incubation of cathepsins without Rhcyst-1pep leads to 100% enzyme activity.

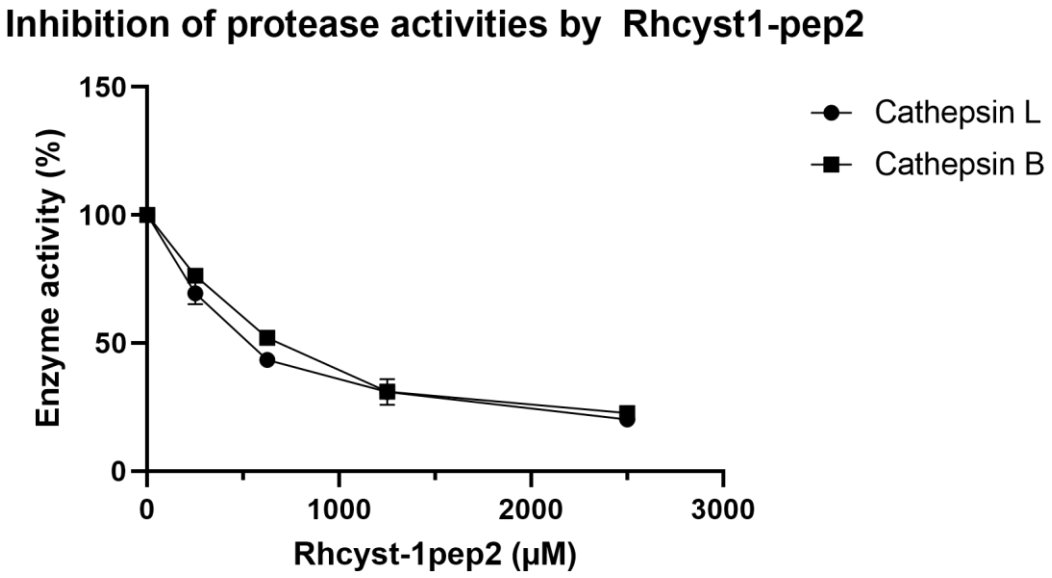

Fig. 5. Inhibition of protease activities by Rhcyst-1pep2. Cathepsins B and L were incubated with the substrates in the presence of different concentrations of Rhcyst-1pep2.

4. Discussion

Different studies that investigate tumor cysteine proteases such as cathepsins have been conducted in the last years, demonstrating the role of these enzymes in tumor progression and metastasis (GOCHEVA; JOYCE, 2007; JOYCE; HANAHAN, 2004; NOMURA; KATUNUMA, 2005; OLSON; JOYCE, 2015; SOOND et al., 2019). These results had encouraged the idea that the imbalance between cysteine proteases and cystatins, their natural inhibitors, can be further explored in the fight against cancer (OLSON; JOYCE, 2015). Therefore, the use of cystatins or other cathepsin inhibitors have been considered a potential therapeutic strategy against cancer (EATEMADI et al., 2017).

Rhcyst-1 is a tick cystatin with great biotechnological potential already demonstrated in vitro and in vivo (WEI et al., 2018). It significantly inhibited the proliferation, migration, and invasion of four different tumor cells in vitro including lung, ovarian, melanoma and cervical cancer cells. Furthermore, when tested in vivo, in a murine model, it inhibited tumor growth and improved the animals survival (WEI et al., 2018). In the present study, we investigated the structural characteristics of Rhcyst-1 and its interaction with two human cathepsins with important roles in cancer invasion and metastasis (JOYCE; HANAHAN, 2004; NOMURA; KATUNUMA, 2005). Based on the enzyme-inhibitor interaction model, two peptides derived from the cystatin RHcyst-1 from the tick *R. haemaphysaloides* were proposed and had its *in silico* and *in vitro* cathepsin inhibitory capacity confirmed against cathepsin B and L. Additionally, the peptides Rhcyst-1pep and Rhcyst-1pep2 showed a reduced immunogenicity compared to the Rhcyst-1 full molecule, which is positive thinking in drug development.

