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REVIEW

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Recent advances in vascular thiol isomerases: insights into structures, functions in thrombosis and antithrombotic inhibitor development

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Abstract

Vascular thiol isomerases (VTIs) encompass proteins such as protein disulfide isomerase (PDI), endoplasmic reticulum protein 5 (ERp5), ERp46, ERp57, ERp72, thioredoxin-related transmembrane protein 1 (TMX1), and TMX4, and play pivotal functions in platelet aggregation and formation of thrombosis. Investigating vascular thiol isomerases, their substrates implicated in thrombosis, the underlying regulatory mechanisms, and the development of inhibitors targeting these enzymes represents a rapidly advancing frontier within vascular biology. In this review, we summarize the structural characteristics and functional attributes of VTIs, describe the associations between these enzymes and thrombosis, and outline the progress in developing inhibitors of VTIs for potential antithrombotic therapeutic applications.

Keywords Vascular thiol isomerase, Thrombosis, Structures, Functions, Inhibitors

Background

Vascular Thiol Isomerases (VTIs) are multifaceted enzymes possessing oxidoreductase, isomerase, and chaperone functions, essential for a variety of cellular processes [1]. Characterized by the presence of endoplasmic reticulum (ER) retrieval motifs (exemplified by the KDEL sequence in PDI), these enzymes are predominantly localized within the ER, where they facilitate the formation of native disulfide bonds in newly synthesized proteins and contribute to quality control of client proteins [2]. In response to vascular injury, activated platelets and endothelial cells release several VTIs into the extracellular milieu [3, 4]. These released VTIs exert a significant influence on thrombus formation by modulating the activities of vascular cell receptors [5–7], adhesive proteins [8–10], and coagulation factors [11–13]. In this review, we summarize the structures and functions of VTIs, elucidate their intricate role in thrombus formation, and highlight progress in the development of anti-thrombotic compounds specifically targeting VTIs.

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Structures and functions of VTIs

PDI represents the first VTIs recognized for its pivotal role in vascular biology [14, 15]. Subsequently, ERp5, ERp46, ERp57, ERp72, TMX1, and TMX4 were found to serve important roles in the regulation of thrombosis

[16, 17]. The unique structures and redox potentials of these VTIs contribute to their different functions in thrombosis.

Structurally, these VTIs are characterized by the inclusion of at least one thioredoxin-like domain, which is comprised of a four-stranded antiparallel β-sheet encircled by three α-helices. These thioredoxin-like domains are subclassified into catalytic domains (a^0, a, and a' domains) containing the CXXC motif (where X denotes any amino acid), and non-catalytic domains (b and b' domains) [18]. The CXXC motif within the catalytic domain is indispensable for the functional activity of VTIs (Fig. 1). Specifically, the two cysteine residues within this motif endow the enzyme with diverse redox capabilities [19]. The N-terminal cysteine of the CXXC forms a mixed heterodimer with the substrate, while the C-terminal cysteine facilitates substrate release. The amino acid residues positioned between the cysteine residues determine the redox potential and functional specificity of the enzyme [20]. Conversely, the b and b' domains serve as spacers and are primarily involved in substrate recruitment.

In addition to their intricate structural features, VTIs exhibit a diverse range of functions that are crucial for cellular homeostasis and disease processes. Within the endoplasmic reticulum (ER), these enzymes are indispensable for the proper folding and assembly of nascent proteins by facilitating the correct formation of disulfide bonds. However, their functions extend beyond the ER, as thiol isomerases have been implicated in various extracellular and cytosolic processes. For instance, thiol isomerases such as PDI have been shown to possess reductase and oxidase activities, which are vital for regulating the cellular redox status and protecting against oxidative stress [21]. Furthermore, recent studies have highlighted the role of thiol isomerases in pathological conditions such as thrombosis, where they interact with platelet receptors and coagulation factors to modulate blood clot formation [22, 23]. These findings underscore the complex and multifaceted nature of VTIs and their importance in maintaining cellular health and preventing disease. By gaining a deeper understanding of their functions and regulatory mechanisms, researchers can potentially identify novel therapeutic targets for the treatment of disorders associated with VTIs dysregulation.

PDI

PDI contains four thioredoxin-like domains, namely a, b, b', and a'. The a and a' domains are the catalytic domains, and their characteristic CGHC motifs (WCGHCK) are responsible for forming, breaking, and rearranging disulfide bonds [24, 25]. The catalytic domains are separated by b and b' domains, with the b' domain serving a dominant role in substrate binding [26]. A 19-amino acid linker (X-linker) connects the b' and a' domains and affects substrate binding [27]. The C-terminal of PDI contains a large number of negatively charged amino acids, which play an important role in the chaperone function of PDI [28]. Crystal structural studies show that PDI is arranged in a U-shape. The catalytic domains a and a' are located at the two ends of the U-shaped structure. The non-catalytic domains b and b' are distributed in the bottom area, with a hydrophobic pocket in the b' domain for substrate binding [29]. PDI not only functions as a single individual protein, but is also the β subunit of prolyl 4-hydroxylase and microsomal triglyceride transfer protein (MTP) [30]. The structure of MTP reveals the molecular mechanisms of PDI interaction with large protein substrates [31]. The b' domain of PDI provides a primary binding site for MTPα, together with both catalytic domains, mainly through hydrophobic interactions, further supporting the premise that the b' domain of PDI is the principal substrate-binding site, but all domains together contribute to the binding of substrate [18].

