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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 competitivode 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 thispanorama. 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 (Figura1).

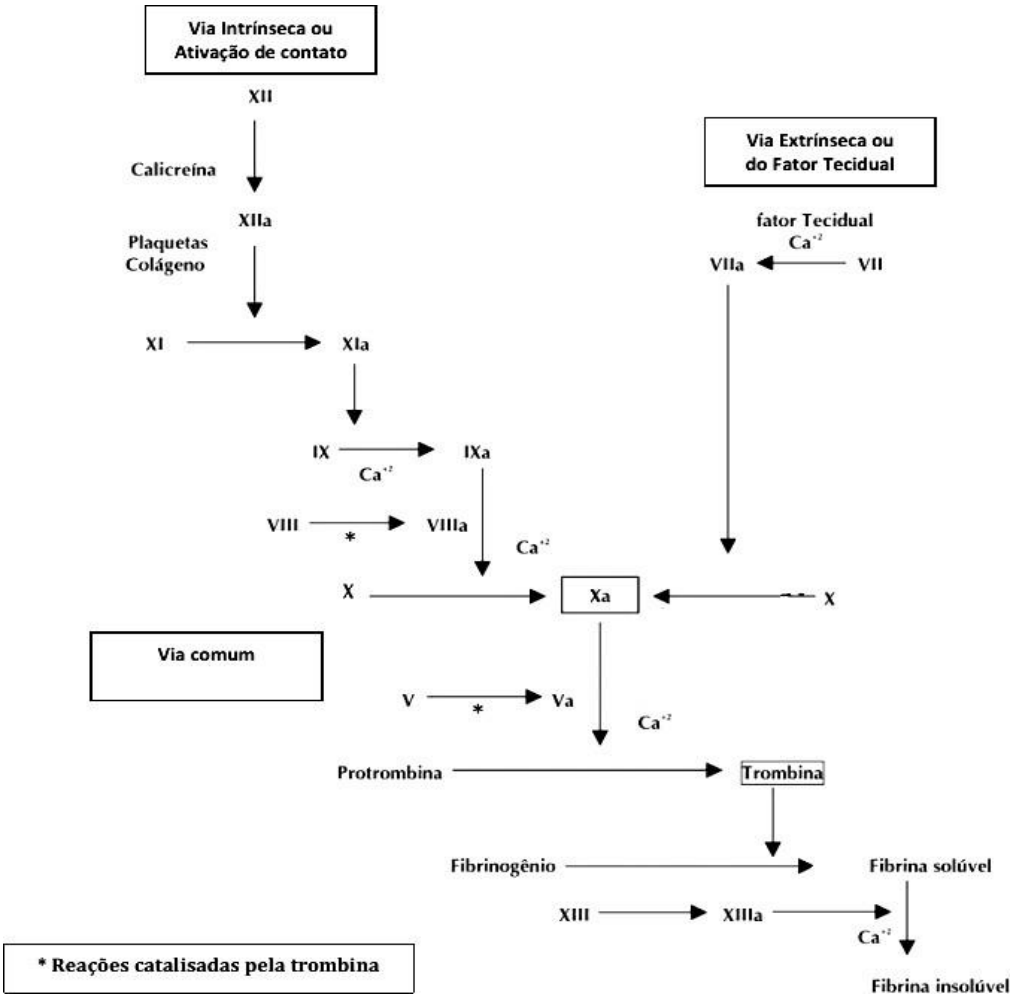

Figura 1. Representação diagramática da cascata de coagulação sanguínea. Adaptado de Mauro *et al*. (2004).

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, problemasmais 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 Virchowtambém 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 paraa 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 diminuindoa 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 eduração 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 HNFtem 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, inibea 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 comoanticoagulantes orais diretos (OADs).

A dabigatrana é inibidor direto e reversível da trombina, impedindo a conversãode 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 comfarmacocinética 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).

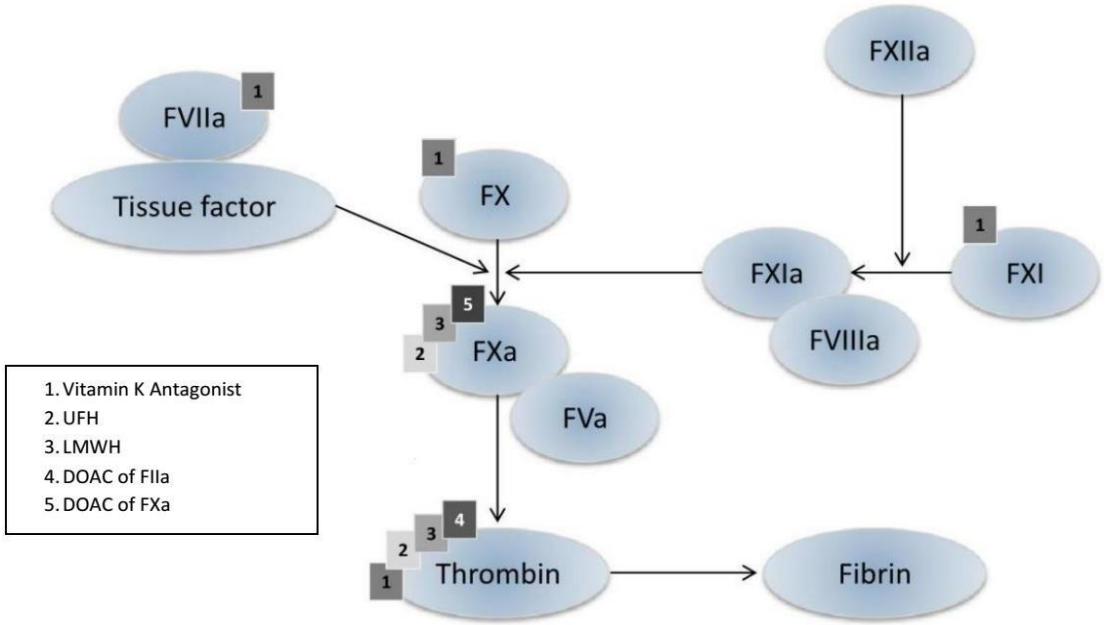

Figura 2. Representação esquemática da cascata de coagulação e fatores de coagulação que são inibidos por diferentes terapias anticoagulantes. Adaptado de LEENTJENS *et al*. (2017).

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, assuas 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 deserino 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 clássicosda 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 emdissulfeto 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 ativoda protease.

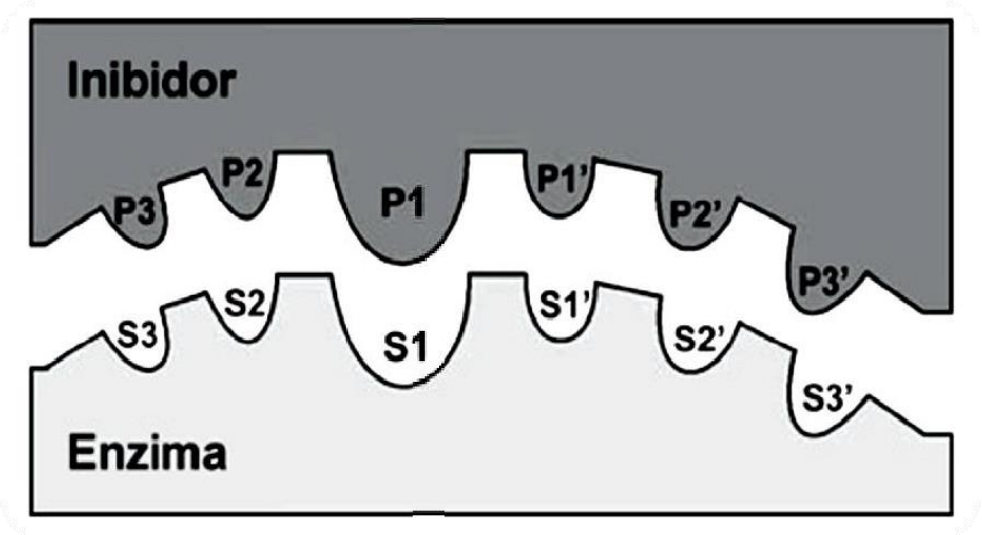

Figura 3. Representação da interação dos aminoácidos catalíticos (S) do sítio ativo da enzima (serinoprotease alvo) com os aminoácidos que sofrerão hidróliseno peptídeo (P) (inibidor). Adaptado de SILVA-LOPEZ (2009).

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 comoreguladores 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 atividadefoi 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 avia 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,* quetambém 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).

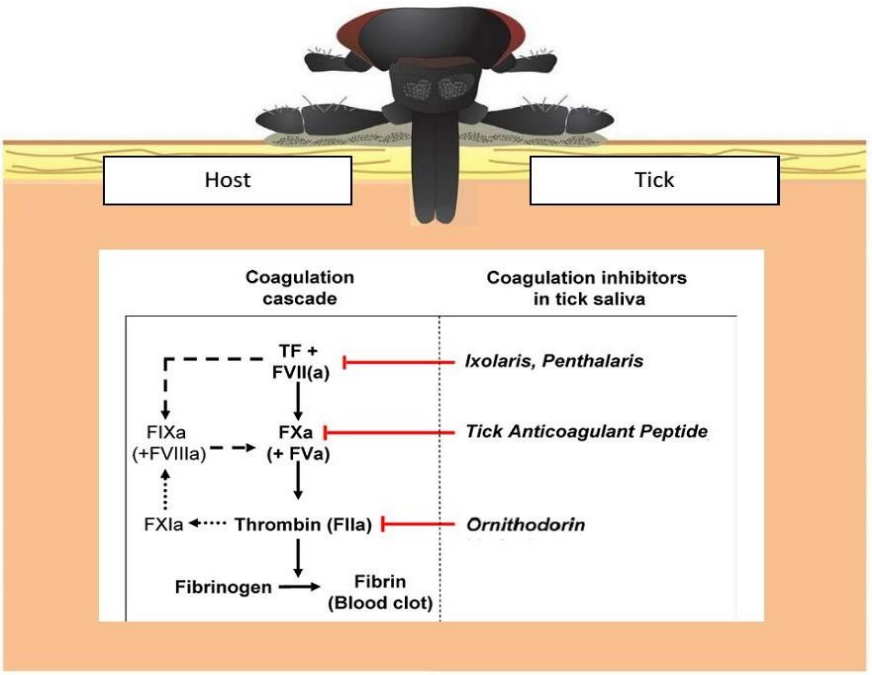

Figura 4. Diagrama mostrando uma visão geral da cascata de coagulação e pontos onde atuam inibidores oriundos de carrapatos de diferentes espécies. Adaptado de HOVIUS *et al*. (2008).

