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Sheila Rosa da Mata

Clonagem, Expressão e Caracterização de um inibidor de serino proteinases do tipo Kunitz (Persulcatina) do carrapato *Ixodes persulcatus*

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“É muito melhor lançar-se em busca de conquistas grandiosas, mesmo expondo-se ao fracasso, do que alinhar-se com os pobres de espírito, que nem gozam muito nem sofrem muito, porque vivem numa penumbra cinzenta, onde não conhecem nem vitória, nem derrota. ” (Theodore Roosevelt)

RESUMO

Doenças Tromboembólicas estão entre as principais causas de mortalidade e morbidade em todo mundo. A terapêutica anticoagulante é de fundamental importância para reduzir este panorama. Entretanto, os anticoagulantes de uso tradicional apresentam desvantagens como interações farmacológicas, variabilidade dose-resposta, margem terapêutica estreita, hemorragias frequentes e complicações tromboembólicas. Com o intuito de ultrapassar essas desvantagens, novos fármacos têm sido desenvolvidos com farmacocinética e farmacodinâmica mais previsíveis. Estes novos fármacos têm como alvo a inibição seletiva de fatores específicos da coagulação, usualmente a trombina e o fator X ativado. Neste contexto, a saliva dos animais hematófagos é uma fonte potencial de componentes farmacologicamente ativos capazes de interferir no sistema hemostático e imunológico de seus hospedeiros. A caracterização dessas substâncias tem mostrado uma grande variedade de estruturas e funções úteis à terapêutica. Muitos compostos bioativos isolados de animais hematófagos, ou análogos projetados a partir deles, têm sido testados para o desenvolvimento de anticoagulantes mais específicos e seguros. Neste trabalho, foi identificada uma sequência que codifica a proteína, nomeada persulcatina, oriunda de uma biblioteca de cDNA de fêmeas do carrapato *Ixodes persulcatus* (ectoparasita humano). A persulcatina é uma nova molécula multifuncional, expressa em glândulas salivares e em outros tecidos, que tem como alvo a plasmina e a trombina, caracterizando-se como um inibidor clássico competitivo de ligação forte. Moléculas como a persulcatina apresentam grande potencial biotecnológico na área da saúde, podendo ser modelo para o desenvolvimento de novos fármacos com atividade anticoagulante.

Palavras-chave: *Ixodes persulcatus*; inibidor de trombina; inibidor do tipo Kunitz; persulcatina; anticoagulantes;

ABSTRACT

Thromboembolic diseases are among the leading causes of mortality and morbidity worldwide. Anticoagulant therapy is of fundamental importance to reduce this panorama. However, traditional anticoagulants present disadvantages such as pharmacological interactions, dose-response variability, narrow therapeutic margin, frequent bleeding and thromboembolic complications. In order to overcome these disadvantages, new drugs have been developed with predictable pharmacokinetics and pharmacodynamics. These new drugs target selective inhibition of specific coagulation factors, usually thrombin and activated factor X. In this context, the saliva of hematophagous animals is a potential source of pharmacologically active components capable of interfering in the hemostatic and immune system of their hosts. The characterization of these substances has shown a wide variety of structures and functions useful for therapeutics. Many bioactive compounds isolated from hematophagous animals, or analogues designed from them, have been tested for the development of more specific and safe anticoagulants. In this report, the coding sequence of the protein named persulcatin, derived from a cDNA library of the *Ixodes persulcatus* female ticks (human ectoparasite). Persulcatin is a new multifunctional molecule, expressed in salivary glands and other tissues, which targets plasmin and thrombin, characterizing itself as a classic competitive inhibitor of strong binding. Molecules such persulcatin present great biotechnological potential in the health sciences area and can be used as model for the development of new drugs with anticoagulant activity.

Keywords: *Ixodes persulcatus*; thrombin inhibitor; Kunitz-type inhibitor; persulcatin; anticoagulants;

LISTA DE ILUSTRAÇÕES

TABELA DE AMINOÁCIDOS

Abreviação		Aminoácido	Abreviação		Aminoácido
Ala	A	Alanina	Leu	L	Leucina
Arg	R	Arginina	Lys	K	Lisina
Asn	N	Aspergina	Met	M	Metionina
Asp	D	Ácido Aspártico	Phe	F	Fenilalanina
Cys	C	Cisteína	Pro	P	Prolina
Glu	E	Ácido glutâmico	Ser	S	Serina
Gln	Q	Glutamina	Thr	T	Treonina
Gly	G	Glicina	Trp	W	Triptofano
His	H	Histidina	Tyr	Y	Tirosina
Ile	I	Isoleucina	Val	V	Valina

LISTA DE ABREVIATURAS E SIGLAS

AT: Antitrombina

AVC: Acidente Vascular Cerebral

AVK: Antagonista de Vitamina K

BPTI: Inibidor de tripsina bovina

cDNA: DNA Complementar

AODs: Anticoagulante orais diretos

DCV: Doença Cardiovacular

EP: Embolia Pulmonar

ESTs: Marcador de Sequência Genética

FAB: Anticorpo Monoclonal Humanizado

FDA: Administração de Alimentos e Medicamentos

FT: Fator tecidual

HBPM: Heparina de Baixo Peso Molecular

HNF: Heparina não Fracionada

IDT: Inibidor Direto de Trombina

IgG: Imunoglobulina G

RCL: Alça do centro de reação

SCA: Síndromes Coronárias Agudas

TAP: Peptídeo Anticoagulante de carrapato

TEA: Tromboembolismo Arterial

TFPI: Inibidor da via do Fator Tecidual

TIH: Trompocitopenia Induzida por Heparina

TP: Tempo de Protrombina

TTPA: Tempo de Tromboplastina Parcial Ativada

UFCSPA: Universidade Federal de Ciências da Saúde de Porto Alegre

UFRGS: Universidade Federal do Rio Grande do Sul

SUMÁRIO

1. REVISÃO DA LITERATURA

1.1 Hemostasia e Cascata da Coagulação sanguínea

O sistema hemostático é composto por uma sequência de eventos integrados que envolvem vasos sanguíneos, plaquetas, proteínas da coagulação, anticoagulantes naturais e proteínas do sistema fibrinolítico, em um processo dinâmico e complexo, envolvendo um conjunto de mecanismos finamente regulados com máxima eficiência. Esse processo fisiológico está envolvido no controle de sangramento frente a uma lesão vascular, evitando hemorragia prolongada. Esse sistema também é responsável por manter a fluidez do sangue na circulação sanguínea, evitando a formação de trombos (CLARK *et al*., 1982; FURIE & FURIE, 1988, GONDIM *et al*., 2017).

A injúria vascular desencadeia três processos principais para evitar perda descontrolada de sangue: a vasoconstrição, responsável por limitar o aporte de sangue no local de lesão endotelial; a agregação plaquetária, que forma um tampão no local da lesão através dos processos de adesão, ativação e agregação de plaquetas; e a coagulação sanguínea, a qual, por meio da ativação sequencial de fatores presentes no plasma, forma o coágulo de fibrina responsável pela formação do coágulo final (MINORS, 2007). Uma vez formado, o coágulo de fibrina é rapidamente digerido pela plasmina, importante enzima do sistema fibrinolítico. A ação da plasmina sobre o fibrinogênio e a fibrina origina os produtos de degradação da fibrina. Esses mecanismos atuam no sentido de impedir a formação de trombos em vasos intactos além de desencadear uma série de reações que resultam no reparo tecidual da parede vascular (OLDENBURG & SCHWAAB, 2001). Portanto, o sistema hemostático requer um equilíbrio biológico entre mecanismos pró-coagulantes e anticoagulantes, aliado a um processo de fibrinólise (AFONSO *et al*., 2016; BOUTITIE *et al*., 2011; HOFFBRAND *et al*., 2011; MATHEWS & ISSAC, 2016).