Our *in silico* results do not demonstrate that the difference in the number of amino acids used in pep 1 and 2 clearly influenced the peptides inhibitory capacity. Once for cathepsin L the peptide 1 was more efficient and for cathepsin B the peptide 2 was more efficient. Besides to consider a short peptide more promising for medical application, we supposed that the peptide with more amino acids could have a better anchoring in the enzyme active site region resulting in a higher affinity. When we compare the full Rhcyst-1 protein with the derived peptides, the parameters analyzed *in silico* demonstrate satisfactory results with a lower activity of the peptides when compared to the sequence of the full protein Rhcyst-1. Otherwise, the IC 50 values achieved *in vitro* in this study for both peptides were in the micromolar order, and the IC 50 previously achieved with the recombinant protein for the same enzymes were in the nanomolar order (WANG et al., 2015). To better compare the molecules, in future *in vitro*

studies we intend to work with all molecules together and to analyze Ki constants instead of IC50.

Enzymatic proteolysis in the vicinity of a tumor is a hallmark of cancer invasion and metastasis (VASILJEVA et al., 2006). Alteration in cathepsins expression levels, processing, and localization, starting by the abundant cathepsins L and B, in tumoral cells when compared to the normal cells of the same lineage have made these enzymes interesting cancer prognostic and diagnostic markers (CHAUHAN; GOLDSTEIN; GOTTESMAN, 1991; GONDI; RAO, 2013). The main cause of cancer-related deaths in some types of cancer, as breast cancer, is the migratory and invasive behavior of cancer cells, which results in metastasis and scattering of the disease to different tissues and organs (LI et al., 2021; LÚCIA DE ALMEIDA et al., 2005). Therefore, there is a great interest in discovering or developing new and effective molecules to avoid the spread of metastatic cancer cells, putting the enzymes involved in these processes as targets for inhibitors development which have being focus of many studies (LI; FANG; AO, 2017; SUDHAN; SIEMANN, 2015c).

As far as we know, until the moment there are no inhibitors of cathepsin L or B being used in clinics. However, during the last few years, some novel inhibitors of cathepsin L or B with new scaffolds from small molecules library, natural products as well as reasonable drug design were developed and tested as new therapeutic agents. Their potency, selectivity and reversibility have been improved by structural optimization to match corresponding sites of cathepsin B or L (LI; FANG; AO, 2017). The therapeutic proteins, initially approved for use in general medicine, were simple replacement proteins (as insulin). In recent years, a proportional increase in biopharmaceuticals development using modified proteins or analogues has been observed (MAHMOOD; GREEN, 2005). In this context, RHcys-1 or its derived peptides are molecules with great biotechnological potential. Among the advantages of using peptides as drugs, we can mention the chemical synthesis capacity, the small molecular size, the lower capacity to generate an immune response and a less complex structure that can be improved and modified more easily to increase the inhibitory capacity and specificity (KURRIKOFF; APHKHAZAVA; LANGEL, 2019).

Some cystatin-like protein inhibitors are not as effective in inhibiting cathepsin B as they are to papain or other cathepsins L (MUSIL et al., 1991). Structural studies showed that it happens because cathepsin B has an “occluding loop” that reduces inhibitors affinity to the active site of the protein. Interestingly, in our study both peptides demonstrated a similar ability to inhibit cathepsin L and cathepsin B, which indicates that the cathepsin B loop is not a problem for these small inhibitors. Considering that cathepsin L and B usually are involved in similar

processes assuming similar importance in tumor progression, this dual activity is very desirable once cases of compensation among cysteine cathepsin are reported (AKKARI et al., 2016).

It is well known that, besides to the most abundant cathepsins L and B, other cathepsins are also involved in the process of tumor invasion, proliferation, and metastasis (TAN et al., 2013). So, it is important during the development of cathepsin inhibitors to privilege molecules that can inhibit more than one of human cathepsins. In future works, we intend to test the inhibitory capacity of the peptides against a greater number of human cathepsins and work on sequence and number of amino acids to make modifications in order to improve the peptides' inhibitory potential together with their plasticity.