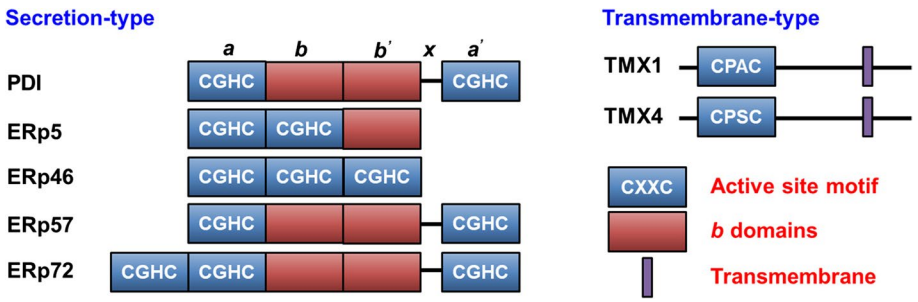


Fig. 1 B Representative vascular thiol isomerases (VTIs) and their domain organization, with a-type domains in darkblue, b-type domains in darkred, and transmembrane domains in purple. A 19-amino acid linker (X-linker) connects the b' and a' domains and affects substrate binding

ERp57

Similar to PDI, ERp57 consists of four thioredoxin-like domains and forms a similar U-shaped structure [32, 33]. Unlike the b' domain of PDI, which has a hydrophobic pocket to bind substrate, the corresponding pocket of the ERp57 b' domain is positively charged. This pocket is critical for ERp57 to interact with negatively charged substrates, such as the arm-like P-domain of lectin chaperones calnexin (CNX)/calreticulin (CRT) [34] to facilitate the correct folding and the quality control of neo-synthesized glycoproteins [35]. The b domain enhances ERp57's binding to the substrate [36], and the basic C-terminus of ERp57 is also responsible for the specific interactions with negatively charged partners. ERp57 engages in the peptide-loading complex (PLC) formation by interaction with CRT and tapasin [37]. The b' domain of ERp57 interacts with CRT to form a flexible belt around tapasin and major histocompatibility complex class I (MHC-I), which play key roles not only in catalyzing the oxidative folding of glycoproteins, but also in stabilizing the PLC to control peptide loading [33]. ERp57 interacts with tapasin, particularly with a and a' domains via hydrophobic interactions, analogous to how the a and a' domains of PDI interact with MTP [31]. However, in this case, a mixed disulfide bond forms between ERp57 and tapasin.

ERp72

ERp72 is the only VTI with five thioredoxin-like domains, which is due to an additional N-terminal catalytic domain a^0 to give an a^0-a-b-b'-a' architecture. Small-angle X-ray scattering (SAXS) studies show that the overall structure of ERp72 is crescent-shaped [38]. Site-directed mutagenesis in the N-terminal Cys of these catalytic domains indicates that the a^0 domain is primarily involved in catalysis, the a domain has intermediate roles in catalysis and binding, and the a' domain functions primarily to bind substrates [39]. The X-ray crystal structure of ERp72 bb' reveals that these two domains form a rigid pair due to the lack of a flexible interdomain linker. The b' domain of ERp72 has a negatively charged patch. The residues Arg398 and Glu459 form a salt bridge to occlude this potential substrate-binding cavity, making it neither binding to hydrophobic substrates like PDI nor negatively charged partners like ERp57 [38]. Alternatively, the X-ray crystal structure of the ERp72 a^0a fragment reveals both two domains contain a small hydrophobic patch adjacent to the catalytic sites [40]. A structural model of full-length ERp72, using the a^0a domains together with bb' and a' domains, shows that all three catalytic sites can be positioned to face each other, and the hydrophobic patches adjacent to the catalytic sites are available for protein substrate binding [40]. Such hydrophobic patches are also found in other VTIs, which cooperatively bind to substrate [41, 42]. Similar to PDI, ERp72 also contains a large number of negatively charged amino acids at its terminus, but these residues are located at the N-terminus of ERp72, which are considered to be the binding site of Ca^{2+}, and mediate ERp72's interaction with chaperones such as BiP, GRP94, and cyclophilin B [43]. Also, one well-defined substrate for ERp72, NADPH oxidase 1 (NOX1), specifically interacts with the first 105 residues of ERp72, which contains this putative Ca^{2+} binding sequence and part of the a^0 domain [44].

ERp46

ERp46 belongs to the protein disulfide isomerase (PDI) family, characterized by the presence of three surface-exposed CGHC motifs located within the a^0, a, and a' domains, which are delineated by flexible linker loops [45]. ERp46 has a radically different molecular architecture compared to other PDIs. This architectural feature distinguishes ERp46 from the more compact, U-shaped conformations of PDI, ERp57, and ERp72, where the active sites are juxtaposed, facing each other across the substrate-binding cleft. Notably, the active sites of ERp46 function independently, contrasting with the cooperative activity displayed by the active sites of PDI. ERp46 exhibits a rapid and promiscuous capacity to introduce disulfide bonds into unfolded substrates, followed by a subsequent, slower, and more precise disulfide bond formation mediated by PDI [46]. Expression of ERp46 on the platelet surface increases with thrombin stimulation. Functionally, ERp46 serves a protective role against apoptosis and is implicated in the production of immunoglobulins and insulin, as well as in the growth of prostate cancer cells [47]. Despite the observations by Holbrook et al. that ERp46 is expressed in human megakaryocytes and human umbilical vein endothelial cells [48] and by Zhou et al. that ERp46 functions in platelet activation and thrombus formation [49], the precise role of ERp46 in the context of thrombosis remains elusive.

ERp5

ERp5 contains two catalytic domains (a^0 and a'), and one noncatalytic b domain, organized in a unique a^0-a'-b arrangement. Although the overall structure of ERp5 is still not determined, ERp5 may form an opened and extended conformation like ERp46, which allows easy access for clients, facilitating disulfide bond introduction into clients during the early stages of oxidative protein folding [50]. The crystal structure of ERp5 a^0 domain in a complex with the Prx4 C-terminal peptide [51] showed the peptide bound a hydrophobic groove of ERp5 a^0 adjacent to the redox-active cysteine, which also exists in other VTI family proteins [41, 42].