A cascata de coagulação envolve uma ativação sucessiva e coordenada de pró-enzimas plasmáticas inativas da classe das serino proteases e seus cofatores (com exceção do fator XIIIa, o qual é uma transpeptidase), que culminam na gênese da trombina (fator II) (FERREIRA *et al*., 2010). A coagulação se inicia quando o fator tecidual (FT) é exposto no meio intravascular, onde, na presença de fosfolipídios e

cálcio, liga-se ao fator VII formando o fator VII ativado (VIIa), o qual ativa o fator X em fator Xa, formando o complexo tenase (FT-VIIa), que caracteriza a via extrínseca da coagulação (DAVIE *et al*., 1981). Já a via intrínseca, inicia-se com a exposição do fator XII ao colágeno ou à superfície subendotelial e, em seguida, a ativação do fator XI. O fator XI, por sua vez, ativa o fator IX. O fator XIa, na presença do fator VIII, fosfolipídios plaquetários e Ca^{2+}, ativa o Fator X. Nesta proposta, a via extrínseca e a via intrínseca convergem para uma via comum, a partir da ativação do fator X. A ativação do fator X, na presença do cofator V, formando o complexo protrombinase, desencadeia a geração de trombina. A trombina converte o fibrinogênio em fibrina por proteólise. Para estabilizar a rede de fibrina, o fator XIIIa polimeriza os monômeros de fibrina formando uma rede estável que juntamente com plaquetas e hemácias formam o trombo (Figura 1).

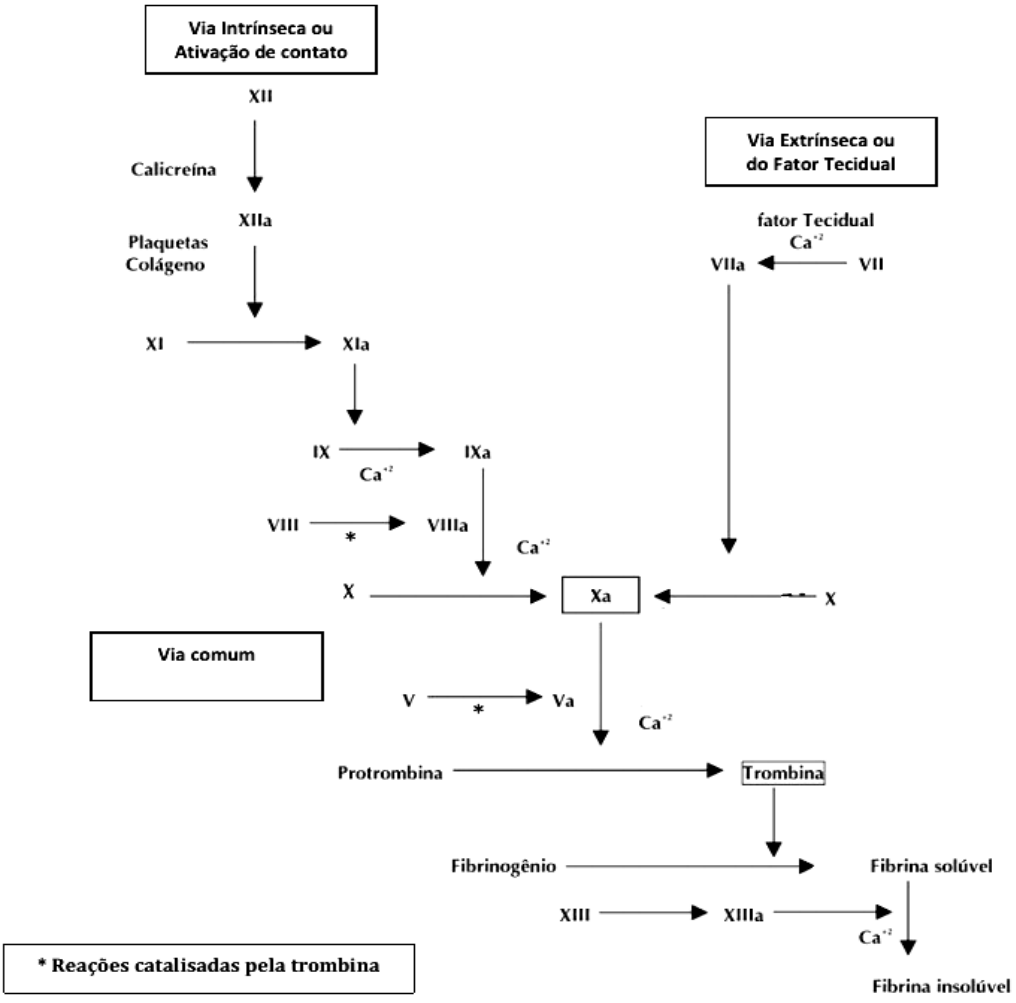

Figura 1. Representação diagramática da cascata de coagulação sanguínea. Adaptado de Mauro *et al*. (2004).

A trombina é o mediador comum final de ambas as vias, que por proteólise, converte o fibrinogênio solúvel em fibrina insolúvel. Assim, uma estratégia pontual e eficiente para inibir a cascata da coagulação sanguínea é bloquear a atividade do fator Xa e/ou trombina (FRANCO, 2001; FERREIRA *et al*., 2010; SILVA & MELO, 2016).

1.2 Desordens do Sistema Hemostático

Uma ativação patológica do sistema de coagulação, em que um componente qualquer esteja alterado, pode resultar em uma produção excessiva de trombina e teria como efeito o fenômeno da trombose (DAVIE *et al.*, 1991). Portanto, a trombose compreende a formação de um coágulo no interior de um vaso sanguíneo. Esse coágulo bloqueia o fluxo de sangue e causa inchaço e dor local. Entretanto, problemas mais graves ocorrem quando um coágulo se desprende e se movimenta na corrente sanguínea, processo chamado de embolia. A embolia pode acontecer no cérebro, nos pulmões, no coração ou em outras áreas, impedindo a passagem de sangue e, consequentemente, levando à morte progressiva da região afetada (ARTEAGA *et al*., 1989; SCANAVACCA & DARRIEUX, 2016).

Esse fenômeno está relacionado a fatores de riscos, que predispõem o indivíduo à referida patologia: idade acima de 40 anos, obesidade, tabagismo, câncer, contraceptivos orais entre outros (ICOLMAN *et al*., 2001). As principais indicações da tromboprofilaxia são tromboembolismo venoso (TEV), síndrome pós-trombrótica, hipertensão pulmonar tromboembólica crônica, fibrilação auricular (FA), síndrome coronária aguda. Além disso, também é indicado para portadores de substitutos de válvulas cardíacas (BASTOS *et al*., 2011).

1.3 Doenças Tromboembólicas

Doenças cardiovasculares (DCV) estão entre as principais causas de mortalidade e morbidade em todo mundo (TOTH *et al*., 2017). Apesar dos importantes esforços para controlar o aumento das DCVs, estas permanecem liderando as causas de morte nas sociedades modernas, atingindo todas as faixas etárias (LESNIEWSKI *et al*., 2013; TOTH *et al*. 2017). Em 2013, os índices anuais de mortalidade registrados demonstraram valor superior a 17,3 milhões de óbitos, numerário este, que representa

aproximadamente 31% das mortes no mundo. Tem-se a expectativa de que, até 2030, o número de mortes decorrentes de DCV aumentará em torno de 23,6 milhões (LEE *et al*., 2017; TOTH *et al*., 2017). Essas doenças cardiovasculares estão relacionadas intimamente com o aumento da coagulação sanguínea dentro dos vasos, resultando em trombose venosa ou arterial. Aproximadamente 90% das DCV ocorrem em consequência de eventos trombóticos e 10% devido a eventos hemorrágicos (CORREIA-DA-SILVA *et al*., 2013). Assim, a patogênese central das doenças tromboembólicas é a perturbação do equilíbrio hemostático normal (CASTELLUCCI *et al*., 2013; WEITZ, 2010).