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 ixolarisse liga ao complexo TF/FVIIa, inibindo o início da coagulação. Além disso, ele se ligaao FX não ativado, impedindo, assim, a ativação de FX. A forma recombinante inibea 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 openthalaris, 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 aativação 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 decDNA. 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, enzimaenvolvida na cascata da coagulação. Moléculas com alta identidade a persulcatina jáforam 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

3.4 Geral

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

3.5 Específicos

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

4. ARTIGO

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A 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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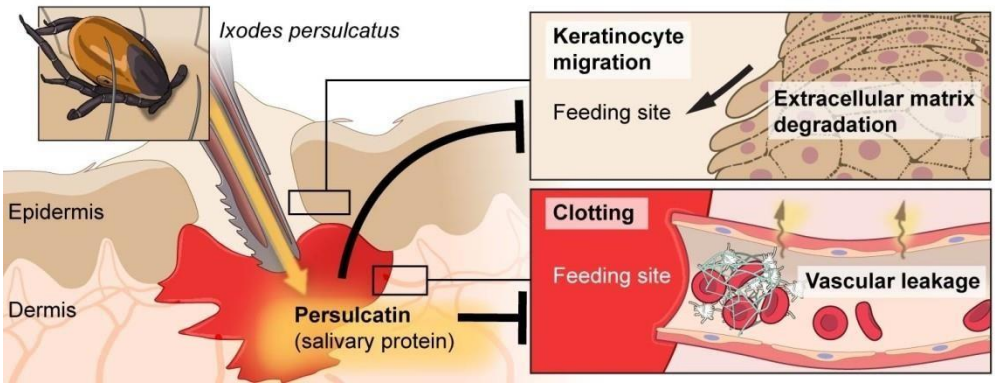

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A Kunitz-type inhibitor from the taiga tick *Ixodes persulcatus* interacts with plasmin and thrombin impairing keratinocyte migration, coagulation and endothelial cell permeability

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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 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 and is a classical tight-binding competitive inhibitor of proteases, 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 plasminogen-plasmin system and thrombin 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: ticks; tick saliva; wound healing; coagulation; protease inhibitors

INTRODUCTION

Ticks are ectoparasites that feed on blood from various vertebrate hosts and are vectors of a wide variety of pathogens with veterinary and medical importance (Rochlin & Toledo, 2020). They are blood pool feeders, meaning that they feed by lacerating small blood vessels located under the skin and ingesting blood directly from the wound (Bartíková et al., 2020). The tick feeding cycle can be divided in three main phases: the preparatory phase, when the tick attaches onto host`s skin, creating the feeding lesion; the slow feeding phase, during which the tick ingests moderate amounts of blood and begins to transmit pathogens through saliva; and the rapid feeding phase, when the tick feeds to repletion (Franta et al., 2010). Depending on the stage and the tick species, it might take several days for ticks to complete their blood-feeding process (Saraiva et al., 2014).

The feeding style utilized by ticks triggers several host-derived responses, and they face the challenge of wound healing, which is achieved through four precisely and highly programmed phases: hemostasis, inflammation, cell proliferation and migration, and remodeling (Qu & Chaikof, 2010), altogether representing barriers to the acquisition of a blood meal. To overcome these barriers, tick salivary glands have evolved a complex and sophisticated pharmacological armamentarium, consisting of bioactive molecules, to assist blood feeding. Tick saliva contains hundreds of compounds that have anti-coagulant, vasodilatory, anti-inflammatory, and immunomodulatory functions, supporting the blood feeding (Ali et al., 2022; Francischetti, 2009). While helping the vector to feed, its saliva also modifies the site where pathogens are injected and, in many cases, facilitates the infection process (Nuttall, 2023).

The skin serves as the first host tissue that the tick mouthparts, tick saliva, and a tick-borne pathogen contact during feeding. Consequently, tick saliva has evolved with various

molecules capable of modulating the wound healing (Bartíková et al., 2020). In this study, we characterized a new Kunitz-type serine protease inhibitor obtained from *Ixodes persulcatus*, a tick species geographically distributed in Far East Asia and South Eurasia and known to be a vector for several human pathogens (Alekseev et al., 1999; Konnai et al., 2011; Randolph, 2008). Named persulcatin, this inhibitor was found to effectively inhibit plasmin and thrombin, modulate keratinocyte migration, affect blood coagulation, and impact endothelial cell function. The biological functions of persulcatin emerge as a novel tick salivary molecule that impairs wound healing.

RESULTS

Persulcatin is a Kunitz-type inhibitor of plasmin and thrombin isolated from *I. persulcatus* ticks

The persulcatin cDNA consists of 438 bp, coding for a protein of a 145-amino acid protein, including a 15-amino acid signal peptide. Sequence and predicted structural analyzes revealed the presence of two Kunitz-type domains containing six conserved cysteine residues, which are predicted to form three disulfide bonds per domain. The P1 residue, located in the inhibitory reactive site, is an arginine residue in the first domain and a lysine in the second domain (Fig 1A). Alignment with other tick proteins belonging to the Kunitz-type serine protease inhibitor family demonstrated conserved amino acid residues and similarity (Figure 1A). The phylogenetic tree constructed using sequences of Kunitz-type inhibitors from different species indicated that persulcatin is evolutionarily related to boophilin and hemalin, previously identified in *Rhipicephalus microplus* and *Haemaphysalis longicornis* ticks, respectively (Figure 1B). The

predicted tertiary structure of persulcatin further confirmed the presence of the two inhibitory domains commonly found in Kunitz-type inhibitors (Figure 1C).

Persulcatin expression was examined in different tick stages and organs, suggesting its potential role in tick feeding. An increase in transcription was observed after blood feeding in larvae and nymphs. Adult females in the fed stage showed higher expression levels in the salivary gland, midgut, and ovary (Figure 1D).

Recombinant persulcatin was successfully expressed in HEK293 cells and subsequently purified using affinity, ion-exchange, and gel-filtration chromatography (Figure 1E). Protein homogeneity was confirmed through reducing SDS-PAGE, showing a single band at approximately 25 kDa (Figure 1E, inset). The specificity of persulcatin was verified in a screening assay optimized to detect protease inhibitors. Persulcatin blocks partially or entirely the amidolytic activity of α-thrombin, activated protein C, pancreatic chymotrypsin, plasmin, and pancreatic trypsin (Figure 1F). Since the screening with the peptide-derived amidolytic substrate for α-thrombin indicated a partial inhibition, we proceed to confirm this activity using the macromolecular substrate, fibrinogen. As shown in Figure 1G, persulcatin (at 1µM) inhibits over 80% of α-thrombin clotting potential.

Persulcatin is a classical fast, tight-binding competitive inhibitor of plasmin

The Figure 2 shows the detailed mechanism of human plasmin inhibition by persulcatin, utilizing the peptide-derived chromogenic substrate S-2251. Progress curves demonstrated that plasmin inhibition occurs in a dose-dependent manner (Figure 2A). Preincubation time between the enzyme and inhibitor did not significantly change the progress curves behavior, suggesting that plasmin inhibition by persulcatin occurs in a fast-binding mode (Figure 2B). Plotting the fractional

velocity as a function of persulcatin at different enzyme concentrations resulted in directly proportional IC_{50} values, indicating that persulcatin is a tight-binding inhibitor for plasmin (Figure 2C). This tight-binding inhibition was confirmed by plotting the IC_{50} values as a function of enzyme concentrations at a single fixed substrate concentration, yielding an apparent K_i (K_i^{app}) of 13 nM (Figure 2D). Considering that persulcatin is a tight binding inhibitor, the Morrison equation (Morison et al., 1950) was used to fit fractional velocities as a function of inhibitor at different substrate concentrations (Figure 2E). The IC_{50} values showed a linear relationship with substrate concentrations, indicating a competitive inhibition mode of action (Figure 2E, inset). Additionally, the classical graphic method of Dixon (Dixon, 1953) for tight-binding competitive inhibitors was utilized to determine the real K_i, resulting in a K_i of 28 nM (Figure 2F).

Persulcatin inhibits keratinocyte migration induced by plasmin

The plasminogen/plasmin system plays a crucial role in regulating pericellular proteolysis events involved in cell migration and wound healing (Li et al., 2003). To investigate whether keratinocyte surfaces can generate active plasmin, triggering cell migration, we conducted experiments as shown in Figure S1. The results demonstrate that plasminogen (PLG) dose-dependently accelerates keratinocyte migration in the scratch-induced assay (Figure S1A and B). Furthermore, active plasmin was also generated on keratinocyte surface after the addition of PLG, showing a dose- (Figure S1C) and time- (Figure S1D) dependent manner.

In Figure 3 and Figure S2, we present evidence that persulcatin hinders PLG/plasmin-induced keratinocyte migration. The scratch-induced revealed reduced keratinocyte migration in PLG stimulated cells when persulcatin was present (Figure 3A and B). Additionally, persulcatin-treated cells generated less active plasmin, as detected in the conditioned culture media obtained

from keratinocytes (Figure 3C). This observation was further confirmed by zymography using fibrin as substrate (Figure 3D) and fibrin clot degradation assays (Figure 3E), where persulcatin clearly blocked fibrin degradation induced by formed plasmin. Moreover, persulcatin down-regulated MMP-2 and MMP-9 secretion (Figure 3F and G) and inhibited fibronectin (Figure 3H) and collagen type 1A (Figure 3I) degradation in keratinocyte conditioned media. These findings suggest inhibitory effects of persulcatin on the plasminogen/plasmin system may play a critical role in modulating keratinocyte migration and proteolysis during the wound healing process.

Persulcatin binds to exosite-1 of thrombin impairing blood coagulation and endothelial cell permeability

Once persulcatin also targets α-thrombin, we proceeded with its characterization on blood coagulation. Persulcatin significantly delayed the human plasma recalcification time (Figure 4A), prolonged activated partial thromboplastin time (Figure 4B) and prothrombin time (Figure 4C), confirming its function as an inhibitor of the common pathway of blood coagulation. In a cell-based coagulation assay, persulcatin prolonged the coagulation time measured directly on the surface of LPS-activated endothelial cells (Figure 4D) and significantly inhibited the *ex-vivo* thrombin-induced tissue factor protein expression in mouse aortic rings (Figure 4E).

The Figure 5 (A-F) elucidates the mechanism of α-thrombin inhibition by persulcatin using fibrinogen as substrate. Progress curves demonstrated the impact of persulcatin on α-thrombin-mediated fibrinogen coagulation (Figure 5A). Similar to plasmin, thrombin inhibition by persulcatin was not time-dependent, occurring in a fast-binding mode (Figure 5B). Persulcatin tightly binds to α-thrombin, as indicated by dose-response plots (Figure 5C) and IC_{50} (Figure 5D)

analyses. The competitive inhibition mechanism was confirmed by Morrison's (Figure 5E) and Dixon's (Figure 5F) plots, resulting in a K_i value of 115 nM.