TMX1

TMX1 (thioredoxin-related transmembrane protein 1, alternative name TXNDC1) is the first identified transmembrane member of the VTI family [52], and the best-known member of the TMX family [53]. TMX1 is a single-pass type I membrane protein of 280 residues with a large luminal N-terminal region harboring a TRX-like domain and a short cytosolic tail. TMX1 displays a non-canonical CPAC active site in its type-a TRX-like domain [53]. The cytosolic tail of TMX1 also contains both palmitoylation and phosphorylation sites [54]. TMX1 does not contain the b or b' domain and typical ER-retention sequence as other VTIs. In contrast, the di-arginine (RQR) motif in the cytosolic tail may promote its retention within the ER. TMX1 is critical for maintaining platelets in a quiescent state and counterbalancing the effect of ERp46 to prevent platelet overactivation [55]. TMX1 has been also found to interact with vitamin K epoxide reductase (VKOR), an enzyme involved in the process of blood coagulation working with membrane-tethered TRX-like proteins, which serve as redox partners [56].

TMX4

TMX4 (thioredoxin-related transmembrane protein 4, alternative name TXNDC13) is a single-pass type I glycoprotein of 349 amino acids that was identified in 2010 during a database search for TRX-like domains containing proteins [57]. Phylogenetic analysis showed that TMX4 represents the paralogue of TMX1, with whom it shares high similarity within the N-terminal luminal regions despite the presence of an N-glycosylation site and a di-arginine RQR retention motif within the C-terminal domain [58]. Consistently with the lack of an ERSE motif within its promoter region, TMX4 is not up-regulated during ER stresses. TMX4 has one luminal type-a TRX-like domain, which contains a non-canonical CPSC active site. Supporting an involvement in protein folding, TMX4 interacts with CNX and with ERp57 [59]. In this functional complex, TMX4 could enzymatically modify clients directly promoting their oxidative maturation, or it could indirectly contribute to protein folding by reducing the ERp57 catalytic site thus promoting its function as a glycoprotein-specific oxidase.

VTIs and substrates in thrombosis

VTIs are localized to the endoplasmic reticulum of megakaryocytes [60, 61] and the dense tubular system in platelets [61, 62]. In endothelial cells, VTIs are localized to vesicles distinct from Weibel-Palade bodies [63, 64]. Activating platelets or endothelial cells releases VTIs onto cell surfaces and into circulation [22, 48]. Circulating VTIs are thought to regulate the activity of vascular cell receptors, adhesive proteins, and clotting factors on activated platelets and endothelial cells by affecting their allosteric disulfide bonds and regulating vascular events, including blood coagulation, platelet activation, and thrombosis [14].

Studies using targeted knockout mice, specific antibodies, and inactive recombinant proteins have provided hints regarding the role of VTIs in thrombosis. Thrombus formation is impaired in genetically engineered mice with either deletions or function-blocking mutations in PDI, ERp46, ERp57, and ERp72 [49, 65–67]. Inhibitory antibodies to PDI, ERp46, ERp57, ERp72, or ERp5 substantially reduced thrombus accumulation and fibrin generation in mice [49, 68–72]. Several inactive recombinant VTIs with mutations at the cysteines in the CXXC motifs were shown to interfere with the formation of thrombus [65, 66, 70, 73]. In contrast, TMX1 was shown to have a negative effect on thrombosis [74]. In TMX1-knockout mice, platelet aggregation, ATP release, activation ofαIIbβ3, and P-selectin expression increased. Inhibitory anti-TMX1 antibody increased platelet aggregation and ATP release induced by agonists of the GPVI and thrombin receptors. The recombinant extracellular domain of wild-type TMX1 (rTMX1) inhibits platelet aggregation and ATP release, while the inactive rTMX1 mutant potentiated these processes [75].

Extensive experiments were carried out to identify the substrates of VTIs. VTIs were shown to regulate the activity of integrins ($\alpha_{IIb}\beta_3$) [76], glycoprotein Ibα (GPIbα) of the GPIb-IX-V complex [77], thrombospondin-1 (TSP-1) [8], vitronectin, coagulation factors, and other proteins which are secreted from platelet α-granules and participate in the formation of thrombus [78]. VTIs represent a critical mechanism regulating the functions of hemostatic proteins. Histidine-rich glycoprotein (HRG) has been reported as a substrate of PDI and the procoagulant and anticoagulant activities of HRG were enhanced by the neutralization of endothelial heparan sulfate (HS) and inhibition of factor XII (FXIIa) activity in a recent study [79]. Thus, the PDI-HRG pathway fine-tunes thrombosis by promoting its rapid initiation by neutralizing HS and preventing excessive propagation by inhibiting FXIIa.

Platelet surface receptors

Integrin $\alpha_{IIb}\beta_3$ is the most abundant receptor on the platelet surface [76]. On resting platelets, inactive $\alpha_{IIb}\beta_3$ adopts a bent conformation burying the headpiece to prevent ligand interaction. Upon activation by agonists, $\alpha_{IIb}\beta_3$ undergoes a conformational change from a bent to an extended form [80], exposing its headpiece that recognizes RGD and KQAGDV peptides present on fibrinogen, von Willebrand Factor (vWF) and fibronectin, and crosslinking with platelets for their adhesion,

aggregation, and clot formation at the site of vessel injury [81, 82]. The extracellular region of β3 has four epidermal growth factor (EGF)-like domains [83]. The disulfides in the EGF domains are generally important for maintaining the inactive state of β3. For instance, Cys560-Cys583 in EGF-4 was identified as an allosteric disulfide bond for $\alpha_{IIb}\beta_3$ [84].

A recurring theme of the VTIs, including ERp5, PDI, ERp57, ERp72, ERp46, TMX1 and TMX4, is their influence on the function of $\alpha_{IIb}\beta_3$ [23]. One clear mechanism is ERp5 in the regulation of the activity of $\alpha_{IIb}\beta_3$ [85]. ERp5 binds to β3 integrin with a K_D of 21 μM independent of Mn^{2+}. The cysteine residues in the ERp5 active sites are not required for binding to β3 integrin [70]. Interestingly, ERp5 can cleave the disulfide bond between Cys177 and Cys184 at the edge of the fibrinogen binding pocket on the βI domain of $\alpha_{IIb}\beta_3$ in a force-mediated manner, thereby regulating the release of fibrinogen from $\alpha_{IIb}\beta_3$ and weakening the adhesion of platelets [7]. The stretching of the disulfide bond by either ligand binding or mechanical force is necessary for ERp5 activity. It has been proposed that this chemical event may limit thrombus growth and thus prevent vessel occlusion. It seems counter-intuitive that ERp5 promotes the release of fibrinogen from platelets during clot formation, yet ERp5 induces thrombosis in animal models. A possible explanation is fibrinogen released from αIIbβ3 promotes the binding of other ligands such as vWF to form more stable adhesion under high shear force [7, 86].