O TEV inclui a trombose venosa profunda (TVP), e a embolia pulmonar (EP) (BASTOS *et al*., 2011). A formação de trombos dentro das veias ocorre geralmente nos membros inferiores, na área de drenagem entre os músculos profundos, e por isso denominada TVP. Durante curso da TVP pode ocorrer extensão ascendente do trombo para o pulmão, o que poderá obstruir a artéria pulmonar ou seus ramos, causando a EP. A TVP é a patologia que ocorre com elevada incidência e pode ser fatal ou resultar em complicações, tais como a síndrome pós-trombótica e a hipertensão pulmonar tromboembólica crônica (HIRSCH *et al*., 1996; BASTOS *et al*., 2011). O TEV resulta de uma combinação de fatores de risco hereditários ou adquiridos ao longo do tempo, também conhecido como trombofilia ou estados de hipercoagulação, associados à lesão endotelial e a estase venosa (CUSHMAN *et al*.,2001; MARQUES *et al*., 2009). Estes três fatores são condições basilares para a compreensão da trombose e são conhecidos como a tríade de Virchow (SOBREIRA *et al*., 2008, CASTELLUCCI *et al*., 2013; VERSTEEG *et al*., 2013). A tríade de Virchow também pode estar relacionada à formação de trombos durante o episódio de FA. A FA é a taquiarritmia, mais frequente na prática clínica, caracterizada por atividade mecânica e elétrica irregular nos átrios, causando batimentos cardíacos rápidos e irregulares. Os trombos formados no interior dos átrios devido à estase sanguínea e à redução do fluxo podem se deslocar provocando episódio tromboembólico quando retornam ao ritmo sinusal. Embora o tromboembolismo provocado pelo FA seja relativamente incomum, as consequências são graves, levando o indivíduo à morte (SCHVARTZMAN *et al*., 1998; SILVESTRE *et al*., 2012).

Por fim, o tromboembolismo arterial (TEA) geralmente ocorre em locais com formação de placas ateroscleróticas e com maior tensão parietal, o que pode resultar

em um trombo rico em plaquetas (AIRD, 2007; GALE, 2011). As placas ateroscleróticas podem ser alvo de ruptura e fissura, com exposição de substâncias pró-trombóticas e inflamatórias subendoteliais, tais como lipídios oxidados, FT e colágeno. Especificamente, nos pontos de derivação e curvaturas maiores das artérias, onde são, normalmente, expostos ao fluxo perturbado, proporcionando esse fenômeno. A fisiopatologia das doenças trombóticas arteriais está centralizada na aterosclerose, a ativação da cascata da coagulação e a consolidação do trombo de plaquetas pela fibrina, que desempenha um papel importante em sua gênese. No TEA é mais comum a perda da integridade vascular do que no TEV. As regiões vasculares envolvidas na aterotrombose incluem as artérias coronárias, responsáveis pelas síndromes coronárias agudas (SCA), bem como as artérias cerebrais, responsáveis pela maior parte dos acidentes vasculares cerebrais (AVC) isquêmicos (BONHORST *et al*., 2010; CALDEIRA *et al*., 2014; DOUKETIS *et al*., 2011).

1.4 Terapêutica Anticoagulante

A terapêutica anticoagulante é fundamental para a profilaxia e tratamento das doenças tromboembólicas. Com o aumento da expectativa de vida, e o consequente envelhecimento da população, a ocorrência de doenças tromboembólicas tende a crescer, sendo esperado um aumento do número de prescrições de anticoagulantes (ARBIT *et al*., 2006; SILVA, 2012; RAMOS *et al*., 2013). A terapêutica anticoagulante é uma terapêutica antitrombótica utilizada em patologias cardiovasculares, sendo as suas principais indicações a FA, o TEV, a SCA, a utilização de próteses vasculares cardíacas e dispositivos mecânicos, insuficiência cardíaca e situações de trombofilia hereditária (BASTOS *et al*., 2011; HOLBROOK *et al*., 2005; MEMON & SYED, 2016).

De uma maneira geral, os agentes anticoagulantes inibem a geração de fibrina por bloquear um ou mais passos da cascata de coagulação. Desde a década de 1950 até recentemente, os antagonistas de vitamina K (AVK), como a varfarina, eram os únicos anticoagulantes por via oral disponíveis, sendo, sem dúvida, ainda considerada "padrão ouro" para a prevenção de doenças como: fibrilação atrial, acidente vascular cerebral, trombose venosa profunda e embolia pulmonar (ALTMAN, 2014; HART *et al*., 2007; RIBEIRO-DA SILVA *et al*., 2013). Sua eficácia é seriamente reduzida por várias limitações que afetam a sua ampla utilização clínica em termos de controle,

complicações, efeitos colaterais e interações com medicamentos e dieta (AGENO *et al*., 2012; ALQUWAIZANI *et al*., 2013; HIRSH, 1991). A varfarina é uma substância derivada da cumarina e antagonista da vitamina K. Esta vitamina é indispensável para a síntese hepática de vários fatores da coagulação (II, VII, IX e X), assim a atividade biológica da protrombina é reduzida, retardando a formação da trombina e diminuindo a coagulação sanguínea. No entanto, o efeito antitrombogênico da varfarina só ocorre em baixas concentrações funcionais dos fatores II, IX e X (JAFFER & BROTMAN, 2006). Esta redução ocorre normalmente dois a quatro dias após o início da terapêutica oral. Como os efeitos da varfarina não são imediatos ela se torna ineficaz para instauração rápida da anticoagulação (GOLDHABER *et al*., 2015; GUIMARÃES & ZAGO, 2007).

Desde 1930, a heparina é o anticoagulante de eleição quando se necessita de um efeito imediato, utilizando-se os derivados cumarínicos para manutenção do tratamento a longo prazo, ou seja, para pacientes que necessitam o uso crônico da medicação (ALTMAN, 2014). As heparinas são anticoagulantes indiretos, que se ligam aos anticoagulantes endógenos, como antitrombina. Existem dois tipos de heparina: heparina não fracionada (HNF) e heparina de baixo peso molecular (HBPM), administradas por via intravenosa e subcutânea, respectivamente. A HNF é formada por uma mistura heterogênia de cadeias polissacarídicas, que variam de 10 a 80 resíduos, com resíduos alternados de D-glicosamina e ácido urônico, ou ácido glucurônico, ou ácido idurônico. A HNF age pela ativação da antitrombina (AT) endógena por meio de sua sequência pentassacarídica, aumentando significativamente a taxa de inibição de várias enzimas da coagulação em condições fisiológicas, incluindo o FIXa, o complexo tenase, o FXIIa, o FXIa e, principalmente a trombina e o FXa, que são mais sensíveis. A HNF tem início de ação rápida e meia-vida curta, o que torna seu uso vantajoso em caso de emergência (CORREIA-DA-SILVA *et al*., 2010; LEENTJENS *et al*., 2017).

Contudo, as principais desvantagens da varfarina e da heparina são a margem terapêutica estreita, variabilidade da relação dose-resposta para cada indivíduo e, em particular, as hemorragias frequentes e as complicações tromboembólicas, as quais são consequência de um efeito anticoagulante excessivo ou insuficiente, respectivamente (FUSTER *et al*., 2012; HARTER *et al*., 2015; RIBEIRO-DA-SILVA *et al*., 2013). O tempo de protrombina (TP), expresso em INR (índice internacional

normalizado), é medido periodicamente para monitorar o ajuste de dose e manter a intensidade da anticoagulação dentro de níveis seguros e eficazes (GUIMARÃES & ZAGO, 2007; SILVA, 2012; RAMOS *et al.*, 2013). Os AVKs ainda apresentam interações farmacológicas e alimentares, sendo a variabilidade da relação dose-resposta afetada por fatores genéticos (como polimorfismos das enzimas CYP2C9 e VKORC1). Já as HNF, ligam-se inespecificamente a proteínas plasmáticas e, consequentemente, apresentam efeitos colaterais como: hipercalemia ocasional, alopecia, osteoporose, alterações em teste de função hepática (TERRA-FILHO *et al*, 2010; RIBEIRO-DA-SILVA *et al*., 2013). Além disso, o uso contínuo de HNF, por 4 a 5 dias, pode levar o paciente à trombocitopenia induzida por heparina (RIBEIRO-DA-SILVA *et al*., 2013).

Esses problemas, decorrentes do uso da HNF na terapêutica, resultam de sua estrutura molecular, a qual apresenta extensos fragmentos que interagem com outras proteínas plasmáticas, como fatores de crescimento, citocinas, proteínas de adesão (ARLOV & SKJÅK-BRÆK, 2017). Devido a isso, nas últimas décadas foram introduzidas as HBPM, que são derivadas da HNF, as quais sofreram despolimerização, reduzindo o seu peso molecular, entretanto a região funcional de sua estrutura foi mantida. Dessa forma, por serem mais curtas, as HBPM apresentam dificuldade de se ligar à AT e à trombina simultaneamente. Entretanto, mesmo apresentando menor atividade inibitória em relação à trombina, a inibição do FXa é mantida. As HBPM foram aprovadas para um conjunto de indicações, em especial para trombocitopenia induzida por heparina (TIH), por demonstrar interação reduzida em relação às proteínas plasmáticas, e, portanto, maior biodisponibilidade e um efeito dose-reposta mais previsível quando comparada a HNF. A meia-vida das HBPM depende diretamente de seu peso molecular, variando sua atividade anticoagulante e duração de sua ação (CORREIA-DA-SILVA *et al*., 2010).