Considering that α-thrombin interactions with its natural substrates and other tick-derived inhibitors happen mainly through binding to the active site and/or to the anion-binding exosites (Alexander et al., 2012; Corral-Rodríguez et al., 2009), we employed a biochemical approach to study the preferred site for persulcatin (Figure 5G-L). Comparing the dose-response inhibition curves, persulcatin displayed a similar behavior to hirudin, an inhibitor that exclusively binds to the exosite-1 of α-thrombin (Fenton Ii et al., 1991). Persulcatin had inhibitory effects when the macromolecular substrate fibrinogen was used (Figure 5G) but had minor or no effects in the presence of the small peptide-based synthetic substrate S-2238 (Figure 5H). Similarly, persulcatin did not change the exosite-1 deleted γ-thrombin activity upon S-2338, suggesting an important role for this domain (Figure 5I). The interaction with exosite-1 was confirmed by bivalirudin and antithrombin III (ATIII) displacement experiments. Bivalirudin is a synthetic peptide-derived inhibitor engineered to interact with both the active site and exosite-1 simultaneously, while ATIII is a physiological macromolecular inhibitor able to interact with the active site but susceptible to allosteric modulations at the exosite-1 (Trapaidze et al., 2016). Increasing concentrations of persulcatin caused the displacement of bivalirudin (Figure 5J) and ATIII (Figure 5K), significantly reducing their α-thrombin inhibitory potential upon S-2238 hydrolysis. The Figure 5L shows the molecular surface of persulcatin according to its electrostatic potential. Highlighted in red are the negatively charged patched composed by the residues that can potentially interact with the anion-binding exosite-1.

Endothelial cell barrier and permeability at the capillary vessel-tissue interface can be modulated and even disrupted by proteases (Claesson-Welsh et al., 2021). The Figure 6 show cases

that α-thrombin increases endothelial cell permeability to FITC-dextran (Figure 6A and B) by disrupting VE-cadherin and cell-cell contacts (Figure 6C). On the other hand, endothelial cells pretreated with persulcatin revealed reduced permeability when stimulated with α-thrombin, and VE-cadherin immunostaining confirmed the modulatory effects of persulcatin.

DISCUSSION

Ticks, as ectoparasites and blood pool feeders, evolved to produce molecules in their saliva that exhibit high-affinity binding and specificity towards various components involved in skin physiology and wound healing (Ali et al., 2022). In this study, we present a detailed description of a newly discovered tick salivary protein named persulcatin, isolated from *I. persulcatus*, the primary vector of tick-borne encephalitis and Lyme disease in East Asia (Wu et al., 2013). Persulcatin demonstrates multifunctional properties as a Kunitz-type protease inhibitor, influencing host plasmin-induced cell migration on keratinocytes and prothrombotic events mediated by thrombin on endothelial cells.

The skin epidermis serves as the initial point of contact for tick mouthparts, tick saliva, and tick-borne pathogens during the feeding process. This interface plays a critical role influencing the host`s initial response to tick feeding and pathogen transmission (Wikel, 2013). Of particular interest are keratinocytes, as they constitute the majority of cells in the skin, playing a crucial role in skin homeostasis and rapidly responding to mechanical damage by secreting soluble factors such as cytokines, chemokines, proteases, and growth factors, that are permanently involved in the crosstalk with immune cells (Bourke et al., 2015). The role of keratinocytes is somehow a neglected topic in the context of tick feeding and tick-borne pathogen transmission biology.

Keratinocytes express various components of proteolytic systems, including elements of the plasminogen-plasmin system (PPS), such as plasminogen (PLG)-binding proteins, urokinase-type plasminogen activator (uPA), and matriptase (Chen et al., 2016; Jaiswal et al., 2018). These components play vital roles in maintaining the structural integrity and barrier function of the epidermis (Chen et al., 2016; Jennemann et al., 2007). Keratinocyte migration is a fundamental process in wound healing and tissue remodeling, allowing cells to move into the wound bed and facilitate the formation of new tissue (Seeger & Paller, 2015). Plasmin is not only essential for dissolving fibrin clots and removing necrotic tissue during keratinocyte migration in wound healing but also contributes to keratinocyte and inflammatory cell migration through by activating and releasing growth factors, activating matrix metalloproteases, promoting angiogenesis, and facilitating extracellular matrix remodeling (Deryugina & Quigley, 2012). Plasmin is initially synthesized and secreted as an inactive precursor, plasminogen (PLG), which is subsequently cleaved and activated by soluble tissue type-plasminogen activator (tPA), membrane-associated uPA, and/or matriptase resulting in the generation of active plasmin (Chen et al., 2016; Lund et al., 2006). Therefore, the regulation of the PPS serves critical roles at every stage of the wound healing process, and any disruption in this system can have significant impacts on the process. Persulcatin functions as a classical fast, tight-binding competitive inhibitor, targeting plasmin with a K_i value of 28 nM. Notably, persulcatin appears to operate as a down-modulator of the plasminogen-plasmin system (PPS), effectively inhibiting the activation of plasminogen and consequent generation of plasmin on keratinocytes. This inhibition consequently leads to the reduction in the migratory ability of keratinocytes. Beyond its direct influence on plasmin, persulcatin takes on the additional role of downregulating matrix metalloproteinase expression within keratinocytes, resulting in a decrease in their capacity to degrade essential extracellular

matrix proteins, such as fibronectin, fibrin, or collagen 1A. This dual-pronged mechanism highlights how persulcatin orchestrates a complex regulatory milieu, curtailing the signaling pathways that typically encourage keratinocyte migration into the wound bed. Therefore, the presence of persulcatin in tick saliva can inhibits the signaling that promotes keratinocyte migration into the wound bed, helping to difficult tissue damage repair and at the same time facilitating the blood meal acquisition (Pham et al., 2021), highlighting the importance of persulcatin characterization and its functional effects on keratinocytes.

Besides healing mechanisms, hemostasis is another host-derived response that occurs locally at the bite site (Chmelar et al., 2012). Ticks have evolved to produce salivary molecules that counteract specific targets involved in vasoconstriction, platelet aggregation, and the coagulation cascade (Tirloni et al., 2014, 2016, 2020). These molecules include inhibitors of clotting factor such as factors Xa, XIIa, XIa, IXa, thrombin, tissue factor/VIIa, and plasma kallikrein (Parizi et al., 2018). An example of such tick salivary inhibitors is persulcatin, which in addition to its role targeting plasmin and keratinocyte migration, targets α-thrombin, inhibiting its ability to clot fibrinogen competitively with a K_i of 115 nM. As a result, persulcatin prolongs classic coagulation time parameters in human plasma, inhibits coagulation in LPS-activated endothelial cells, and down-regulates tissue factor expression in mice aorta. From a molecular standpoint, persulcatin features two Kunitz-like domains, containing six cysteine residues per domain. It shares structural similarity to other tick thrombin inhibitors such as hemalin from *H. longicornis* (Liao et al., 2009), amblin from *Amblyomma hebraeum* (Lai et al., 2004), and boophilin from *R. microplus* (Macedo-Ribeiro et al., 2008; Soares et al., 2012). Persulcatin is evolutionary closely related to boophilin, a two Kunitz-type inhibitor derived from *R. microplus* midgut, previously characterized as an anticoagulant and antithrombotic molecule (Assumpção et

al., 2016). Unlike boophilin, which behaves as a non-competitive α-thrombin inhibitor interacting with both catalytic site and exosite-1 (Assumpção et al., 2016), persulcatin is a competitive inhibitor that seems to exclusively interact with α-thrombin exosite-1. The function of α-thrombin depends on its active site and two anion binding exosites that mediate enzyme interaction with various macromolecular substrates and inhibitors (Krishnaswamy, 2005). Exosite-1 binds to fibrinogen, PAR-1 receptor, thrombomodulin, heparin cofactor II, and factors V/Va, while exosite-2 is the heparin-binding site (Abdel Aziz & Desai, 2018). Inhibitors binding to exosite-1 can physically obstruct macromolecular substrate recognition or induce allosteric changes in the catalytic site. Consequently, the ability of the enzyme to cleave small synthetic peptide substrates could remain partially unchanged, but its activity against macromolecular substrates, such as fibrinogen, is severely impaired (Grzegorski et al., 2020). This phenomenon is observed with persulcatin and hirudin, both pure exosite-1 inhibitors. These inhibitors do not block S-2238 hydrolysis but exhibit potent anticoagulant effects. At a 500-fold ratio to α-thrombin, persulcatin inhibits around 30% of the S-2238 hydrolysis by α-thrombin. Notably, persulcatin binding to exosite-1 also appears to induce allosteric changes that protect thrombin from serpin inactivation, as indicated by the antithrombin III (ATIII) displacement experiments. Although ATIII recognition by thrombin does not directly rely on exosite-1 interaction, ligand binding or inactivation of exosite-1 can allosterically reduce catalytic site inactivation by ATIII (Lane et al., 2005).

Beyond its role in blood coagulation, thrombin activates the PAR-1 receptor in endothelial cells, initiating downstream pathways of phosphoinositide 3-kinase (PI3K)/Akt pathway, mitogen-activated protein kinase (MAPK) pathway, and the Rho GTPase pathway (Zohrabian et al., 2009). Thrombin-induced Rho GTPase activation triggers actin cytoskeleton reorganization and stress

fiber formation, leading to endothelial cell contraction and barrier disruption (Yang & Chen, 2022). Our data indicates that thrombin disrupts VE-cadherin in endothelial cells, inducing cell contraction, increasing intercellular space, and enhancing cell permeability to FITC-dextran. Persulcatin counteracts thrombin-induced endothelial cell permeability, preserving VE-cadherin structural integrity. Given that increased vascular permeability is essential for primary inflammatory responses and inflammatory cell migration, persulcatin likely contributes to tick feeding process by safeguarding endothelial cell tight junctions from proteases activity.

In conclusion, this study elucidates the multifunctional properties of persulcatin, a novel molecule discovered in the salivary glands of *I. persulcatus* ticks. By inhibiting plasmin-induced cell migration on keratinocytes and thrombin- associated prothrombotic events in endothelial cells, persulcatin effectively regulates keratinocyte migration, blood coagulation, endothelial cell permeability, and extracellular matrix function. This ultimately aids in the tick feeding process. The findings underscore the pivotal role persulcatin plays in modulating the initial host response during tick feeding, facilitating successful blood meals, and potentially assisting in the transmission of tick-borne pathogens. This discovery deepens our comprehension of the intricate interactions among ticks, their saliva, and the host, offering valuable insights for future research and potential applications in combating tick-borne diseases.

MATERIAL AND METHODS

Animals and ethics statement

Ixodes persulcatus ticks were obtained from the Graduate School of Veterinary Medicine, Hokkaido University, Japan, following the standard procedures for tick colony maintenance and management (Konnai et al., 2008). The protocols for all experiments involving animal and ticks

were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Hokkaido University (22-0035). C57BL/6J mice were purchased from Charles River Laboratories and used for the *ex-vivo* aortic ring assays (details are provided in the supplementary methods). The animals were kept under the care of the Rocky Mountain Veterinary Branch at the Rocky Mountain Laboratories (Hamilton, MT, USA). The NIH Guide for the Care and Use of Laboratory Animals was followed in all the procedures, and collection of mice tissue was approved by the Animal Care and Use Committee at the Rocky Mountain Laboratories (2020-045).