Regulatory roles of other VTIs on the ligand-binding activity of $\alpha_{IIb}\beta_3$ have also been demonstrated [3, 23]. However, the detailed molecular mechanism by which these VTIs modulate $\alpha_{IIb}\beta_3$ function is still unclear. PDI, ERp57and ERp72 may reduce or isomerize the disulfide bonds of thrombin-activated or Mn^{2+}-treated $\alpha_{IIb}\beta_3$, and change the conformation of $\alpha_{IIb}\beta_3$ from low affinity to high affinity, strengthening the connection between platelets and fibrinogen, and promoting thrombus formation [3, 71]. PDI interacts with $\alpha_{IIb}\beta_3$ with 1–2 μM affinity mainly using the b' domain, with the cooperation of the a and a' domains [5]. ERp57 regulates $\alpha_{IIb}\beta_3$ activity mainly via the a' domain [65]. As for ERp72, both a and a' active sites can regulate the activity of $\alpha_{IIb}\beta_3$ [66]. ERp46 more strongly reduced disulfide bonds (Cys473-Cys503) in the β3 subunit than other VTIs, implicating a target for ERp46. ERp46-deficient platelets have decreased thiols in β3, implying that ERp46 cleaves disulfide bonds in platelets [49]. TMX1 regulates thrombosis negatively. TMX1 can oxidize the disulfide bonds in $\alpha_{IIb}\beta_3$ to inhibit its activation, thereby inhibiting platelet aggregation and thrombus formation. As an endogenous thrombosis inhibitor, the presence of TMX1 may serve an important role in balancing the activation of $\alpha_{IIb}\beta_3$ by PDI [75]. A recent study reported that recombinant TMX4 enhanced platelet aggregation and reduced integrin αIIbβ3 disulfide bonds, and TMX4 deficiency decreased free thiols of integrin αIIbβ3, consistent with a potent reductase activity of TMX4 [87].

The previous studies showed that PDI colocalizes with $\alpha_V\beta_3$ on the surface of endothelial cells, and both proteins in the presence of Mn^{2+} form a complex that dissociates upon reduction. In addition, Mn^{2+} induces the transition of $\alpha_V\beta_3$ to the ligand-competent state, and this reaction is associated with the formation of free thiols within the integrin [88, 89].

GPIbα is a component of the GPIb-IX-V complex, which can bind to vWF at high shear force and promote platelet adhesion and aggregation at the injured vessel wall [77]. The interaction between GPIbα and VWF can deliver a signal to platelets, which subsequently causes a conformational change of αIIbβ3 to enhance fibrinogen binding [90]. PDI directly binds to GPIbα and cleaves two allosteric disulfide bonds (Cys4-Cys17 and Cys209-Cys248), strengthening the ligand-binding function [6] and promoting platelet-neutrophil interactions and vascular occlusion under thromboinflammatory conditions [91].

Matrix proteins

Several receptors on the platelet surface are important for platelet adhesion, and the ligands of these receptors further promote platelet aggregation. The adhesive proteins of the extracellular matrix can enhance platelet aggregation by crosslinking with their receptors and serve important roles in thrombosis.

TSP-1 is a multidomain glycoprotein formed by three identical 150 kDa subunits that are connected via disulfide bonds [8]. TSP-1 contains interacting domains for fibrinogen, vWF, the scavenger receptor CD36, and the cell surface receptor CD47. TSP-1 functions in platelet aggregation by promoting platelet adhesion to collagen and by protecting vWF strings from degradation. TSP-1 was the first identified cell surface substrate of PDI [92]. PDI regulates the Cys974 in the C-terminal of TPS-1, resulting in the exposure of a cryptic RGD motif of TSP-1 to enhance integrin binding, thus affecting its cell adhesive properties [8, 93].

Vitronectin is an abundant plasma protein with a concentration of 200–400 μg/ml that is present in both monomeric and multimeric forms depending on its association with other molecular species and the activity of the proteolytic enzymes [94]. Vitronectin binds to $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$, plasminogen activator I, urokinase receptor, collagen, and complement C5b-C9, and functions in thrombus formation [94]. PDI can reduce the disulfide bonds Cys137-Cys161 and Cys274-Cys453 of vitronectin,

enabling binding to $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ [9]. In a laser-induced vascular injury model of thrombosis, inhibition of PDI prevented vitronectin accumulation at the site of thrombus formation [9].

Another important protein in thrombus formation is vWF, which is a large glycoprotein that circulates as a series of multimers. The higher multimers are more effective at capturing platelets at high shear forces due to polyvalent properties that enhance binding to collagen, platelet receptors GPIbα and $\alpha_{IIb}\beta_3$ [95]. There are three vWF A domains (A1, A2, and A3) with each containing one disulfide bond. The A1 domain contains binding sites for platelet GPIbα and collagen. The A3 domain binds only to collagen fibrils types I and III, whereas the A2 domain contains the cleavage site for the metalloprotease ADAMTS-13 and Cys1669 and Cys1670 at the C terminus of A2 control autoinhibition of vWF [96]. It is suggested that vWF subunits exist in two functional forms in the circulation based on the redox state of the A2 domain: an oxidized form with high affinity for GP1bα when sufficient shear force unfolds the domain, and a reduced plus glutathionylated form with low affinity for GP1bα, insensitive to shear force [10]. It is unknown whether VTIs regulate the function of vWF like ERp5 on $\alpha_{IIb}\beta_3$ in a force-mediated manner (e.g., in a manner similar to how ERp5 acts on $\alpha_{IIb}\beta_3$), though PDI was reported to regulate the vWF dimerization in the ER [97].