Em 1980, foi desenvolvido uma HBPM com apenas a região pentassacarídica DEFGH da HNF, nomeado fondaparinux. Este inibidor indireto é um análogo sintético que potencializa a taxa de inibição do FXa e, ao contrário das HNF e HBPM, não inibe a trombina. Essa inibição seletiva tornou o fondaparinux melhor tolerado e com menos efeitos colaterais. Sua eficácia, segurança, dose única diária e a falta de necessidade de monitorização conduziram a recomendação desse inibidor para pacientes com TIH (SCHINDEWOLF *et al*., 2017). No entanto, o fondaparinux apresenta um risco maior

de hemorragia, mesmo em doses mínimas, e também é dependente da AT como todas as HBPM. Além disso, não inibe o fator Xa ligado ao complexo protrombinase (CORREIA-DA-SILVA *et al*., 2010; LEENTJENS *et al*., 2017; SCHINDEWOLF *et al*., 2017). Portanto, varfarina, HNF e HBPM são anticoagulantes indiretos e fazem parte da terapêutica tradicional. Essas, por sua vez, possuem limitações relevantes (ARBIT *et al*., 2006).

Nesse contexto, com o intuito de ultrapassar as desvantagens da terapêutica anticoagulante em uso, têm sido desenvolvidos novos fármacos anticoagulantes com farmacocinética e farmacodinâmica previsíveis, maior margem terapêutica e doses fixas, sem necessidade de monitorização periódica (ALTMAN, 2014; GÓMEZ-OUTES *et al*., 2010). Múltiplos fármacos têm surgido e alguns ainda estão em fase de ensaios clínicos, outros já foram autorizados e são utilizados na prática clínica, continuando a serem testados para outras indicações. Estes novos fármacos antitrombóticos têm como alvo a inibição seletiva de fatores diretos da coagulação como a trombina e o FXa, usualmente (ALTMAN, 2014; SILVA, 2012; RAMOS *et al.*, 2013). Esses fármacos foram considerados superiores aos inibidores indiretos, uma vez que a HNF tem ação inibitória similar entre a trombina e o FXa, enquanto a HBPM possui maior capacidade inibitória relativa contra o FXa do que a trombina. Os antitrombínicos diretos têm pouco efeito na geração de trombina, mas são inibidores potentes da trombina que foi formada, ou seja, eles atuam sobre a trombina ativa e/ou ligada ao coágulo (O'BRIEN & MUREEBE, 2001).

Os anticoagulantes diretos ligam-se ao sítio ativo de fatores da coagulação específicos, inibindo a sua atividade enzimática (ALQUWAIZANI *et al*., 2013). Os inibidores diretos de trombina (IDTs) ligam-se a trombina sem a necessidade da presença de cofatores e sem interferir em outros mecanismos pró-trombótica, impedindo a formação do coágulo e a ativação de plaquetas. Os IDTs podem ser monovalentes, por interagirem apenas com o sítio ativo da trombina, e bivalentes, por interagirem com o sitio ativo e o exosítio (TERRA-FILHO *et al*., 2010; CORREIA-DA-SILVA *et al*. 2013). O protótipo do grupo dos inibidores específicos da trombina é a hirudina, isolado da glândula salivar da sanguessuga europeia *Hirudo medicinalis* (WALSMANN *et al*., 1985). A hirudina é um potente e específico inibidor bivalente de trombina, que inibe todas as ações proteolíticas da trombina de forma irreversível. Entretanto, é importante considerar a necessidade de monitoramento dos pacientes,

a administração parenteral e a ocorrência e hemorragias graves. Além disso, a hirudina é eliminada pelo sistema renal, sendo necessária cautela na administração em pacientes com insuficiência renal. Várias formas comerciais da hirudina foram desenvolvidas a partir da tecnologia do DNA recombinante. A lepirudina é um exemplo de hirudina recombinante dissulfatada, indicada para pacientes com TIH. Os estudos clínicos iniciais realizados nos Estados Unidos e Europa mostraram seus benefícios na diminuição de óbitos e amputação de membros (MAURO *et al*., 2004; TERRA-FILHO *et al*., 2010). O risco de hemorragia não é superior às heparinas, entretanto apresenta outros efeitos como reações cutâneas, aumento das enzimas hepáticas e seu uso por mais de 5 dias pode ocorrer a formação de anticorpos IgG anti hirudina, causando uma elevação do tempo de tromboplastina parcial ativada (TTPA). A bivalirudina, conhecida previamente como hirulog, também derivada da hirudina, inibe a trombina temporariamente, por exibir menor afinidade em comparação com a hirudina, resultando em um perfil de segurança mais favorável. Porém, a hirudina, a bivalurina e a lepirudina não apresentam antídoto (CORREIA-DA-SILVA *et al*. 2013; TERRA-FILHO *et al*., 2010). Outro IDT é o inibidor univalente: dabigatrana, que apresenta vantagens em relação aos antagonistas da vitamina K, nomeadamente, a sua farmacocinética, meia-vida mais curta, o que permite uma suspensão segura e metabolismo previsível, dispensando a monitorização da sua ação. A dabigatrana, assim como o rivaroxabada e a apixabana também são nomeados como anticoagulantes orais diretos (OADs).

A dabigatrana é inibidor direto e reversível da trombina, impedindo a conversão de fibrinogênio em fibrina (EBNER *et al*., 2010). Foi aprovado pela União Europeia, Canadá, Estados Unidos e Brasil para prevenção de TEV após cirurgia de substituição de quadril e joelho (MELNIKOVA, 2009) e AVC após FA não valvar (MARTINS *et al*., 2017). O etexilato de dabigatrana é uma pró-fármaco, metanosulfonato de éster etílico, que é convertida em dabigatrana ativo por meio de hidrólise catalisada por esterase (EBNER *et al*., 2010). Trata-se de um medicamento de uso oral, sendo a sua excreção por via renal. Assim, não é recomendado para pacientes com insuficiência renal moderada a grave. A dabigatrana, por sua vez, apresenta interações com medicamentos, principalmente associado a betabloqueadores (CORREIA-DA-SILVA *et al*., 2010; MAHAN, 2015; MARTINS *et al*., 2017).

Os inibidores diretos do FXa atuam no local de amplificação da cascata de coagulação sanguínea, sem a necessidade da participação da AT, inibindo a geração de trombina. O FXa, em comparação com a trombina, exerce menos funções fora da cascata de coagulação e, consequentemente, apresenta menos efeitos colaterais, razão pela qual se torna um alvo promissor para uso como medicamentos em várias patologias. A rivaroxabana é um derivado da oxazolidinona, composto orgânico heterocíclico, aprovado para uso em TEV, FA não valvular, AVC e pacientes submetidos à artoplastia de quadril e de joelho (SILVA, 2012; MAHAN, 2015). Foi aprovado no Brasil, Canadá, União Europeia e alguns países da Ásia e África (FLATO *et al*., 2011). A rivaroxabana tem ação rápida e meia-vida curta, resposta previsível, assim como a apixabana, outro inibidor direto do FXa. A apixabana teve sucesso na prevenção do AVC em pacientes com FA. Outro inibidor de FXa, a endoxabana foi aprovada na Europa para TEV e tromboembolismo pulmonar, AVC e embolismo sistêmico na FA não valvular. Com o avanço dos estudos clínicos o rivaroxabano, a apixabana e a endoxabana têm mostrado excelentes resultados na profilaxia da trombose venosa. As vantagens são numerosas quando postas em comparação com os AVK: não necessitam de monitoramento, a administração é oral e não são afetados por interações medicamentosas e alimentares. Contudo, também têm desvantagens como a ausência de antídoto disponível, possibilidade de ocorrências de hemorragias graves, preço mais elevado e, principalmente, a experiência clínica é reduzida, levando à sua menor prescrição. Segundo GLUND *et al*, 2015, o idarucizumab, fragmento de anticorpo monoclonal humanizado (Fab), está sendo utilizado como antídoto da dabigatrana livre e ligado à fibrina, revertendo a atividade anticoagulante da dabigatrana de forma imediata. Em 2017, entretanto, foi reportado um caso de anafilaxia após o uso de idarucizumab, tendo sido recomendado o monitoramento durante a infusão de idarucizumab (NAM *et al*., 2017). Assim, as heparinas e os AVKs, apesar de suas limitações, continuam sendo os anticoagulantes orais mais prescritos mundialmente (AFONSO *et al*. 2016). Portanto, os esforços para descobrir novos anticoagulantes são importantes para garantir um anticoagulante ideal, eficiente e com farmacocinética e farmacodinâmica previsível, com administração via oral, sem ajuste de dose, ausência de interação alimentar e/ou medicamentosa, ampla janela terapêutica, início de ação rápido, rápida reversibilidade e baixo custo (MARQUES, 2013).