Protein expression, purification, and characterization

Bioinformatic analyzes were employed to characterize the sequence alignment, phylogeny, and specific structural aspects of persulcatin in comparison to other Kunitz-type inhibitors. The protein-coding sequence corresponding to mature persulcatin was cloned and further optimized for mammalian expression using Expi293 human embryonic kidney cells (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the recombinant protein was purified employing conventional protein purification chromatography methods. The specificity of persulcatin was assessed through a comprehensive broad panel of proteases crucial for coagulation, inflammation, and immune response against tick feeding. Further details of these experiments can be found in the supplementary methods section.

Protease and blood coagulation experiments

In-depth investigation was carried out on the effects of persulcatin against human plasmin and α-thrombin (THR) to characterize the mechanisms of inhibition, kinetics, mode of action, and binding aspects within protease-inhibitor interactions. The ability of persulcatin to modulate blood coagulation was explored through the following approaches: *i.* Measurement of coagulation time parameters in human plasma; *ii*. Assessment of clot formation on the surface of LPS-activated

endothelial cells; and *iii*. Evaluation of thrombin-induced tissue factor expression in *ex vivo* aortic rings. Detailed procedures for these experiments can be found in the supplementary methods section.

Cellular and molecular analyses

The scratch-induced wound healing assay was employed on keratinocyte monolayers to estimate cell migration and to explore the mechanisms of plasminogen/plasmin system modulation by persulcatin. The analysis encompassed cell surface plasmin generation, matrix metalloproteinase activation, as well as fibrin and matrix protein degradation. Endothelial cell monolayers were used to investigate the effects of persulcatin on thrombin-induced cell permeability and tight junction protein degradation. Detailed procedures for these and other experiments (immunofluorescence, western-blot and RT-PCR) can be found in the supplementary methods section.

Data analyses

Data analyses were conducted using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). All data are presented as the means ± standard error (SE) of at least three replicates. Significance of differences was assessed by one-way ANOVA, followed by unpaired t-tests with Bonferroni's correction for multiple comparisons. A significance level of P-values < 0.05 was considered statistically significant.

DATA AVAILABILITY STATEMENT

For any data relevant to this study, please contact the corresponding author via email at lucas.tirloni@nih.gov

CONFLICT OF INTEREST STATEMENT

The authors state no conflict of interest.

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CRediT STATEMENT

MB, SRM, NMP, ISV, AS and LT conceived and designed the experiments. MB, SRM, NMP, AS and LT performed the experiments. SK, ISV, AS and LT contributed to reagents, materials, and analysis tools. MB, SRM, ISV, AS and LT drafted the article. MB, SRM, NMP, LFP, SK, ISV, AS and LT contributed to critical revision of the article. All authors contributed to the article and approved the submitted version.

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FIGURE LEGENDS

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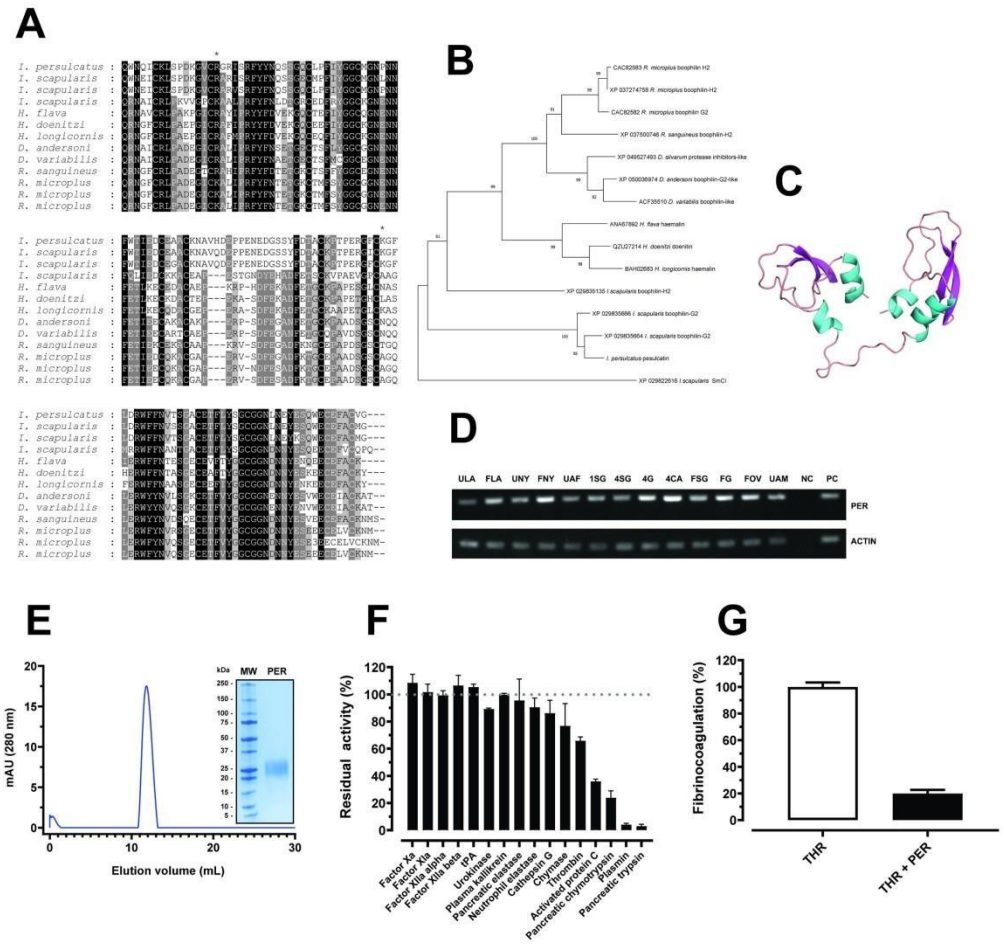

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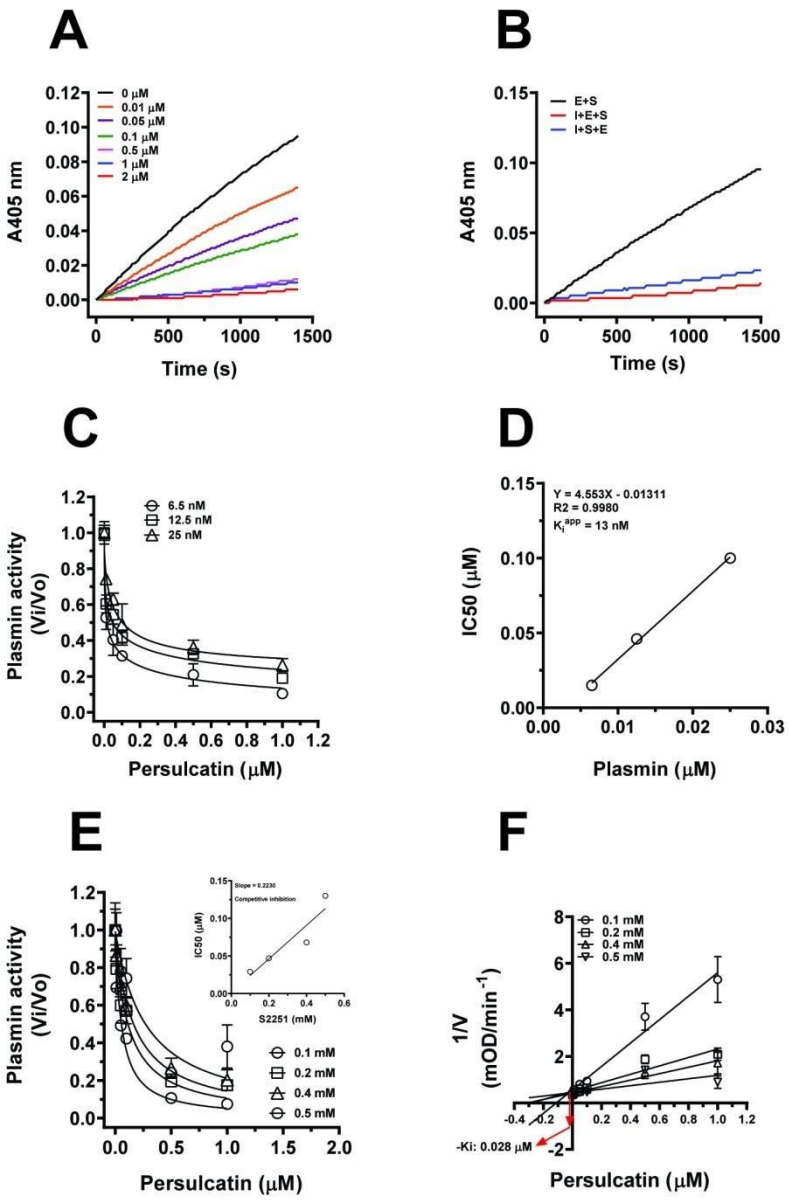

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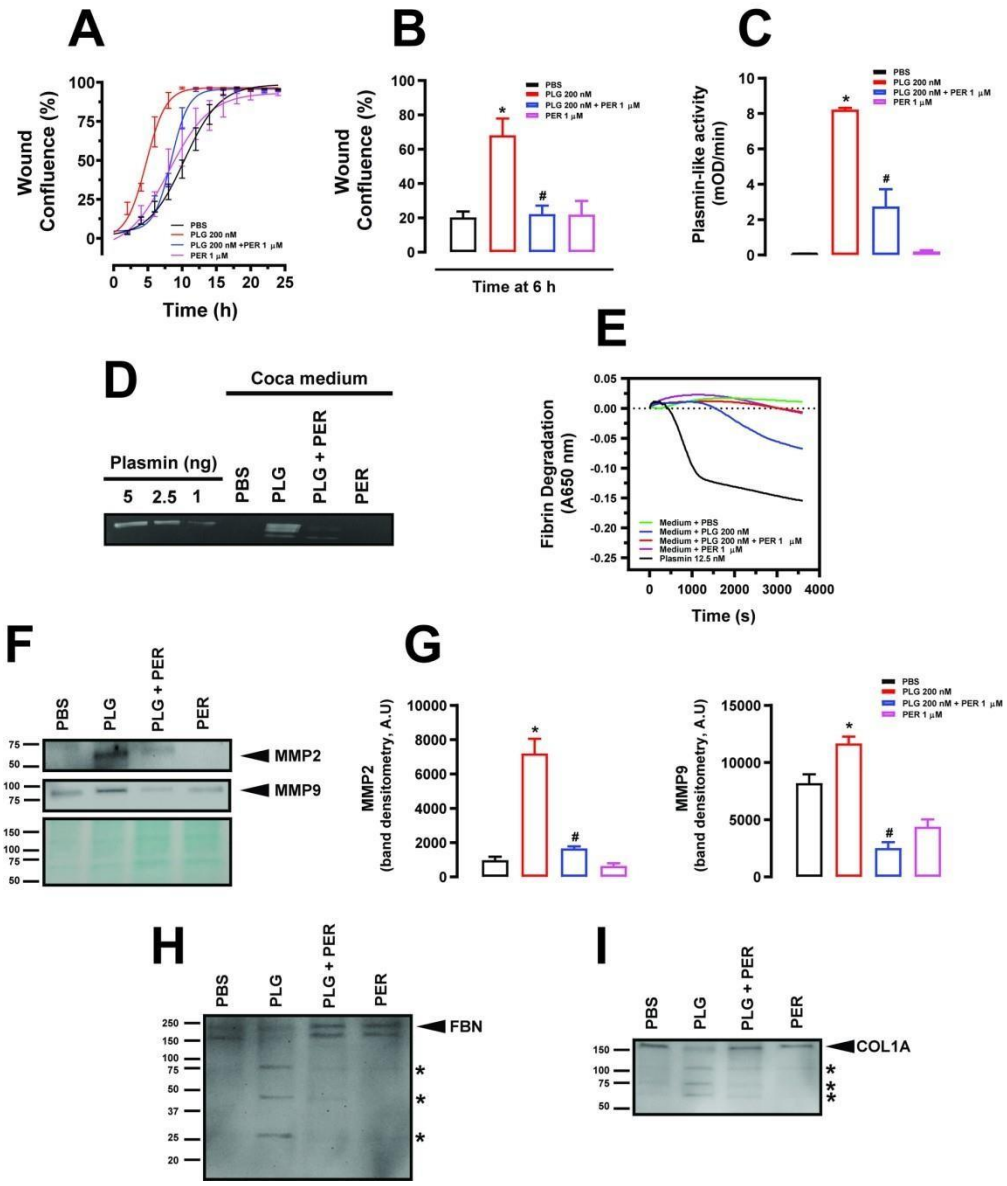