Coagulation factors

PDI directly participates in modulating the activation of coagulation factors, such as factor XI (FXI), factor V (FV), and tissue factor (TF), and regulates platelet and endothelial cell-dependent coagulant activity. Proteolytic activation of coagulation FXI contributes to the consolidation phase of blood coagulation [98]. FXI is a disulfide-linked homodimer consisting of two identical subunits, and each of the subunits contains 19 disulfide bonds. One or more FXI disulfide bonds are reduced in the zymogen, and the Cys362-Cys482 and Cys118-Cys147 disulfides are reduced in the active enzyme [99]. PDI may also cleave the Cys362-Cys482 disulfide bond of FXI. The resultant reduced protein appears to be more efficiently activated by thrombin, factor XIIa (FXIIa), or factor XIa (FXIa) than the oxidized protein [11, 99].

FV is a cofactor in the prothrombinase complex, where factors Xa (FXa) and FVa convert prothrombin to thrombin. A mechanistic-based substrate trapping approach has identified FV as the substrate of PDI [13]. In the initial of thrombosis, PDI may reduce the disulfide bond in FV to regulate its activities. Meanwhile, FV may be released from a complex with multimerin by PDI-mediated cleavage of the disulfide bond that connects the two proteins, providing additional FV for thrombin generation [100].

TF is a transmembrane cofactor for factor VIIa. The TF/factor VIIa complex proteolytically activates FX to initiate blood coagulation and provide the thrombin burst required for a stable thrombus [101]. The formation of a disulfide bond between Cys186 and Cys209, the exposure of phosphatidylserine on the cell surface, and the hydrolysis of sphingomyelin by acid sphingomyelinase have all been proposed to contribute to TF decryption [102, 103]. Although the role of PDI in tissue factor activation remains controversial, experimental evidence debates that PDI has been implicated in TF decryption, and PDI oxidization of reduced Cys186 and Cys209 thiols in soluble TF may regulate the activity of TF [12]. PDI also regulates a critical P2X7 receptor-dependent signaling pathway that is associated with the decryption of TF [104]. Inhibition of PDI has been suggested to increase the activity of TF via the expression of phosphatidylserine [105].

Working model of VTIs in thrombosis

VTIs, including PDI, ERp57, ERp5, ERp72, ERp46, TMX1, and TMX4, all play a distinctive role in regulating thrombosis, but their roles seem unique and do not overlap. In the PDI-, ERp57-, or ERp72-null platelets, platelet aggregation was recovered only when the missing TI was added back to the assay, but not when other types of VTI were added back, showing that they appear to regulate platelet aggregation through differential mechanisms [106, 107]. For example, these VTIs may regulate the activity of platelet surface integrin $\alpha_{IIb}\beta_3$ by catalyzing sequential or simultaneous reactions in $\alpha_{IIb}\beta_3$, which were well-reviewed previously [65, 108]. Because of the redox ability of these VTIs, there might be two ways to catalyze the reaction: one VTI may generate thiols in $\alpha_{IIb}\beta_3$ while another VTI subsequently catalyzes a thiol-disulfide exchange reaction, or one VTI acting on another VTI that in turn acts on $\alpha_{IIb}\beta_3$ [3]. The proposed working model of VTIs suggests that the inactivation of thiols in $\alpha_{IIb}\beta_3$ is initiated through inside-out signaling, leading to the low-affinity binding of fibrinogen to $\alpha_{IIb}\beta_3$. Subsequently, conformational changes in $\alpha_{IIb}\beta_3$ are driven by disulfide isomerase-catalyzed processes. The thiol-disulfide rearrangement within $\alpha_{IIb}\beta_3$ promotes the transition to a high-affinity state. Additionally, the generation of free thiols in this process may involve external thiol/disulfide pairs, such as GSH/GSSG, or NAD(P)H-dependent reductase activity, with $\alpha_{IIb}\beta_3$ potentially amplifying this reaction [109, 110].

Based on the redox potentials of these VTIs, Chiu et al. proposed that VTIs constitute a redox chain [89, 111] such that electrons flow from the most reducing

thioredoxin to the most oxidizing ERp57. ERp72,- ERp5, and PDI consecutively participate in this process. The electrons may flow from any stage to the disulfide bond of the substrates, or the electrons skip some VTIs and flow to the next VTI [111]. As the kinetic trapping experiment identified, both thioredoxin and ERp57 are PDI substrates, and the thioredoxin is biased toward oxidation, and ERp57 favors reduction, which is consistent with the possibility that VTIs act in parallel [25, 107]. As for how TMX1 participates in this redox chain, it remains to be explored. However, TMX1 can oxidize the cysteine residues of αIIbβ3 and is likely to participate in this process near the most oxidizing ERp57 [74]. Recombinant TMX4 protein enhanced platelet aggregation and reduced integrin αIIbβ3 disulfide bonds, and TMX4 deficiency decreased free thiols of integrin αIIbβ3, consistent with a potent reductase activity of TMX4 [87].

VTIs play different roles in thrombosis and affect thrombus formation individually, but how VTIs regulate their substrates and whether there are any other substrates regulated by VTIs in thrombosis remains unclear. In the future, using trapping VTIs mutants containing intervening sequence variants, CGPC and CGRC (instead of CGHC) [25], or active site-variant to find more substrates of TIs will raise important possibilities to study their roles in platelet function and thrombosis.

Antithrombotic inhibitors targeting VTIs

The modulation of disulfide bonds within thrombus-associated proteins by VTIs has created new opportunities for developing new antithrombotics that could exhibit either superior efficacy or enhanced safety profiles compared to those directed against traditional blood coagulation factors. Recently, the implementation of high-throughput screening methodologies has facilitated the identification of small-molecule inhibitors that specifically target VTIs. The structure, inhibitory ability, and mode of action of these small molecule inhibitors are compared in Table 1.