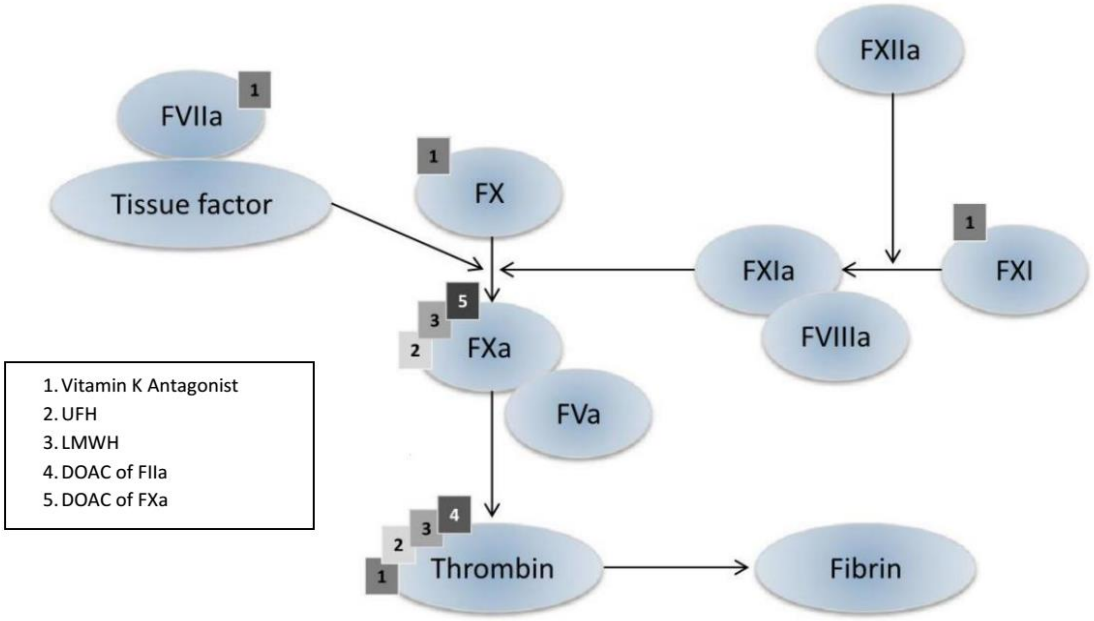

Figura 2. Representação esquemática da cascata de coagulação e fatores de coagulação que são inibidos por diferentes terapias anticoagulantes. Adaptado de LEENTJENS *et al*. (2017).

1.5 Proteases

Proteases ou peptidases são proteínas com atividade proteolítica que atuam na clivagem hidrolítica de diferentes ligações peptídicas entre os aminoácidos de proteínas e peptídeos. As proteases são encontradas em todos os organismos vivos, desde bactérias até mamíferos (BATT *el al*., 2015; PUENTE *et al*., 2003; RANASINGHE & McMANUS, 2013). As peptidases estão envolvidas em múltiplos processos fisiológicos, desde processos simples e não específicos, como a degradação de proteínas durante a digestão alimentar, até processos extremamente regulados e seletivos, tais como a clivagem de substratos específicos da cascata de coagulação, complemento e fibrinólise (BATT *el al*., 2015; PUENTE *et al*., 2003; SILVA-LOPEZ., 2009). Deste modo, uma vez que as proteases estão envolvidas em uma grande variedade de reações metabólicas, é necessário um controle preciso de suas atividades, visto que qualquer perturbação do equilíbrio de seu funcionamento pode causar patologias graves. Nos últimos anos, o campo de desenvolvimento de novos fármacos vem investindo em proteases como alvo de drogas. O FDA (*Food and*

Drug Administration) tem aprovado várias terapias com proteases em sua forma endógena e recombinante para tratamento de doenças cardiovasculares, incluindo trombose, AVC, infarto do miocárdio e hemofilias (BATT *et al*., 2015).

As proteases são classificadas de acordo com o seu mecanismo de catálise, as suas relações evolutivas e a presença de um resíduo específico de aminoácido no sítio ativo: aspartilprotease, metaloprotease, cisteinilprotease, serinoprotease e treoninaprotease (FAN *et al*., 2005; GODBOLE *et al*., 2022). São os resíduos do sitio ativo que atribuem alta especificidade às proteases, juntamente com os domínios presentes na superfície, que estão envolvidos no reconhecimento e interação com seus substratos e inibidores endógenos e exógenos (BATT *et al*., 2015; GOJOBORI & IKEO *et al*., 1994; VERSTEEG *et al*., 2013).

1.6 Inibidores de Serino proteases

As serino proteases são enzimas proteolíticas, que possuem um mecanismo catalítico comum que envolve a presença de um resíduo de serina em seu sítio ativo, participando diretamente na reação de quebra de ligações peptídicas. A maioria dos inibidores de proteases naturais são polipeptídeos, com raras exceções alguns microorganismos secretam pequenos compostos não-peptídicos que inibem a atividade das proteases de seus hospedeiros (SILVA-LOPEZ, 2009). Os inibidores de serino proteases também podem ser agrupados, com base em seu mecanismo de ação, em inibidores canônicos, inibidores não canônicos e serpinas (OTLEWSKI et al.,1999). As enzimas tripsina, quimotripsina e elastase são representantes clássicos da família. A catálise é realizada por um sistema de trocas de cargas, na tríade catalítica ácido aspártico-histidina-serina (Asp-His-Ser), presente no cerne do centro reativo destas enzimas. As serino proteases são encontradas em eucariotos, procariotos e vírus, estando envolvidas em diversos processos fisiológicos: resposta imune, digestão, homeostasia, apoptose (HEDSTROM, 2002). Atualmente, existe um forte investimento em pesquisas de inibidores de serino proteases devido ao seu papel modulador de uma variedade de funções biológicas tais como: coagulação sanguínea, sistema inflamatório e imunológico (LU *et al*., 2008; BORA *et al*., 2017).

Inibidores de serino proteases são classificados em 19 famílias com base em sua similaridade de sequência de aminoácidos, sítio ativo, características estruturais

e mecanismo de ação: Kunitz, Kazal, Bowman-Birk, serpinas, α-macroglobulina e pacifastina (LASKOWSKI & KATO, 1980; LASKOWSKI & QASIM, 2000; PONPRATEEP *et al*., 2017).

1.7 Inibidores do tipo Kunitz

Os inibidores de serinoproteases do tipo Kunitz são ubíquos, estão presentes em plantas, animais e microorganismos. A família de inibidores do tipo Kunitz apresenta estruturas funcionais geralmente constituídas por seis resíduos de cisteínas conservados formando três pontes dissulfeto, baixo peso molecular próximo ou inferior a 20 kDa e apresentam cadeias polipeptídicas de cerca de 60 resíduos de aminoácidos. Os domínios Kunitz são estáveis e tem a capacidade de reconhecer estruturas proteicas específicas, funcionando como inibidores competitivos reversíveis (BROZE & GIRARD, 2012). Esses inibidores podem conter um domínio (como o inibidor de tripsina pancreática bovina - BPTI) ou mais domínios (como TFPI, inibidor da via do fator tecidual, com três domínios). Cada domínio envolve α–hélices ricas em dissulfeto e fitas-β estabilizadas por três pontes dissulfeto altamente conservadas. A natureza dos dobramentos e o posicionamento das três pontes dissulfeto tornam a molécula compacta e estável (BLISNICK *et al*., 2017).