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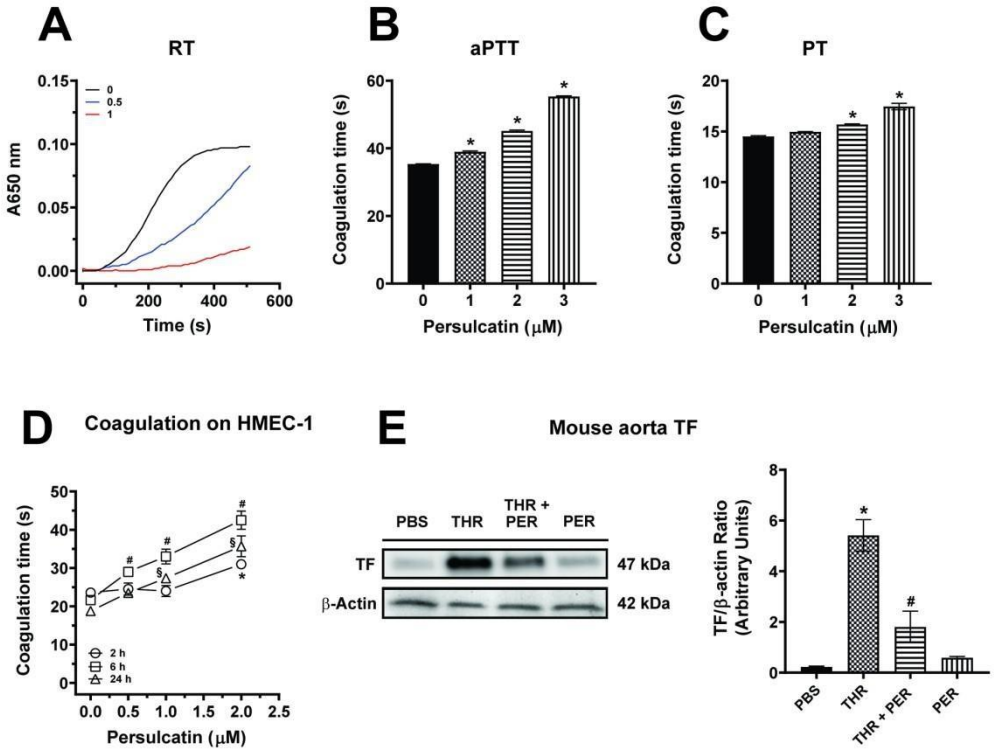

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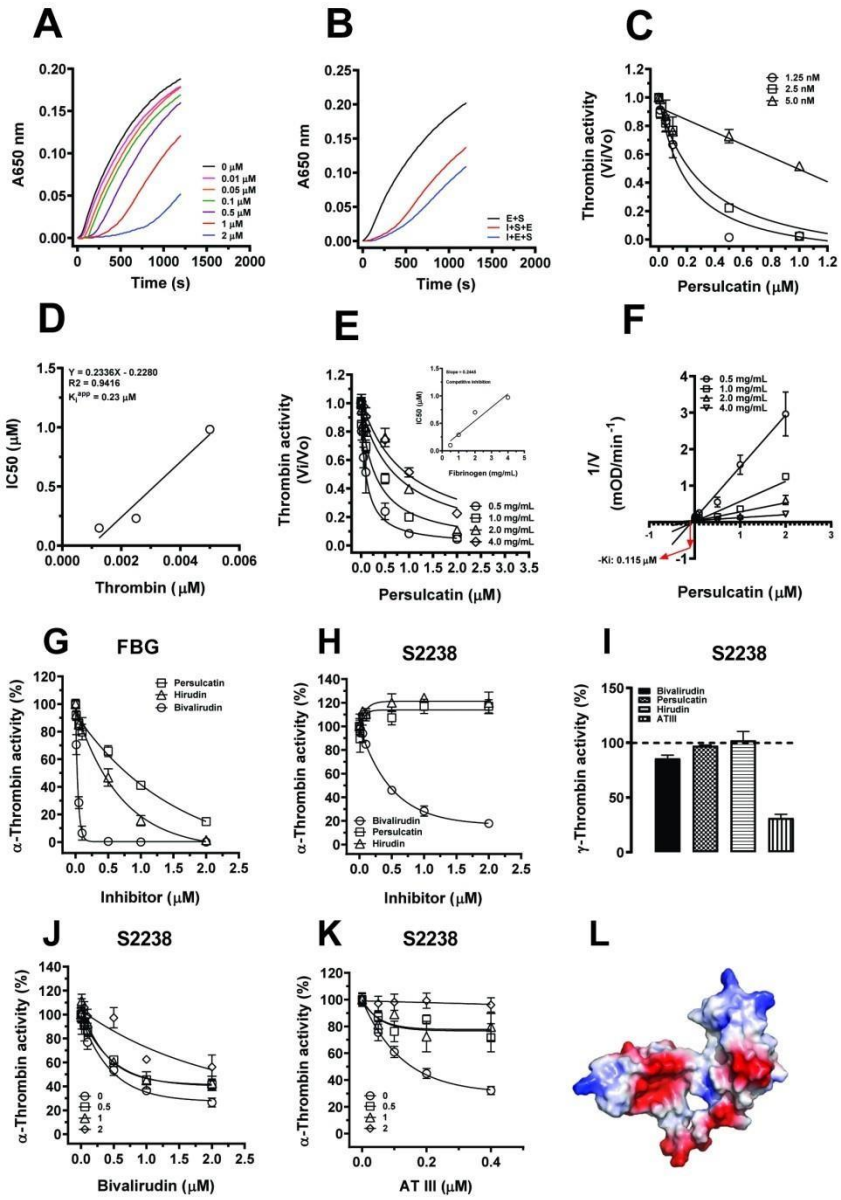

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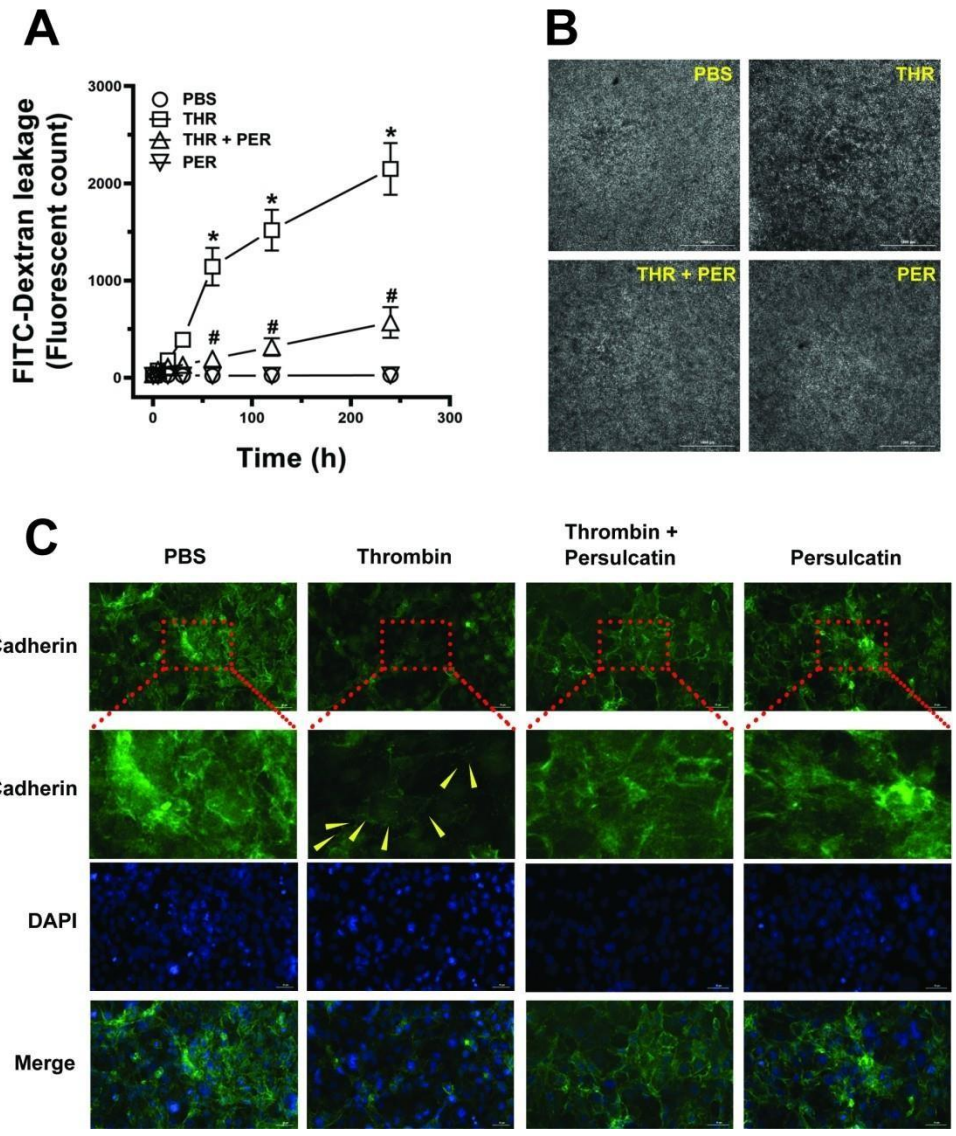

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170x192mm (300 x 300 DPI)

SUPPLEMENTARY MATERIAL

Extended material and methods

Reagents

MCDB 131 (microvascular endothelial cell medium), epidermal growth factor (EGF), hydrocortisone, glutamine, fetal calf serum (FCS), penicillin/streptomycin, trypsin-EDTA (0.25%), and trypsin neutralizer solution were purchased from Life Technologies (Carlsbad, CA, USA). CnT-07 epithelial proliferation medium was obtained from CellnTec Advanced Cell Systems (Bern, Switzerland). Purified human α-thrombin, γ-thrombin, factor XIa, factor XIIa-α, factor XIIa-β, kallikrein, activated protein C, plasmin, factor Xa, and fibrinogen were sourced from Enzyme Research Laboratories (South Bend, IN, USA). Human neutrophil elastase, porcine pancreatic elastase, human urokinase plasminogen activator (uPA), human tissue-type plasminogen activator (tPA), and human cathepsin G were purchased from Molecular Innovations (Novi, MI, USA). Human chymase, bovine α-chymotrypsin, porcine trypsin, bivalirudin, human antithrombin III, Glu-type human plasminogen, [Tyr(SO3H)]63]-Hirudin fragment 54-65, human plasma, E. coli O111:B4 lipopolysaccharide (LPS), and FITC-Dextran 70,000 were procured from Sigma-Aldrich/EMD Millipore Corp. (Saint Louis, MO, USA). A complete list of all chromogenic substrates and their suppliers is provided in Supplementary Table 1 (Table S1). All other chemicals were of analytical grade and were commercially available.