5. Conclusions

In summary, two peptides derived from the RHcyst-1 protein were modeled, chemically synthesized, and tested *in silico* and *in vitro* against human cathepsins B and L. In this study, the three-dimensional structure of RHcyst-1 was modeled and presented. The inhibitory activity of the peptides was measured, and the average inhibitory concentration demonstrated that the peptides have similar inhibitory activity against both proteins. The results of the allergenicity and toxicity evaluation showed that both the RHcyst-1 protein and the derived peptides do not have any allergenic or cytotoxic effects *in silico*. The peptides showed better results regarding the possibility of generating immune responses when compared to RHcyst-1. In future works, to improve the peptide inhibitory activity, changes in amino acids sequence and length will be carried out. We hypothesized that the peptides, even used in higher concentration, could play a role like RHcys-1 in the tumor microenvironment and exhibit potential antitumor and antimetastatic efficacy.

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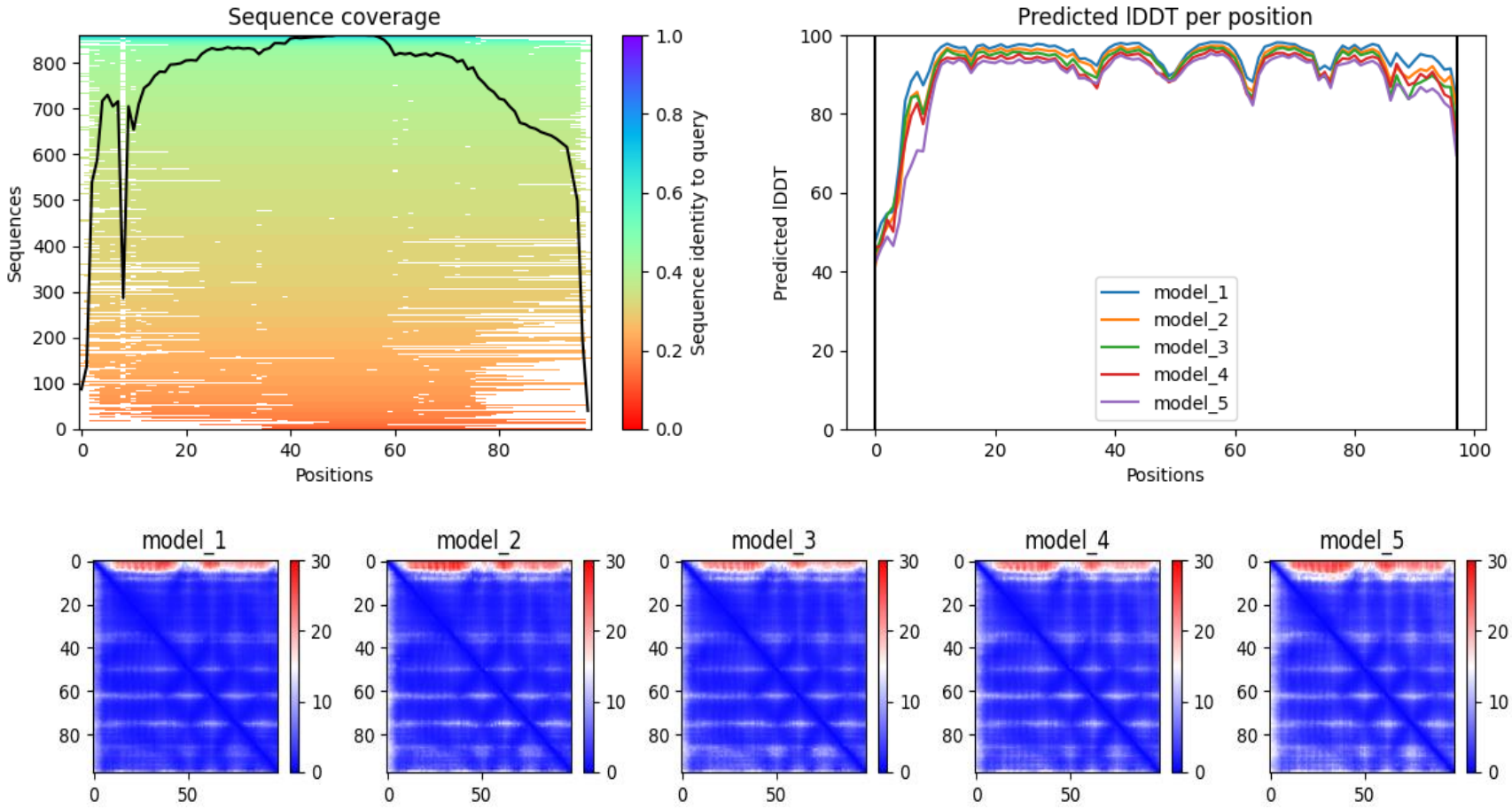
Supplementary material