O mecanismo de inibição dos inibidores do tipo Kunitz ocorre pelo mecanismo canônico. O mecanismo canônico envolve uma forte interação não covalente, formando um complexo enzima-substrato ou enzima-inibidor (Figura 3). Os inibidores bloqueiam diretamente o sítio ativo das serino proteases sem alterações conformacionais e formam fitas-β antiparalelas entre a enzima e o inibidor. O segmento responsável pela inibição da protease é chamado de *loop* de ligação à protease. Este *loop* fica exposto ao solvente e é altamente complementar ao sítio ativo da protease.

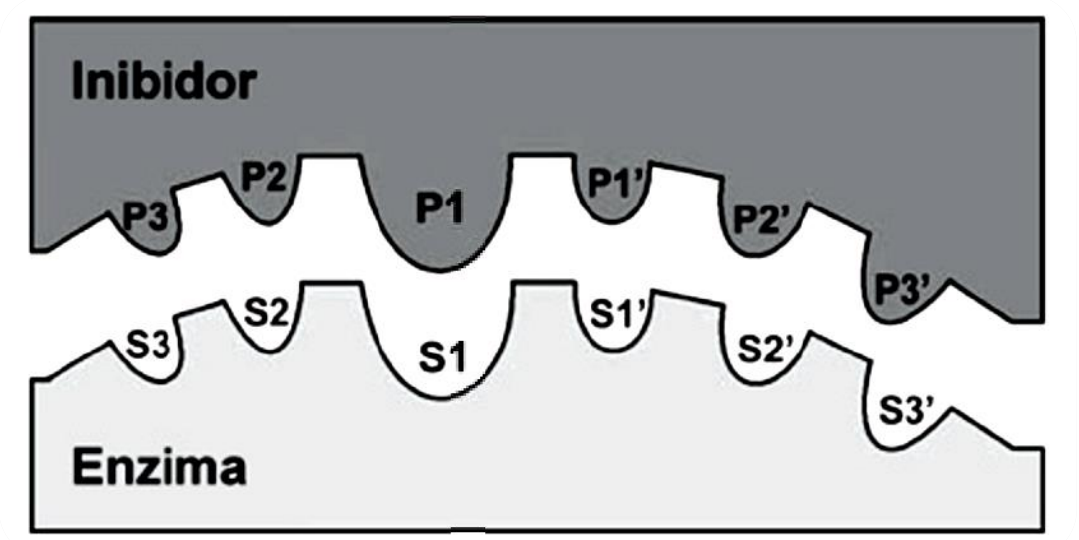

Figura 3. Representação da interação dos aminoácidos catalíticos (S) do sítio ativo da enzima (serinoprotease alvo) com os aminoácidos que sofrerão hidrólise no peptídeo (P) (inibidor). Adaptado de SILVA-LOPEZ (2009).

A interação entre enzima e inibidor pode ser apresentada de forma simplificada como uma reação de hidrólise da ligação peptídica $P_1 – P_1'$, que fica na curva externa do *loop* do centro reativo (RCL), resultando na formação do complexo no qual o *loop* reativo do inibidor está ligado ao sítio ativo da enzima. No complexo da interação enzima-inibidor, entre 10 e 17 resíduos de aminoácidos no sítio ativo do inibidor e entre 17 e 29 resíduos da protease, fazendo numerosas interações de força de van der Waals e ligações de hidrogênio (RANASINGHE & McMANUS, 2013).

O inibidor de tripsina pancreática bovina (BPTI) foi o primeiro inibidor do tipo Kunitz descrito e responsável por dar o nome à família, fazendo parte dos membros clássicos (BLISNICK *et al*., 2017; DREWES *et al*., 2012; KUNITZ & NORTHROP, 1936). Outro inibidor clássico, integrante da via regulatória da coagulação sanguínea, é o TFPI, o qual tem papel central na via extrínseca, por regular a atividade do complexo FVIIa/FT e do FXa. Portanto, ele é o inibidor da formação de trombina pela cascata de coagulação. Nos últimos anos, muitos inibidores do tipo Kunitz vem sendo descritos estruturalmente e funcionalmente, a fim de caracterizar novas moléculas de plantas e animais como alvos para o desenvolvimento de biofármacos (BROZE & GIRARD, 2012).

1.8 Animais Hematófagos

Os inibidores de serino protease desempenham um papel crucial no processo de alimentação de animais hematófagos, que podem ficar fixos por períodos prolongados em seus hospedeiros. Durante esse período de alimentação, esses animais causam lesões teciduais em seus hospedeiros, ativando o processo de homeostase (CHMELAR *et al*., 2016; MANS *et al*., 2011). Sendo assim, esses animais necessitam inibir as reações locais do hospedeiro para manter o sangue fluido no momento da captação, impedindo a coagulação do sangue no seu próprio sistema digestório (MACKMAN, 2009). Devido a essa necessidade, eles desenvolveram, ao longo da evolução, uma grande diversidade de substâncias que são injetadas no hospedeiro através da saliva, as quais permitiram o sucesso do parasitismo. As estratégias utilizadas pelos ectoparasitas são muito variadas, sendo fundamentadas em inúmeras substâncias que interferem em diferentes pontos da coagulação. Essa conquista se deve ao fato dos ectoparasitas terem desenvolvido a capacidade não só de inibir reações hemostáticas, mas também às reações inflamatórias e imunológicas desempenhadas pelo sistema de defesa do hospedeiro (RIBEIRO *et al*., 1997; GILLESPIE *et al*., 2000; MANS *et al*., 2011). A caracterização dessas substâncias tem mostrado uma grande variedade de estruturas e funções úteis à terapêutica, assim como uma ferramenta promissora para estudos da fisiologia dos processos vasculares e hemostáticos (CIPRANDI *et al*., 2003; FRANCISCHETTI *et al*. 2009).

Muitos compostos isolados de animais hematófagos, ou análogos projetados a partir deles, vêm sendo testados para o desenvolvimento de anticoagulantes mais específicos e seguros, com menos efeitos colaterais sistêmicos (CIPRANDI *et al*., 2003). Assim, devido ao estudo dessas moléculas responsáveis por estes efeitos tem se tornado possível desenvolver novos anti-hemostáticos de uso clínico (FRANCISCHETTI *et al*. 2009). A utilidade terapêutica destes compostos tem sido confirmada por estudos pré-clínicos e clínicos (MARCO *et al*., 2008).

A hirudina foi produzida através da técnica de DNA recombinante, resultando em uma proteína recombinante estruturalmente quase idêntica à hirudina natural, exceto pela falta de um grupo sulfato na tirosina. Seu mecanismo de ação consiste na ligação a dois sítios da trombina: a extremidade C-terminal liga-se ao sítio de reconhecimento do fibrinogênio; já a sua extremidade N-terminal liga-se ao sítio catalítico, inibindo a

reação autocatalítica (BLAYA *et al*, 1998; CIPRANDI *et al*. 2003). Derivados de hirudina recombinantes como bivalirudina, desirudina, lepirudina têm sido aplicados no tratamento de pacientes com complicações trombóticas (CANNON *et al*., 1995). A bivalirudina, mais especificamente, é um análogo semissintético da hirudina que possui meia-vida mais curta, o que é considerado uma vantagem sobre a hirudina a qual depende de um antídoto eficaz. Estudos clínicos comprovam sua eficácia durante angioplastia coronariana sem apresentar complicações hemorrágicas e infarto do miocárdio (KASTRATI, *et al*., 2011). Outros inibidores exógenos da coagulação vêm sendo caracterizados a partir da saliva de invertebrados hematófagos, incluindo anophelina de *Anopheles albimanius* (FRANSISCHETI, *et al*., 1999), entre outros.