Ticks

Adult *Ixodes persulcatus* ticks were obtained from a laboratory colony fed on pathogen-free Syrian hamsters maintained in a P3 animal facility at Graduate School of Veterinary Medicine, Hokkaido University, Japan (Konnai et al., 2008). Use of Laboratory Animals, Faculty of Veterinary Medicine, Hokkaido University, as approved by the Association for Assessment and Accreditation

of Laboratory Animal Care International (Frederick, MD, USA). The protocols of all animal experiments were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Hokkaido University (22-0035).

Mice

Adult male C57BL/6J mice aged 4 to 6 weeks and weighing 15 to 20 g were obtained from Charles River Laboratories. Throughout the experiment, the mice were housed in the animal facility at our institution, the Rocky Mountain Veterinary Branch (RMVB), located at Rocky Mountain Laboratories (Hamilton, MT, USA). The mice were kept under controlled conditions, with temperatures maintained between 20-24°C and a relative air humidity of 40-60%. They were subjected to a 12-hour dark/light cycle and provided free access to water and food. All procedures involving the animals were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Our experimental protocol received approval from the Animal Care and Use Committee at Rocky Mountain Laboratories (#2020-045).

Tick dissection, RNA extraction and cDNA library construction

For RNA extraction, both partially and fully engorged adult females of *Ixodes persulcatus* ticks were washed with 70% ethanol and dissected in a Petri dish containing cold 20 mM phosphate-buffered saline (PBS) at pH 7.2. Dissection was performed using a scalpel blade under a light microscope. To analyze the expression profile, we collected unfed and fed larvae (ULA, FLA), unfed and fed nymphs (UNY, FNY), unfed adult females (UAF), 1-day-fed female salivary glands (1SG), 4-day-fed female salivary glands (4SG), gut (4G), and carcass (CA, whole tick without SG, G, and OV), fully fed female salivary glands (FSG), ovary (FOV), and unfed adult males (UAM).

These samples were macerated in TRIzol® reagent (Invitrogen) for RNA extraction. RNA isolation followed the manufacturer's recommendations, and total RNA was treated with DNase I (Invitrogen) to remove any contaminating DNA. RNA yield and quality were assessed using a spectrophotometer (Amersham Biosciences, Pharmacia Biotech, Ultraspec 1000). To construct the *I. persulcatus* cDNA library, total RNA extracted from whole adult female ticks was used. For cDNA synthesis, the Superscript III kit (Invitrogen) was employed following the manufacturer's instructions. Subsequently, the cDNA library was generated using the SMART® cDNA Library Construction Kit (Clontech, Takara Bio Company) as per the manufacturer's guidelines.

Persulcatin sequence amplification and cloning

Sequences encoding Kunitz-type inhibitors from other tick species, which were deposited in the GenBank database, were employed as queries and searched against the *Ixodes scapularis* database using the BLAST (Basic Local Alignment and Search Tool) algorithm (Altschul et al., 1997). To amplify the full-length ORF sequence from *Ixodes persulcatus* corresponding to persulcatin, primers based on the *I. scapularis* sequence XM_002403992, available in the NCBI database, were designed. The PCR reaction was carried out using the Elongase® enzyme (Invitrogen) under the following conditions: initial denaturation at 95ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 58ºC for 30 sec, and extension at 68ºC for 2 min. A final extension step was performed at 68°C for 5 min. The resulting amplification products were separated using 0.8% agarose gel electrophoresis and then purified using the Geneclean kit (BIO 101, Q-BIOgene), following the manufacturer's instructions. The obtained amplicon was cloned into the pGEM-T plasmid (Promega) and introduced into *Escherichia coli* DH5α host strain using the manufacturer's instructions. Verification of successful cloning was accomplished through DNA sequencing.

Bioinformatic analysis

The deduced amino acid sequence was analyzed using Compute pI/Mw in ExPASy molecular biology server (Gasteiger, 2003). The putative domain architecture and functional sites were identified using ScanProsite (Sigrist et al., 2012) and InterProScan (Hunter et al., 2009). Sequences were aligned using the MUSCLE algorithm (Edgar, 2004). The secretory signal peptide and cleavage site for the mature protein were predicted by SignalP server version 4.1 (Bendtsen et al., 2004). The phylogenetic analysis was performed by the neighbor-joining method using the MEGA 11 software (Tamura et al., 2021) with 1,000 replicates bootstrap support. The AlphaFold2 program (Jumper et al., 2021) was used to predict the tertiary structures of persulcatin. The program was run locally on the NIH Biowulf cluster in a Linux environment using the monomer mode. Structures were visualized, and figures were generated using PyMol (The PyMOL Molecular Graphics System, Version 2.6.0a0, Schrödinger, LLC).

Transcriptional profiling analysis of persulcatin gene

The transcriptional profile of the persulcatin gene was evaluated in *Ixodes persulcatus* male and female different tissues and developmental stages by RT-PCR using specific primers. Tick actin was used as internal RT-PCR control (Sajiki et al., 2022). Reaction products were analyzed by agarose electrophoresis.

Recombinant expression and protein purification

The mature protein-coding sequence (without the predicted signal peptide) was codon-optimized for mammalian expression, synthetized, and cloned into VR2001 vector in frame with tPA signal peptide sequence containing a 6x-histidine tag followed by an enterokinase cleavage site (BioBasic

Inc., Markham, ON, Canada). Plasmid DNA was purified using NucleoBond PC 2000 plasmid kit (Takara, Kusatsu, Shiga, Japan). Expi293 human embryonic kidney cells (Thermo Fisher Scientific, Waltham, MA, USA) were transfected with endotoxin-free plasmid. Supernatants were collected 72 h post-transfection, centrifuged (1,000 g per 15 minutes) and frozen. Recombinant protein was purified by affinity, ion exchange, and size-exclusion chromatography. The supernatant containing the secreted recombinant protein was incubated for 1 hour at room temperature with AmMag™ Ni Magnetic beads (GenScript, Piscataway, NJ, USA) previously pre-equilibrated with PBS, pH 7.4 supplemented with Tween-20 0.05%. Then, beads were washed with the binding buffer, followed with PBS, pH 7.4 and an extra wash with PBS, pH 7.4 supplemented with 5 mM imidazole. The recombinant protein was eluted using PBS, pH 7.4 supplemented with imidazole 500 mM. The protein fractions were visualized in a 4-20% SDS-PAGE gel electrophoresis followed by Coomassie blue staining. The fractions of interest were pooled and concentrated and dialyzed against Tris-HCl 20 mM, pH 8.0 using an Amicon® Ultra-15 centrifugal filter units (Millipore Sigma) and used for the subsequent ion exchange chromatography. Sample was applied on a pre-equilibrated HiTrap Q column followed by a washing step with 5 column volumes of Tris-HCl 20 mM, pH 8.0 buffer. The recombinant protein was eluted by passing a linear gradient of Tris-HCl 20 mM, NaCl 1 M, pH 8.0 buffer using an AKTA start chromatography system (Cytiva). Eluted fractions were visualized in a 4-20% SDS-PAGE electrophoresis followed by Coomassie blue staining. The fractions of interest were pooled, concentrated, and dialyzed against Tris-HCl 20 mM, NaCl 150mM, pH 7.4 buffer using Amicon® Ultra-15 centrifugal filter units (Millipore Sigma) and used for the subsequent gel filtration chromatography. Sample was loaded on a Superdex 75 10/300 GL column and proteins were eluted isocratically at a flow rate of 0.5 mL/minute in Tris-HCl 20 mM, NaCl 150 mM, pH 7.4

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Serine protease inhibition screening

The inhibitory potential of persulcatin was tested against the following proteases: Human α-thrombin (2 nM), human γ-thrombin (5 nM), human factor XIa (5 nM), human factor XIIa-α (10 nM), human factor XIIa -β (10 nM), plasma kallikrein (1 nM), human activated protein C (5 nM), human plasmin (10 nM), human factor Xa (5 nM), human chymase (10 nM), bovine pancreatic α-chymotrypsin (10 nM), porcine pancreatic trypsin (5 nM), human neutrophil elastase (20 nM), porcine pancreatic elastase (50 nM), human uPA (10 nM), human tPA (20 nM) and human cathepsin G (50 nM). Briefly, persulcatin (1 μM) was pre-incubated with each protease for 15 min in 20 mM Tris-HCl, 150 mM NaCl, 0.01% Tween-20, pH 7.4 at 37ºC. After incubation, different synthetic chromogenic substrates (200 μM) designed specifically for each protease were added in a final volume of 100 μL using a 96-well plate. A complete list with all substrates used can be found in Supplementary table 1. The substrate hydrolysis rate at 30 ºC was followed at 405 nm in kinetic mode in a Synergy H1 max micro plate reader (Biotek, CA, USA) for 15 min. The observed substrate hydrolysis rate in the absence of persulcatin was considered as 100 % and compared to the remaining enzymatic activity in the presence of persulcatin. Data are presented as mean ± standard error of triplicate readings.