Flavonoids are particularly prominent VTIs targeted anti-thrombotic inhibitors. Quercetin-3-rutinoside (also known as rutin) has attracted significant attention due to its excellent anti-thrombotic effect in vivo and the low tendency to cause bleeding. In various mouse thrombosis models, intravenous injection of rutin inhibited platelet aggregation and fibrin formation during thrombosis, without causing prolonged bleeding time [112, 130]. SAXS showed that rutin bound directly to PDI and forced PDI into a closed conformation [112], while smFRET shows that the binding of rutin results in the twisting of the enzyme [131]. The binding of rutin inhibited the reduction of insulin and the small molecules di-eosin-GSSG or bodipy-labeled L-cysteine by PDI [110, 131]. Small molecular FRET and enzyme kinetic studies show that rutin has an allosteric effect on PDI conformation that blocks, in addition to competitively inhibiting the interaction between PDI and substrate [131]. Rutin also affected the redox activity PDI by inducing conformational changes. Our molecular dynamics simulations and site-directed mutagenesis experiments further showed that rutin is not only bound to the hydrophobic pocket on the PDI b' domain, but also bound to the vicinity of the hydrophobic pocket and the x-linker [112]. The interaction between rutin and PDI x-linker limited the flexibility of x-linker, which in turn affected the catalytic activity of PDI. Recently, we identified H256 as the key residue for PDI interacting with the phenoxyl group at position 7 in rutin is essential for the inhibition of PDI by rutin [132]. However, clinical translation of rutin is restricted due to its low aqueous solubility and oral bioavailability, thus, we formed rutin:Zn complex and fabricated rutin-loaded lipid-based nano-formulation (NanoR), which not only increased rutin aqueous solubility, but also exhibited greatly enhanced antithrombotic activity and good safety performance [133, 134].

We also administrated oral isoquercetin, an analog of rutin with stronger in vivo stability, in anti-thrombotic clinical trials for cancer patients [100, 113]. Patients with advanced cancer received either 500 or 1000 mg of isoquercetin orally every day for 56 days. Evaluation of plasma samples following ingestion of the higher dose showed statistically significant decreases in plasma PDI activity, D-dimer levels (the primary endpoint), platelet-dependent thrombin generation, and soluble P-selectin levels compared to levels obtained before isoquercetin ingestion [13]. It was reported that myricetin also had a good anti-platelet effect by inhibiting platelet activation and reducing the interaction between platelets and collagen, while not affecting the hemostatic process in mice even at a high dose of 50 mg/kg. The molecular docking result showed that myricetin bound to the domain of PDI through non-covalent hydrogen bonds to inhibit its reductive activity. Myricetin also had an inhibitory effect on ERp5 [114].

Some other small molecule inhibitors targeting VTIs were also developed. We discovered the small molecule inhibitors of PDI termed bepristats (bepristat 1a, bepristat 2a [131], and ML359 [117]) through high-throughput screening. Bepristat 1a and bepristat 2a inhibited platelet aggregation and thrombus formation in vivo. In the mice model, infusion of 15 mg/kg of bepristat 1a reduced platelet accumulation by 79.7% at the injured site, and bepristat 2a reduced platelet accumulation by 85.1%, showing an excellent anti-thrombotic effect. SAXS showed that bepristats displaced the x-linker to reversibly bind to the b' domain of PDI, and reduce the

Table 1 Antithrombotic small molecule inhibitors targeting on VTIs

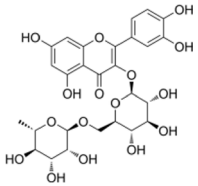
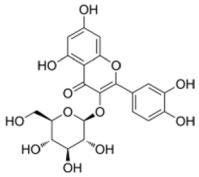
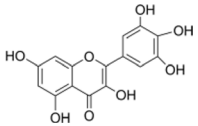
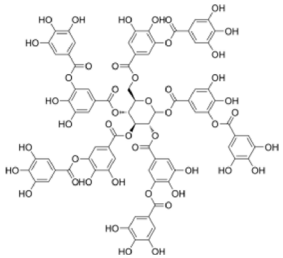
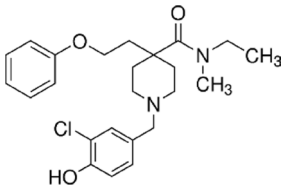
Inhibitor	Chemical formula	IC_{50}/K_D	Binding site	Cell or animal-level inhibition test	Binding target	Ref
Rutin		10 μM	b′ domain and x-linker	Inhibits the aggregation of platelets and the formation of fibrin, and inhibits thrombosis at 0.5 mg/kg in vivo without causing prolonged bleeding time	PDI	[112]
Isoquercetin		2.5 μM	b′ domain	Used in anti-thrombotic clinical trials for cancer patients, it can effectively reduce the formation of thrombus (thrombin formation decreased by 51%) by taking 1000 mg orally every day	PDI	[113]
Myricetin		–-	a domain	Inhibit platelet activation, reduce the interaction between platelets and collagen, inhibit thrombosis, and do not affect the process of hemostasis	PDI, ERp5	[114]
Tannic acid		< 1.68 nM	CXXC motif	Inhibits PDI activity, platelet activation, and thrombus formation	PDI	[115]
Bepristat 1a		0.7 μM	b′ domain	Inhibit platelet aggregation and thrombus formation in a laser-induced thrombosis model in mice at 15 mg/kg	PDI	[116]

Table 1 (continued)