Nesta procura de novas fontes para isolamento de compostos ativos, moléculas com atividade anticoagulante vêm sendo caracterizadas a partir de carrapatos, os quais são artrópodes hematófagos obrigatórios (DE LA FUENTE *et al*., 2008; IWANAGA *et al*., 2003). Inibidores de serinoprotease do tipo Kunitz são os mais abundantemente encontrados na saliva de carrapatos moles e duros (CHMELAR *et al*. 2012). Muitas das proteínas presentes na saliva destes ectoparasitas atuam como reguladores naturais de muitos processos fisiológicos de vertebrados, em especial a hemostasia (CHMELAR *et al*. 2012). Inibidores endógenos do tipo Kunitz tem como alvo várias serinoproteases de etapas diferentes da coagulação como o FVII, FXa, entretanto não inibem a trombina diretamente. Surpreendentemente, alguns inibidores do tipo Kunitz apresentam um mecanismo de inibição não-canônico, o que conferia a estes inibidores a capacidade de inibir a trombina (CORRAL-RODRIGUEZ *et al*., 2009). Esses inibidores seguiam o mesmo mecanismo de inibição da hirudina, inserindo os resíduos presentes no N-terminal no sítio ativo e formando fitas-β paralelas com a trombina (RANASINGHE & McMANUS, 2013).

Estudos com o carrapato da espécie *Ixodes ricinus*, pertencente à família *Ixodidae* (carrapatos duros), pela primeira vez em 1899, demonstraram que extratos proteicos deste parasita evitavam a coagulação sanguínea. Mais tarde, essa atividade foi caracterizada como um inibidor de FXa e denominada de ixodina. O *I. ricinus* apresenta também um inibidor de trombina denominado ixina, que inibe também a agregação plaquetária induzida por trombina (HOFFMANN *et al*. 1991). A partir disso, outros estudos envolvendo também outras espécies de carrapato levaram a descobertas de outros inibidores de carrapato com potencial uso terapêutico em

doenças cardiovasculares, coagulação, fibrinólise e angiogênese (FRANCISCHETTI *et al*. 2009; PARIZI *et al*., 2017).

A inibição da trombina é uma maneira eficiente de reprimir a coagulação sanguínea, agregação plaquetária e atividade de outros fatores da cascata de coagulação. É importante ressaltar que a trombina também é responsável por ativar a via do sistema complemento ou inflamação. Consequentemente, vários inibidores de trombina já foram caracterizados em diferentes espécies de carrapato (CHMELAR *et al*., 2012; LAI *et al*., 2004). A ornitodorina é um inibidor de serinoprotease do tipo Kunitz do carrapato *Ornithodoros moubata* (Figura 4), que se diferencia do BPTI clássico, pois foi o primeiro inibidor do tipo Kunitz caracterizado estrutural e funcionalmente apresentando modo de inibição do tipo não-canônico semelhante ao modelo de inibição da hirudina (CORRAL-RODRIGUEZ *et al*., 2009; VAN DE LOCHT *et al*., 1996; VELDÉS *et al*., 2013). A diferença entre o mecanismo canônico e não-canônico pode ser explicado, neste caso, comparando a ornitodorina com o BPTI: o BPTI insere seu único domínio em forma de RCL da trombina; já a ornitodorina, por sua vez, possui dois domínios, formando *loops* com as fitas-β antiparalelas, que não entram em contato com o sítio ativo da trombina. Deste modo, a inibição da trombina ocorre por inserção do N-terminal no sítio ativo da trombina, enquanto o C-terminal interage com o exosítio I hidrofóbico (CORRAL-RODRÍGUEZ *et al*., 2009).

O mesmo mecanismo de inibição não-canônico ocorre em outros inibidores de carrapatos, como a savignina do carrapato *Ornithodoros savignyn* (NIENABER *et al*., 1999), a boofilina do *Rhiphicephalus (Boophilus) microplus* (MACEDO-RIBEIRO *et al*., 2008). A boofilina, além de inibir a trombina, também exibe a propriedade de, quando complexada à trombina, inibir a atividade de uma segunda serinoprotease, do tipo tripsina, com mecanismo canônico (MACEDO-RIBEIRO *et al*., 2008; CEREIJA *et al*, 2012). O mesmo ocorre com a hemalina do carrapato *Haemaphysalis longicornis,* que também apresenta atividade semelhante à boofilina de inibição da trombina (LIAO *et al*. 2009). Outro inibidor de trombina é a amblina (LAI *et al*, 2004), que apresenta alta similaridade com o TFPI e Ixolaris, primeiro inibidor de trombina encontrado na hemolinfa do carrapato *Amblyomma hebraeum* (CARNEIRO-LOBO *et al*., 2009). A Rhipilin-2 do *Rhipicephalus haemaphysaloides* prolonga o tempo de protrombina (PT) e ativa o tempo tromboplastina parcial ativada (TTPA) (CAO *et al*., 2013). Cumpre informar, que o PT é usado como teste para diagnosticar deficiências nas vias

extrínseca e comum da coagulação, já o TTPA é usado para testar a via intrínseca. A especificidade do rhipilin ainda não foi determinada. No entanto, como ele inibe ambas as vias, pode ser um inibidor de FXa e/ou trombina (BOAS *et al*., 2014; DIAS *et al*., 2007).

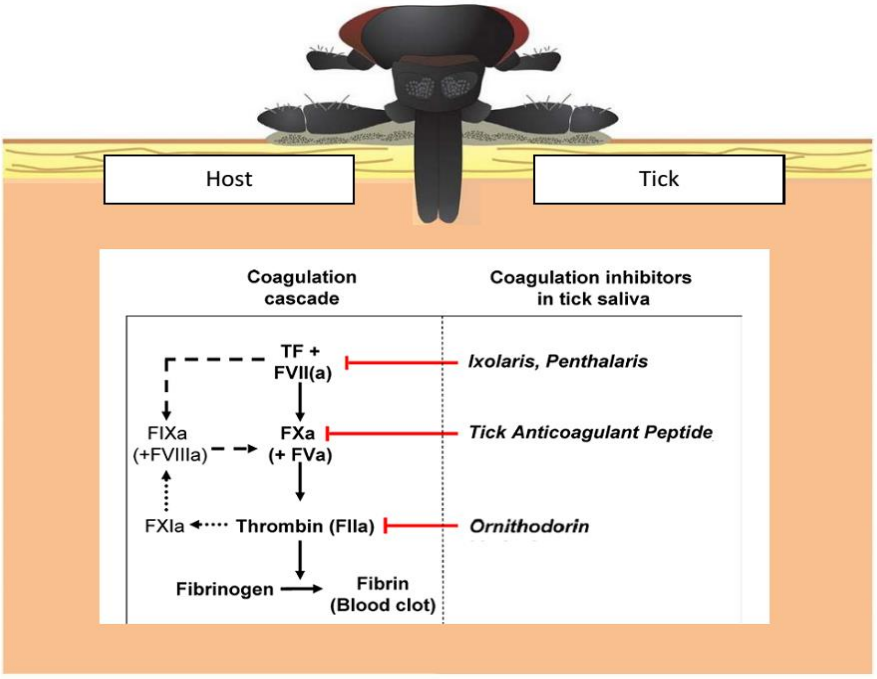

Figura 4. Diagrama mostrando uma visão geral da cascata de coagulação e pontos onde atuam inibidores oriundos de carrapatos de diferentes espécies. Adaptado de HOVIUS *et al*. (2008).

O FXa é responsável por ativar o complexo protrombinase para geração de trombina. O primeiro inibidor de serino protease do tipo Kunitz de FXa identificado foi o peptídeo anticoagulante de carrapato (TAP) (Figura 4), com apenas 60 aminoácidos do carrapato *O. moubata* (WAXMAN *et al*., 1990). TAP forma um complexo estequiométrico com o FXa. O TAP se liga tanto ao sítio ativo quanto ao exosítio do FXa. O ixolaris presente na saliva do carrapato *I. scapularis* possui dois domínios Kunitz, à semelhança do inibidor da via do fator tecidual (Figura 4). O inibidor ixolaris se liga ao complexo TF/FVIIa, inibindo o início da coagulação. Além disso, ele se liga ao FX não ativado, impedindo, assim, a ativação de FX. A forma recombinante inibe a coagulação na faixa de picomolar, além disso, apresenta uma atividade antitumoral

inibindo o crescimento de glioblastoma humano (CARNEIRO-LOBO *et al*., 2009; FRANCISCHETTI *et al*., 2002; MCEACHRON *et al*., 2009). Outro inibidor potente é o penthalaris, homólogo do TFPI foi caracterizado da saliva do carrapato *I. scapularis* (Figura 4). O penthalaris apresenta atividade anticoagulante dentro da faixa nanomolar (FRANCISCHETTI *et al*., 2004). Assim como o ixolaris, o penthalaris inibe a cascata de coagulação ao se ligar ao complexo tenase da via extríseca e impedir a ativação do FX (FRANCISCHETTI *et al*., 2004). O amblyomina-X é um inibidor do tipo Kunitz que foi identificado no transcriptoma de *Amblyomma cajennense*, conhecido como carrapato-estrela, através da análise de sequências ESTs de uma biblioteca de cDNA. Essa proteína é capaz de inibir o fator FXa na presença de fosfolipídios e de prolongar os tempos globais de coagulação como o tempo de trombolastina parcial ativado (TTPA) e o tempo de protrombina (TP). Recentemente, foi demonstrado que a proteína possui atividade pró-apoptótica em células tumorais e, em experimentos *in vivo*, promove regressão da massa tumoral e redução de metástases de alguns tumores testados (DREWES *et al*., 2012; CHUDZINSKI-TAVASSI *et al*., 2016).