Mechanisms of plasmin inhibition

Plasmin inhibition by persulcatin was characterized using classical methods of enzyme kinetic analysis. The enzyme (10 nM) was preincubated at 37° C by 15 min with different concentrations of persulcatin (0, 0.01, 0.05, 0.1, 0.5, 1 and 2 µM) in a reaction media containing 20 mM Tris-HCl, 150 mM NaCl, 0.01% Tween-20, pH 7.4. The remaining plasmin activity was measured by the addition of S-2251 substrate (200 µM) in a volume of 100 µL. The amount of *p*-nitroaniline produced was monitored at 405 nm in intervals of 14 s for 30 min using a Synergy H1 max micro plate reader (Biotek, CA, USA). The progress curves were obtained by plotting absorbance values (A405 nm) versus time (s). For all calculations the initial rate of hydrolysis was chosen to determine the steady-state kinetics (Vmax mode) of persulcatin-plasmin interaction. Slow- or fast-binding mode was determined analyzing the progress curves with or without 45 min preincubation time between enzyme plus inhibitor. To determine the type of inhibition, binding mode, and kinetic constants (IC_{50}, apparent K_i and real K_i) a dose-response plot of fractional velocity (V_i/V_o) as function of persulcatin (0 – 1 µM) was constructed in the presence of different plasmin (6.5, 12.5 and 25 nM) or S-2251 (0.1, 0.2, 0.4 and 0.5 mM) concentrations. In these cases, V_i was the inhibited steady-state velocity and V_o was the control (uninhibited) velocity. A positive linear regression obtained by plotting IC_{50} values as function of enzyme concentration was used to determine the tight binding mode of inhibition, while the y-intercept indicated the apparent K_i (K_i^{app}). The tight binding behavior was also confirmed by fitting the fractional velocity in the presence of different substrate concentrations to the Morrison equation. The type of inhibition (competitive, noncompetitive or uncompetitive) was determined by the slope of IC_{50} plotted as function of S-2251 concentration and the real K_i value was estimated by the Dixon´s plot ($1/V_i$ vs inhibitor concentration)(Dixon, 1953). All data points from kinetic measurements were fitted using

GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). Data are the mean of three determinations, each performed in triplicates.

Mechanisms of thrombin inhibition

Human α-thrombin (2.5 nM) was preincubated at 37° C by 15 min with different concentrations of persulcatin (0, 0.01, 0.05, 0.1, 0.5, 1 and 2 µM) in a reaction media containing 20 mM Tris-HCl, 150 mM NaCl, 0.01 % Tween-20, pH 7.4. The remaining thrombin activity was measured by the addition of human fibrinogen as substrate (2 mg/mL) in a volume of 150 µL. Fibrinocoagulation process was then followed by the increase in optical density at 650 nm in intervals of 14 s for 30 min using a Synergy H1 max micro plate reader (Biotek, CA, USA). The type of inhibition, binding mode, and kinetic constants were all determined following the same method described above for plasmin. α-thrombin inhibition was also compared in the presence of specific α-thrombin exosite-1 (hirudin) or active-site (bivalirudin and antithrombin III) inhibitors. Briefly, 2.5 nM α-thrombin was incubated in the presence of different concentrations (0, 0.01, 0.05, 0.1, 0.5, 1.0 and 2.0 µM) of persulcatin, hirudin, bivalirudin or antithrombin III. The remaining enzyme activity was then measured by the addition of macromolecular substrate fibrinogen or the peptide-based chromogenic substrate S-2238 following the procedure described above. The displacement of bivalirudin or antithrombin III from its binding site was also evaluated in the presence of persulcatin. In these cases, bivalirudin (0 – 2 µM) or antithrombin III (0 – 0.4 µM) was preincubated (30 min) with α-thrombin (2.5 nM) followed by the addition of increasing concentrations of persulcatin (0, 0.5, 1.0 and 2.0 µM) and the substrate S-2238 (200 µM). Similarly, in some experiments γ-thrombin activity (a thrombin variant that has its exosite-1 deleted) was also measured in the presence of bivalirudin (1 µM), persulcatin (1 µM), hirudin (1

μM) or antithrombin III (0.2 μM) using S-2238 as substrate. For all the experiments data represents the mean of three determinations, each performed in triplicates.

Blood coagulation *in vitro*

The recalcification profile of normal citrated human plasma was evaluated by kinetic assay using a Synergy H1 max micro plate reader (Biotek, CA, USA). Briefly, plasma (50 μL) was incubated at 37° C with 2 μM persulcatin in Tris-buffered saline, pH 7.4 (TBS) (150 μL, final volume). Then, coagulation was triggered by 10 mM $CaCl_2$ and the increase in optical density monitored at 650 nm during 30 min. Partial thromboplastin time (aPTT) and prothrombin time (PT) were evaluated on a STart 4 coagulometer (Diagnostica Stago, NJ, USA). For the aPTT assay, plasma (50 μL) was incubated with different concentrations of persulcatin (1- 3 μM volume of 10 μL) or the same TBS volume (control) and placed in the coagulometer for 10 min at 37 °C. After, 50 μL of prewarmed aPTT reagent (STA PTT; Diagnostica Stago) was added and incubated for 3 min at 37 °C. $CaCl_2$ (50 μL at 25 mM) was added to start reactions. Time for clot formation was recorded in quadruplicate in two independent replicates. For the PT, plasma (50 μL) was incubated with different concentrations of persulcatin (1 - 3 μM volume of 10 μL) or the same TBS volume (control) and placed in the coagulometer for 10 min at 37 °C. Then, 100 μL of the PT reagent (NEOplastine CI plus; Diagnostica Stago) was added. Time for clot formation was recorded in quadruplicate in two independent replicates.

Endothelial cell-based coagulation assay

Human dermal microvascular endothelial cells (HMEC-1, ATCC® CRL-3243) were cultured in MCDB 131 supplemented with epidermal growth factor (10 ng/mL), hydrocortisone (1 μg/mL),

glutamine (10 mM) and fetal bovine serum (10 %). The cells were maintained without antibiotics in a 37°C/5 % CO_2 environment following the standard procedures for cell culture. Cells ($1x10^3$) were cultured in 96-well plates until reached a confluent monolayer. A procoagulant profile was induced by activating the endothelial cells for 2, 6 and 24 h with 1 µg/mL LPS (O111:B4) in the absence of fetal bovine serum. After LPS treatments, culture media was removed, and human plasma (50 µL) was added in the presence or absence of different concentrations of persulcatin (0 – 2 µM) in a final volume of 150 µL. The coagulation process was triggered by 10 mM $CaCl_2$ and the clot formation was followed directly on endothelial cell monolayers at 650 nm during 40 min using a Cytation 5 multimode reader (Biotek, CA, USA). The coagulation time (measured in seconds (s) was defined as the time taken for reaching the 0.05 absorbance value (onset O.D). All data points represent the mean of three determinations, each performed in triplicates.

Endothelial cell permeability assay

Endothelial cell permeability was studied by the FITC-dextran method using transwell inserts (12 mm x 0.4 µm) (Millicell Cell Cuture Insert, Merck, NJ, USA). HMEC-1 ($5x10^4$ cells per transwell insert) were plated on 1 % gelatin-coated inserts (24-wells) and cultured for 7 days to form a 100 % confluent monolayer inside each insert. Then, the inserts were divided and treated according to the following groups (n = 4/group/plate): *i*. PBS; *ii*. A-thrombin (50 nM); *iii*. A-thrombin (50 nM) + persulcatin (1 µM); and *iv*. Persulcatin (1 µM). The upper chamber received the treatments in Krebs-Ringer bicarbonate buffer containing 2 g/L bovine serum albumin and FITC-dextran (70-kDa, 1 mg/mL) in a total volume of 200 µL. The lower chamber received only Krebs-Ringer buffer in a total volume of 500 µL. FITC-dextran leakage to the lower chamber was measured in duplicates for each insert at 15, 30, 60, 120, and 240 min using the Cytation 5 multimode reader

(Biotek, CA, USA) (485 nm excitation/528 nm emission). During the experiment, cells were kept in a 37ºC/5 % CO_2 environment. The alteration of membrane permeability was also detected by crystal violet stain. In brief, inserts containing cells were fixed with 4 % paraformaldehyde for 15 min, stained with 1 % crystal violet for 30 min, and finally washed three times with PBS. Images were captured with the Cytation 5 multimode reader (Biotek, CA, USA). Alternatively, endothelial cell permeability was evaluated by VE-cadherin immunocytochemistry. In this case, one hundred percent confluent HMEC-1 cells were treated with PBS, thrombin (50 nM), thrombin (50 nM) plus persulcatin (1 µM), and persulcatin (1 µM). After 4 h at 37ºC/5 % CO_2 environment, cells were fixed in methanol, permeabilized in TBS/0.1 % Tween, blocked in normal goat serum (10 %) and processed for overnight immunolabeling with VE-cadherin antibody (working dilution 5 µg/mL) (Life Technologies, Carlsbad, CA, USA). The secondary antibody was a 448-fluorescence conjugated goat anti-rabbit IgG at a dilution of 1:1000 (Life Technologies, Carlsbad, CA, USA) and nuclei was counter stained with Hoechst. Fluorescent labelled images were captured using the Cytation 5 multimode reader (Biotek, CA, USA).

***Ex vivo* aortic ring assay**

Adult male C57BL/6J mice weighting 15-20 g (n = 8) were euthanized by isoflurane overdose followed by cervical dislocation. Abdominal aorta was dissected from surrounding tissues and the aortic rings (approximately 2 mm) were prepared, washed, and resuspended in Krebs-Ringer buffer. Three rings per animal were individually placed in each well of a 96-well plate, incubated at 37 ºC for 5 min in Krebs-Ringer buffer and divided into four treatment groups: *i*. PBS; *ii*. α-thrombin (50 nM); *iii*. α-thrombin (50 nM) plus persulcatin (1 µM); and *iv*. Persulcatin (1 µM). The rings were incubated during 4 h in a 37ºC/5 % CO_2 environment and then processed for tissue

factor (TF) protein expression analysis by western blot. The following antibody was used: anti-TF antibody (H-9) (working dilution 1:200) (#SC-374441, Santa Cruz Biotechnology, TX, USA).

Plasminogen-plasmin system activation on keratinocyte surface

Mouse epidermal keratinocytes (COCA cell line, ECACC 10112001) were maintained in CnT-07 epithelial proliferation medium (CellnTec, Bern, Switzerland) following the manufacturer instructions. Plasminogen activation was estimated directly on the surface of confluent COCA cells cultured on 24-well plates in complete medium. In a first set of experiments, different concentrations of human plasminogen (PLG) (0, 100, 200 and 400 nM) was added to the cells and plasmin generation was monitored at 0, 15, 30, 60, 120, and 240 min. Plasmin-like activity at the different time-points was estimated in conditioned medium through S-2251 hydrolysis following the procedure described above for enzyme activity measurements. A similar experiment was also designed but, in this case, keratinocytes were treated with PBS, 200 nM PLG, 200 nM PLG plus 1 µM persulcatin, and 1 µM persulcatin before plasmin determinations. Plasmin formation was also demonstrated by fibrin degradation assays. For this purpose, conditioned media obtained from keratinocytes treated with PLG and/or persulcatin were submitted to a zymogram using fibrin as substrate, in which a polyacrylamide gel was co-polymerized with a mixture containing 12 mg/mL human fibrinogen plus 50 nM thrombin. After SDS removal with 2.5 % Triton X-100 solution, formed plasmin was detected as a single clear band of fibrinolysis against a blue background of undigested fibrin substrate. Alternatively, fibrin degradation was also followed in a kinetic assay. In this case, a fibrin clot was produced by incubating (30 min/37 ^{o}C) fibrinogen (2 mg/mL) + thrombin (50 nM) in a final volume of 150 µL in a 96-well plate. Then, conditioned media (20 µL) from keratinocytes were carefully added to the top of each fibrin clot. Human purified plasmin

(12.5 nM) was used as a positive control for fibrin degradation. The fibrin degradation process was monitored by optical density decrease at 650 nm.