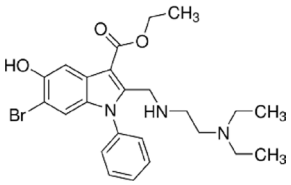
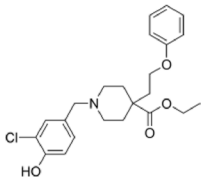
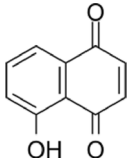
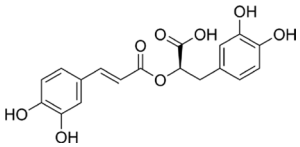
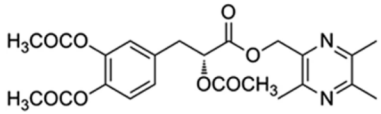
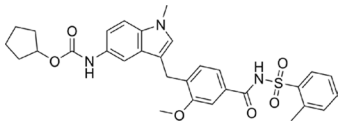
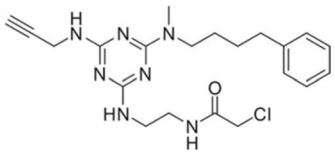
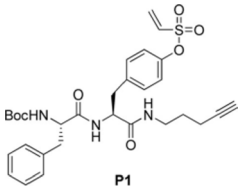
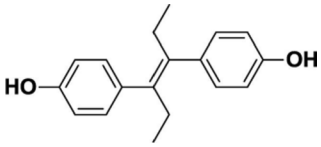
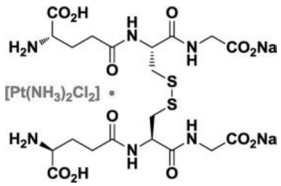
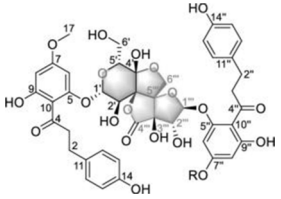
Inhibitor	Chemical formula	IC_{50}/K_D	Binding site	Cell or animal-level inhibition test	Binding target	Ref
Bepristat 2a		1.2 μM	b′ domain	Inhibit platelet aggregation and thrombus formation in a laser-induced thrombosis model in mice at 15 mg/kg	PDI	[116]
ML359		250 nM	b′ domain	Inhibit platelet aggregation and thrombosis, and has no cytotoxicity to HEK 293, HepG2, HeLa cell lines	PDI	[117, 118]
Juglone		1.61 ± 0.11 μM	a′ domain	Juglone (1—5 μM) concentration-dependently inhibited platelet aggregation caused by all three agonists with IC50 values of 1.46 ± 0.19 μM, 1.70 ± 0.14 μM, and 2.51 ± 0.19 μM, respectively	PDI	[119]
Rosmarinic acid		176.82 ± 11.7 μM	a′ domain	Inhibit platelet aggregation	ERp57	[120]
ADTM		100–300 μM	–-	Inhibit the expression of P-selectin and the activation of αIIbβ3, inhibit platelet aggregation and thrombosis in vivo	ERp57, ERp72, ERp5, PDI	[121]
Zafirlukast		5–10 μM	a and a′ domain	Platelet aggregation in response to collagen had an IC_{50} value of 1.66 μM	PDI, ERp57, ERp72	[122, 123]

Table 1 (continued)

Inhibitor	Chemical formula	IC_{50}/K_D	Binding site	Cell or animal-level inhibition test	Binding target	Ref
RB-11-ca		10 ± 1.1 μM	a and a′ domain	The effect of RB-11-ca on HeLa cell proliferation demonstrates that RB-11-ca shows micromolar inhibition (23.9 μM) of cell proliferation	PDI	[124, 125]
P1		1.7 ± 0.4 μM	a′ domain	P1 shows low-micromolar inhibition of cell proliferation against six cancer cells	PDI	[126]
Diethylstilbestrol		–-	–-	Diethylstilbestrol cause a more pronounced inhibition of receptor-mediated than of voltage-dependent Ca^{2+} channels	PDI	[127]
NOV-002		–-	–-	The mechanism of action of NOV-002 is the modulation of cellular redox balance	PDI	[128]
Piericones A		0.15 ± 0.04 μM	b′ and xa′ domain	Piericone A significantly inhibited platelet aggregation and fibrin formation in vitro and thrombus formation in vivo by inhibiting extracellular PDI without increasing the bleeding risk	PDI	[129]

cysteines at the active site, increasing its reductive activity [16, 116]. Compared to rutin, bepristat-2a promotes a more compact conformation of PDI, in which a greater enzymatic activity is achieved by accelerating the nucleophilic step of the a domain, leading to faster formation of the covalent enzyme–substrate complex [131].

Recently, several galloylated polyphenols were shown to inhibit PDI [135]. Tannic acid (TA), a galloylated polyphenol from plants, was found to bind to PDI with a high affinity. A molecular docking study showed that TA was likely bound to the active site of PDI, and a single dose of 5 mg/kg TA inhibited the activity of platelet and thrombus formation in the laser-induced mouse thrombosis model without causing bleeding [115]. A natural compound named juglone also inhibited the activity of PDI. 1–5 μM juglone inhibited platelet aggregation and glycoprotein (GP) IIb/IIIa activation, exhibiting potent anti-platelet and anti-thrombotic effects in vitro [119]. Rosmarinic acid is a water-soluble phenolic compound and showed a specific inhibition on ERp57. Molecular docking showed that it bound to the a' domain of ERp57 through hydrogen bonding. The compound displayed a significant inhibitory effect on ERp57 activity and inhibited arachidonic acid-induced platelet aggregation [120].

Some potent PDI inhibitors with unprecedented chemical structure have been isolated from *Pieris japonica* [129]. Among these inhibitors, Piericones A was a nanomolar noncompetitive PDI inhibitor possessing an unprecedented 3,6,10,15-tetraoxatetracyclo[7.6.0.0^{4,9}.0^{1,12}]pentadecane motif with nine contiguous stereogenic centers. Piericone A significantly inhibited platelet aggregation and fibrin formation in vitro and thrombus formation in vivo by inhibiting extracellular PDI without increasing the bleeding risk.

The broad-spectrum inhibitors of VTIs were also used for antithrombus. ADTM [121] and zafirlukast [122, 123] both inhibited the activity of PDI, ERp5, ERp57, and ERp72. 20 mg/kg ADTM significantly inhibited platelet aggregation and thrombus formation. Meanwhile, zafirlukast diminished platelet aggregation and showed an excellent anti-thrombosis effect in the presence of 20 μM/mL of blood volume, and without affecting hemostasis.