Assim, neste trabalho, foi caracterizado uma nova molécula multifuncional a partir de uma espécie de carrapato que tem capacidade de parasitar humanos, o *Ixodes persulcatus.* Este carrapato é transmissor da doença de Lyme no leste da Europa e na Ásia (STEERE *et al*., 2004). O ectoparasita *I. persulcatus* pode apresentar mecanismos interessantes para controlar a cascata da coagulação. Deste modo, este trabalho visou clonar, expressar e realizar a caracterização da sequência codificante da proteína, nomeada persulcatina, e caracterizar sua ação sobre a trombina, enzima envolvida na cascata da coagulação. Moléculas com alta identidade a persulcatina já foram caracterizadas em outras espécies de carrapato como o inibidor do tipo Kunitz da trombina, boofilina (MACEDO-RIBEIRO *et al*., 2008).

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3. OBJETIVOS

3.1 Geral

Estudar inibidores do tipo Kunitz do ectoparasita hematófago *I. persulcatus* e obter na forma recombinante uma molécula com provável atividade anticoagulante.

3.2 Específicos

- Identificar inibidores do tipo Kunitz no ectoparasita hematófago *I. persulcatus*.
- Estudar as características estruturais e a interação de um inibidor tipo Kunitz de *I. persulcatus* com a trombina.
- Obter uma proteína com potencial anticoagulante na forma heteróloga para estudos futuros.

4. ARTIGO

O presente artigo foi elaborado em língua estrangeira e está formatado conforme as normas da revista Journal of the American Academy of Dermatology (Fator de Imacto: 7,59 (2023).

A novel Kunitz-type inhibitor from the taiga tick *Ixodes persulcatus* interacts with plasmin and thrombin impairing keratinocyte migration, coagulation and endothelial cell permeability

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Abstract

The skin is the first host tissue that the tick mouthparts, tick saliva, and a tick-borne pathogen contact during feeding. Tick salivary glands have evolved a complex and sophisticated pharmacological armamentarium, consisting of bioactive molecules, to assist blood feeding and pathogen transmission. In this work, persulcatin, a new multifunctional molecule that targets keratinocyte function and hemostasis, was identified from *Ixodes persulcatus* female ticks. The recombinant persulcatin was purified and found to be a 25 kDa acidic protein with two Kunitz-type domains. Persulcatin is expressed in salivary glands and other tissues. It is a classical tight-binding competitive inhibitor, targeting plasmin (K_i: 28 nM) and thrombin (K_i: 115 nM). It blocks plasmin generation on keratinocytes and inhibits its migration, matrix protein degradation, down-regulates MMP-2 and -9, and causes a delay in blood coagulation, endothelial cell activation, and thrombin-induced fibrinocoagulation. It interacts with exosite I of thrombin and reduces thrombin-induced endothelial cell permeability by inhibiting VE-cadherin disruption. The multifaceted roles of persulcatin as an inhibitor and modulator within the thrombin and plasminogen-plasmin system not only unveils new insights into the intricate mechanisms governing wound healing but also provides a fresh perspective on the intricate interactions between ticks and their host organisms.

Keywords: *Ixodes*; persulcatin; thrombin; coagulation; kunitz inhibitor

5. CONCLUSÂO

A terapia anticoagulante é, cada vez mais, usada na prática clínica para a profilaxia e o tratamento de doenças tromboembólicas, que são responsáveis por uma alta mortalidade em todo mundo. Uma das alternativas para minimizar os efeitos adversos dessas terapias, foi a busca por anticoagulantes naturais. Assim, visando a caracterização de novas moléculas com protencial anticoagulante, neste estudo foi realizada a clonagem da sequência codificante da proteína persulcatina, um inibidor de serino proteinase do tipo Kunitz do ectoparasita hematófago I. persulcatus, em vetor de expressão e sua expressão em culturas de células Expi293. Os resultados obtidos por meio das análises enzimáticas mostraram que a persulcatina é um inibidor competitivo da α-Trombina, capaz de inibir mais de 80% do seu potencial de coagulação, causando um aumento do tempo de coagulação do plasma humano. A persulcatina realiza essa inibição por meio do seu exosítio-1, assim como a hirudina, que é, reconhecidamente, considerada como um potente e específico inibidor de trombina (WALSMANN et al., 1985)

Além disso da sua capacidade de inibição da trombina, constatou-se que a persulcatina é um inibidor clássico competitivo de ligação forte e rápida da plasmina, funcionando como um modulador negativo do sistema plasminogênio-plasmina, causando a redução da capacidade migratória dos queratinócitos. Ademais, a persulcatina também apresenta regulação negativa na expressão MMP-2 e -9, reduzindo a degradação de proteínas da matriz celular. Esse mecanismo duplo, induzido pela persulcatina, revela um complexo meio regulatório de inibição da sinalização, reduzindo a capacidade de reparo tecidual.

Essas descobertas representam o importante papel da persulcatina na modulação inicial dos sistemas de coagulação e inflamação do hospedeiro, que podem contribuir com pesquisas futuras, não apenas relacionadas ao reparo tecidual e à terapia anticoagulante, mas também fornecer uma nova perspectiva sobre as interações entre carrapatos e seus organismos hospedeiros.

6. ANEXO- Normas de Publicação da revista

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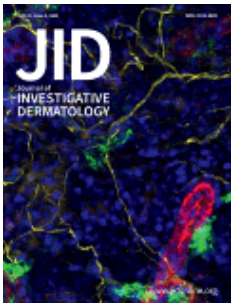

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Reporting sex- and gender-based analyses

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Sex generally refers to a set of biological attributes that are associated with physical and physiological features (e.g., chromosomal genotype, hormonal levels, internal and external anatomy). A binary sex categorization (male/female) is usually designated at birth ("sex assigned at birth"), most often based solely on the visible external anatomy of a newborn. Gender generally refers to socially constructed roles, behaviors, and identities of women, men and gender-diverse people that occur in a historical and cultural context and may vary across societies and over time. Gender influences how people view themselves and each other, how they behave and interact and how power is distributed in society. Sex and gender are often incorrectly portrayed as binary (female/male or woman/man) and unchanging whereas these constructs actually exist along a spectrum and include additional sex categorizations and gender identities such as people who are intersex/have differences of sex development (DSD) or identify as non-binary. Moreover, the terms "sex" and "gender" can be ambiguous—thus it is important for authors to define the manner in which they are used. In addition to this definition guidance and the SAGER guidelines, the resources on this page offer further insight around sex and gender in research studies.

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Appendix 1

Matthew Marek[a], Manthoula Tucker[b], Yinghong Le[c], Browning Schwarz[c], Norris Fisher[a], Edward Yilmaz[d], Heidi Chan[d], Phyllis Bruno[a]

[a]Department of Medical and Morphological Sciences, Example University, Milan, Italy; [b]Department of Aging, Sample University, New York, NY USA; [c]Department of Dermatology, University of Examples, London, UK; [d]Medical University of Examples, San Francisco, CA USA

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Line Numbers: Do not number lines of text.

Order of Sections: Title page, Abstract, Introduction, Results, Discussion (Results and Discussion may be combined into one section), Materials & Methods, Conflict of Interest, Acknowledgements, References, Tables, Figure Legends, Supplementary Material.

Heading Styles: First level: **ALL CAPS, BOLD**; Second level: **Sentence Case, Bold Underline**;Third level: ***Sentence Case, Bold Italics***

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