Supplementary Fig. 1. AlphaFold2 evaluation results. The IDDT result shows that all models were above the values considered confident

6 CONCLUSÃO

Os peptídeos Rhcyst-1pep e Rhcyst-1pep2 projetados a partir da estrutura da Rhcyst-1 foram capazes de inibir as catepsinas L e B e apresentaram menor capacidade de gerar resposta imune *in silico*. Esta atividade pode ser comprovada *in vitro* através de experimentos realizados com o peptídeo sintético. A capacidade de síntese química dos peptídeos desenvolvidos é uma vantagem, visto que podemos proceder alterações ou inserções na sequência de aminoácidos a fim de melhorar a capacidade inibitória dos mesmos.

Assim como a RHcyst-1, os peptídeos obtidos neste estudo são inibidores de cisteíno proteases, sendo prováveis candidatos a apresentarem aplicabilidade como agentes antitumorais e antimetastáticos. Em estudos futuros vamos avaliar a capacidade dos peptídeos como possíveis antitumorais, realizar mais estudos estruturais visando aprimorar a capacidade inibitória de catepsinas L e B e investigar o potencial de inibição frente aoutras catepsinas cisteínicas envolvidas no câncer e em outras patologias humanas.

Pensando no desenvolvimento futuro de novos biofármacos, moléculas como as propostas neste estudo podem ser usadas junto aos quimioterápicos atuais como auxiliares na contenção de metástase, contribuindo para a redução da progressão tumoral.

7 ANEXOS

7.1 Normas para submissão na revista *Process Biochemistry*

DESCRIPTION

Process Biochemistry is an application-orientated research journal devoted to reporting advances with originality and novelty, in the science and technology of the processes involving bioactive molecules and living organisms. These processes concern the production of useful metabolites or materials, or the removal of toxic compounds using tools and methods of current biology and engineering. Its main areas of interest include novel bioprocesses and enabling technologies (such as nanobiotechnology, tissue engineering, directed evolution, metabolic engineering, systems biology, and synthetic biology) applicable in food (nutraceutical), healthcare (medical, pharmaceutical, cosmetic), energy (biofuels), environmental, and biorefinery industries and their underlying biological and engineering principles.

Main topics covered include, with most of possible aspects and domains of application:
• Fermentation, biochemical and bioreactor engineering
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• Mass transfer, mixing, scale-up and scale-down, bioprocess monitoring, bio-manufacturing
• Cell, tissue and antibody engineering: animal and plant cells/tissues, algae, micro-algae, extremophile, antibody screening and production
• Environmental biotechnology: biodegradation, bioremediation, wastewater treatment, biosorption and bioaccumulation
• Bio-commodity engineering: biomass, bio-refinery, bio-energy
• Bioseparation, purification, protein refolding.
• Other new bioprocess and bioreactor related topics especially on application to healthcare sectors

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INTRODUCTION

Process Biochemistry is an application-orientated research journal devoted to reporting advances with originality and novelty, in the science and technology of the processes involving bioactive molecules and living organisms. These processes concern the production of useful metabolites or materials, or the removal of toxic compounds using tools and methods of current biology and engineering. Its main areas of interest include novel bioprocesses and enabling technologies (such as nanobiotechnology, tissue engineering, directed evolution, metabolic engineering, systems biology, and synthetic biology) applicable in food (nutraceutical), healthcare (medical, pharmaceutical, cosmetic), energy (biofuels), environmental, and biorefinery industries and their underlying biological and engineering principles.

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