In addition to small molecule inhibitors, peptides are also used to inhibit the activity of VTIs. CXXC is a 12-amino acid (VEFYAPWCGHCK) peptide inhibitor derived from a partial sequence of the PDI C-terminal. It is an irreversible inhibitor that is covalently bound to the Cys400 of PDI a' domain to inhibit PDI activity, thereby inhibiting the activity of $\alpha_{IIb}\beta_3$ [136].

Estrogenic compounds have been reported to be inhibitors of PDI, including diethylstilbestrol and estrone [127]. In addition, NOV-2002 is a novel glutathione-disulfide mimic complexed to platinum that was studied for use as a chemotherapeutic for lung cancer [128]. It was reported to inhibit PDI, but it was also reported to be a substrate for glutathione reductase. Its likely toxicity, irreversibility, and lack of selectivity make it undesirable for antithrombotic studies.

Perspective on the development of anti-thrombotic inhibitors targeting VTIs

VTIs participate in many disease processes. Important attributes of potential VTIs targeted anti-thrombotic inhibitors include reversibility, cell membrane permeability, and specificity.

Reversibility is important for avoiding the risk of hemorrhage. VTI inhibitors like RB-11-ca [124, 125] and P1 [126] that act on the cysteine of CGHC motif and exhibit irreversible binding have good anti-tumor effects. Still, their mechanism of action may be less conducive to their application in anti-thrombotic therapy. Permeability is another key parameter for anti-thrombotic inhibitors development. VTIs exist in both intracellular and extracellular space, and only extracellular VTIs are associated with thrombosis. Developing inhibitors that are non-permeable to the cell membrane can block extracellular VTIs to inhibit thrombus formation without affecting the intracellular VTIs, thus maybe less toxicity to cells. Rutin has demonstrated no toxicity to endothelial cells even at high concentrations, which may be due to 3-O-glycosidic linkage impairing its cell permeability [130, 137].

It was recently reported that the mice deficient in VTIs, including PDI, PDIp, ERp57, PDIr, ERp5, ERp27, ERp29, TMX4, and ERdj5, had no effects on venous thrombosis in the inferior vena cava (IVC) stenosis model [138]. However, the mice deficient in ERp18 developed significantly less venous thrombosis compared with WT mice, which demonstrates the novel role of ERp18 in enhancing the development of venous thrombosis. Although mice deficient in thiol isomerases such as PDI did not affect venous thrombosis in the IVC stenosis model, this does not mean that these proteins are completely not involved in the regulation of thrombosis. These thiol isomerases may play a role in other physiological or pathological conditions, or affect thrombosis in specific cell types or environments. In addition, inhibition of these thiol isomerases in clinical trials may demonstrate antithrombotic effects by affecting platelet function, inflammatory response, or other mechanisms associated with thrombosis. Therefore, the findings in cancer patients are not necessarily directly related to those observed in mouse models, and further research is needed to clarify the mechanism.

Furthermore, inhibitors must possess robust selectivity and specificity to mitigate adverse effects arising from the

inhibition of non-target proteins. The a and a' domains of VTIs exhibit high homology, rendering the acquisition of specific inhibitors for these domains particularly challenging. It is quite challenging to develop specific VTIs inhibitors targeting the active motif. Besides the specificity issue, such inhibitors are often covalent binders and may have strong reactivity along their delivery route and suffer from the problem of bioavailability. Conversely, the b and b' domains display low homology and emerge as primary candidates for creating specific inhibitors. Numerous antithrombotic agents that are currently being studied are indeed targeted to these domains. However, these inhibitors generally exhibit low potency, exemplified by isoquercetin's binding affinity to PDI, progressing through clinical phases II/III, which are merely 2.5 μM. Furthermore, the precise molecular mechanisms underlying the binding of these inhibitors to their targets and the subsequent impairment of enzymatic function remain elusive, highlighting potential avenues for future research endeavors.

Conclusion

The review of the literature on vascular thiol isomerases (VTIs) and their related family members, highlights their crucial roles in thrombus formation and platelet function. VTIs, such as PDI, ERp46, ERp57, ERp72, and TMX proteins, exhibit diverse structural features and functional mechanisms. These proteins not only catalyze disulfide bond formation and isomerization but also act as chaperones and play significant roles in stabilizing protein complexes. The inhibitory effects of various small molecule inhibitors targeting VTIs demonstrate their potential as antithrombotic agents. By binding to specific domains of VTIs, these inhibitors effectively inhibit platelet aggregation and thrombus formation without causing prolonged bleeding times. However, the development of specific inhibitors for VTIs remains challenging due to the high homology between their domains. Future research should focus on identifying more potent and selective inhibitors for VTIs to mitigate adverse effects and enhance their therapeutic potential in treating thrombosis and related disorders. Overall, the understanding of VTIs and their inhibitors provides new insights into the regulation of thrombus formation and platelet function, with promising implications for antithrombotic therapy.

Abbreviations

CNX	Calnexin
CRT	Calreticulin
ER	Endoplasmic reticulum
ERp5	Endoplasmic reticulum protein 5
FV	Factor V
FXa	Factors Xa
FXI	Factor XI
FXIIa	Factor XIIa
GPIbα	Glycoprotein Ibα
MHC-I	Major histocompatibility complex class I
MTP	Microsomal triglyceride transfer protein
HRG	Histidine-rich glycoprotein
HS	Heparan sulfate
NOX1	NADPH oxidase 1
PLC	Peptide-loading complex
PDI	Protein Disulfide Isomerase
IVC	Inferior vena cava
TA	Tannic acid
TF	Tissue factor
TMX1	Thioredoxin-related transmembrane protein 1
TSP-1	Thrombospondin-1
SAXS	Small-angle X-ray scattering
IVC	Inferior Vena Cava
VKOR	Vitamin K epoxide reductase
VTIs	Vascular thiol isomerases
vWF	Von Willebrand factor

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Authors' contributions

Mingdong Huang and Robert Flaumenhaft provided the subject matter and direction of this manuscript and offered guidance in its preparation. Longguang Jiang wrote, edited, and revised the manuscript. Cai Yuan, Robert Flaumenhaft and Mingdong Huang revised the manuscript. All authors have read and approved the final draft of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare no competing interests.

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