Plasminogen-plasmin-induced keratinocyte migration

Keratinocyte (COCA cell line) migration was evaluated by the scratch-induced wound healing assay directly on cell monolayers. One hundred percent confluent COCA cells cultured on 24-well plates were used and kept in complete CnT-07 medium during all the experiment. A minimum of 4 plates were run for each experiment. A scratch wound was induced on cell monolayers using the BioTek AutoScratch equipment (Biotek, CA, USA), allowing to create automatically reproducible scratch wounds per well. Next, the wounded cells were treated with increasing concentrations of human plasminogen (PLG) (100, 200 and 400 nM) or PBS. Optical bright field images were captured at the beginning (0 h) and every 2 h during a total time of 24 h in a Cytation 5 multimode reader. The wound confluence was estimated by the area of cells recovering the gap induced by the scratch. Gap areas were measured and compared by the AutoScratch app (Biotek, CA, USA). In another set of experiments scratch-induced wound healing assay was also performed in the presence of PBS, 200 nM PLG, 200 nM PLG plus 1 µM persulcatin, and 1 µM persulcatin. Plasmin-like activity generated after the scratch-induced wound healing experiments (total time 24 h) was estimated in keratinocyte-conditioned media through S-2251 hydrolysis following the procedure described above for enzyme activity measurements.

Matrix protein analysis

Conditioned media from keratinocytes treated with PLG and/or persulcatin as obtained in the item 10.1.18 were used to analyze the protein content of matrix metalloproteinases (MMP) – 2 and 9,

fibronectin and collagen-1A1 by western-blot. The following antibodies were used: Mouse/rat MMP – 2 (working dilution 1:500) (#AF1488, R&D Systems, MN, USA); Mouse/rat MMP – 9 (working dilution 1:500) (#AF909, R&D Systems, MN, USA); fibronectin (working dilution 1:200) (#610077, BD Biosciences, NJ, USA) and collagen-1A1 (working dilution 1:200) (#SC-59772, Santa Cruz Biotechnology, TX, USA).

Western-blot

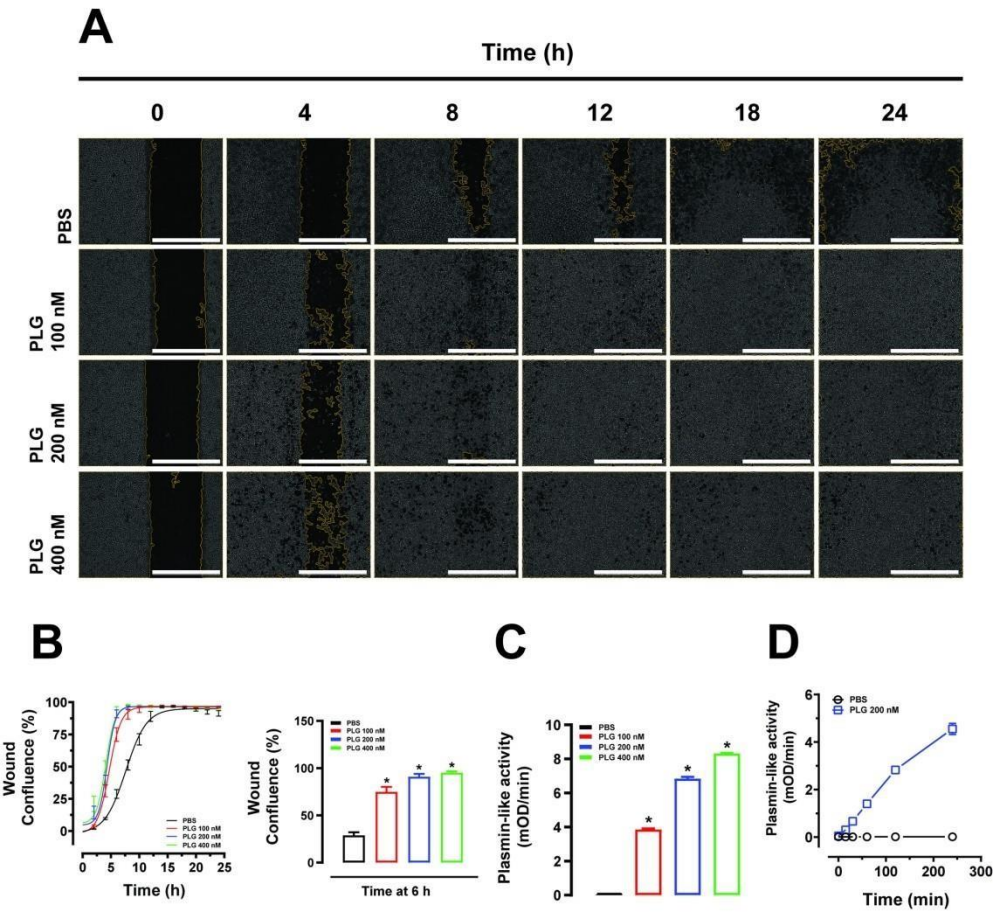
For western-blot analysis protein samples were reduced with 10 % β-mercaptoethanol, separated using Mini-PROTEAN TGX stain-free precast gels (Bio-Rad, Hercules, USA) and transferred in a Trans-Blot Turbo transfer system (Bio-Rad, Hercules, USA). Blots were probed using the respective antibodies described above and processed by chemiluminescence detection following the classical standard procedures. Images were acquired in a ChemiDoc MP Imaging System (Bio-Rad, Hercules, USA).

Supplementary figure legends

Figure S1. Plasminogen is activated on the surface of keratinocytes generating plasmin and inducing cell migration. (A) Keratinocyte (COCA cell line) migration was estimated in the presence of phosphate-buffered saline (PBS) and increasing concentrations of human plasminogen (PLG) using the classical scratch-induced wound healing assay in cell monolayers. Representative images are shown indicating the wound closure over time until 24 h. (B) Quantitative analysis of keratinocyte migration after PLG administration. (C) Plasmin-like activity generated in the keratinocyte conditioned media as estimated based on S-2251 hydrolysis. (D) Time-point kinetics for plasmin generation on the surface of keratinocytes. Cells were treated with 200 nM PLG and

after different time-points (0, 5, 15, 30, 60, 120, 240 min) formed plasmin was estimated by S-2251 hydrolysis.

Figure S2. Persulcatin inhibits the keratinocyte migration induced by plasmin. Representative images from the experiment are shown in figure 3. Keratinocyte (COCA cell line) migration was estimated in the presence of phosphate-buffered saline (PBS), 200 nM plasminogen (PLG) and/or 1 μM persulcatin (PER) using the classical scratch-induced wound healing assay in cell monolayers. The images are showing the wound closure over time until 24 h.



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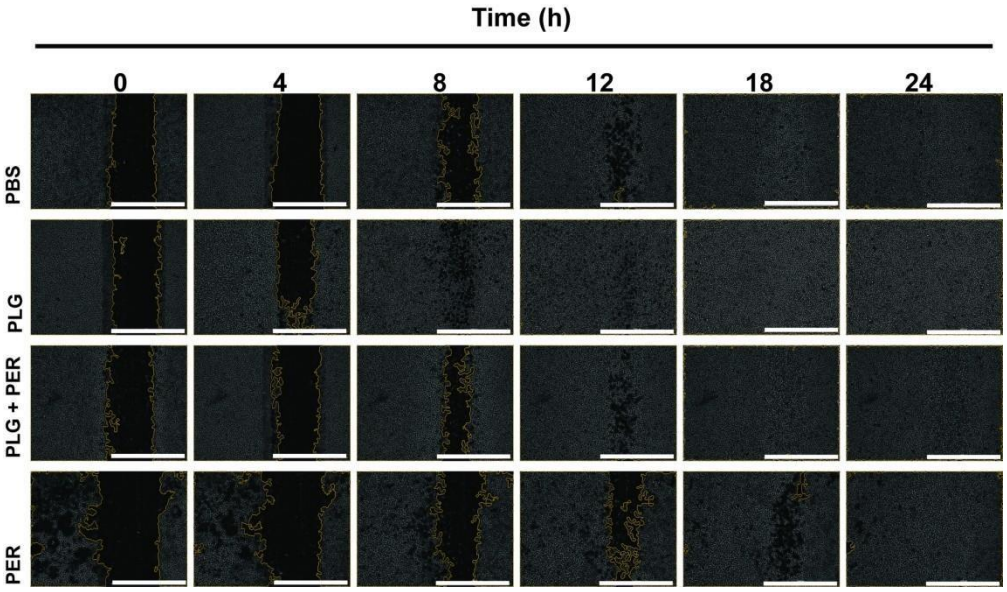
177x164mm (300 x 300 DPI)



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163x95mm (300 x 300 DPI)

Table S1. List of chromogenic substrates used in this work.

Substrate*	Sequence	Protease specificity	Company
Chromogenix S-2238	H-D-Phe-L-Pip-Arg-pNA	α-thrombin, γ-thrombin	Diapharma
Chromogenix S-2222	Bz-Ile- Glu(γ-OR)-Gly-Arg-pNA	Factor Xa	Diapharma
Chromogenix S-2302	H-D-Pro-Phe-Arg-pNA	Factor XIa, Factor XIIa-α, Factor XIIa-β and plasma kallikrein	Diapharma
Chromogenix S-2251	H-D-Val-Leu-Lys-pNA	Plasmin	Diapharma
Calbiochem 324696	MeOSuc-Ala-Ala-Pro-Val-pNA	Human neutrophil elastase	Calbiochem
Chromogenix S-2288	H-D-Ile-Pro-Arg-pNA	Tissue type plasminogen activator and trypsin	Diapharma
Chromogenix S-7388	N-Succinyl-Ala-Ala-Pro-Phe-pNA	Cathepsin G, chymotrypsin and chymase	Diapharma
Chromogenix S-2366	L-PyroGlu-Pro-Arg-pNA	Activated protein C	Diapharma
Chromogenix S-4760	N-Succinyl-Ala-Ala-Ala-pNA	pancreatic elastase	Diapharma
Chromogenix S-2444	pyroGlu-Gly-Arg-pNA	Urokinase-type plasminogen activator	Diapharma

* In general experiments the final concentrations used for each substrate was 200 µM. Different concentrations were used just to determine thrombin and plasmin mechanisms of inhibition, as detailed in the extended material and methods

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 acaracterização 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 inibidorcompetitivo da α-Trombina, capaz de inibir mais de 80% do seu potencial decoagulação, 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 dasinalização, 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

JOURNAL OF INVESTIGATIVE DERMATOLOGY

AUTHOR INFORMATION PACK

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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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