Thiol isomerases orchestrate thrombosis and haemostasis

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Authorship

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SUMMARY

Since protein disulphide isomerase (PDI) was first described in 1963, researchers have shown conclusively that PDI and sibling proteins are quintessential for thrombus formation. PDI, ERp5, ERp57 and ERp72, which in most cells are located in the endoplasmic reticulum and function to assist the folding of nascent protein, are released from platelets and vascular cells and interact with integrin αIIbβ3 on the outer surface of platelets. At the cell surface they continue to influence protein folding and function, propagating thrombosis and maintaining haemostasis. TMX1, which is a transmembrane thiol isomerase, is the first family member shown to negatively regulate platelets known to date. Targets of thiol isomerases have been indentified including integrin α2β1, Von Willebrand Factor (VWF), GpIbα, Nox-1, Nox-2 and tissue factor, all of which are pro-thrombotic, and several of which are on the cell surface. In spite of this, PDI can paradoxically catalyse the delivery of nitric oxide to platelets, which inhibits their function and decreases thrombus formation. Although the overall effect of PDI is to positively regulate platelet activation, it is still unclear how thiol isomerases function in pro-thrombotic states, such as obesity, diabetes and cancer. In parallel, there has been a surge in the development of novel thiol isomerase inhibitors, which display selectivity, potency and modulate thrombosis and haemostasis. The availability of selective thiol isomerase inhibitors has culminated in clinical trials with promising outcomes for the prevention of cancer-associated thrombosis. Altogether, thiol isomerases are perceived as an orchestrating force that regulates thrombus development. In the current review we will explore the history of PDI in cardiovascular biology, detail known mechanisms of action and summarise known thiol isomerase inhibitors.

KEYWORDS: Protein disulphide isomerase, platelet, thrombosis, thiol isomerase, history, inhibitors

1. INTRODUCTION

Platelets were first discovered in the 19th century as 'small plates' derived from either neutrophils or red blood cells [1]. Interests in platelets were rekindled in the 1960s, almost 100 years after their initial description, when several groups began to investigate their functions [2-4]. These cells are regarded as anucleated fractions of megakaryocytes and great scientific effort has been employed to understand their vital task: to prevent and stop mammals from bleeding [5] – which makes platelets a remarkable evolutionary adaptation necessary for human survival (reviewed in [6]).

In order to maintain haemostasis, platelets rely on several molecules to either inhibit (e.g. nitric oxide, prostacylin) or induce (e.g. thrombin, thromboxane, etc) platelet activation [7]. Thrombus development initiates upon exposure of sub-endothelial extracellular matrix proteins following blood vessel injury, and particularly collagens to which von Willebrand factor (VWF) binds. Platelet receptors GPVI and integrin α2β1 bind to collagen, while glycoprotein (GP) Ib-V-IX binds to VWF [8,9]. Together these receptors start intracellular (inside-out) signalling that culminates in conformational changes in integrin αIIbβ3, which initiates ligand binding. Fibrinogen and VWF then bind to the integrin, creating a second wave of signalling events, termed outside-in signalling (reviewed in [10]). This latter chain of signals created by integrin αIIbβ3 binding and clustering results in irreversible platelet adhesion, aggregation, pseudopodia formation and reinforces degranulation of dense- and α-granules. In this way key activatory molecules such as ADP and serotonin are secreted, while metabolism of arachidonic acid liberated from platelet membranes results in the production of thromboxane A2 (TxA_2). Through the actions of these secondary activators on platelet receptors, a positive feedback loop is initiated, resulting in the activation and recruitment of further platelets and the growth of a platelet thrombus, or haemostatic plug, to stem the loss of blood (reviewed in [11]). A summary of inside-out and outside-in signalling is presented in **Figure 1**.

Platelet activation and endothelial damage have been shown to result in the release of a number of normally endoplasmic reticulum (ER)-resident proteins into the blood [12,13]. These include members of the thiol isomerase family of oxidoreductase enzymes, namely protein disulphide isomerase A1 (herein referred to as PDI), ERp5, ERp57, ERp72 and TMX1 (negative regulator), which were previously thought to be restricted to the ER [12-15]. PDI is the prototype of the thiol isomerase family, also known as thioredoxins, which catalyse reduction, oxidation and isomerisation of disulphide bonds, as well as nitric oxide (NO) transfer through transitrosation (**Figure 2**) [16]. PDI is the product of the P4HB gene, with a

molecular mass of 57 kDa and comprises five modules: two thioredoxin-like domains (a and a') that catalyse oxidoreductase reactions, two substrate-binding domains (b and b'), a C-terminal extension domain and a cross-linker sequence between b' and a'. Upon activation soluble, non trans-membrane thiol isomerases (PDI, ERp5, ERp57 and ERp72) are released from platelets whereupon they bind to the outer surface of the plasma membrane, being important for platelet function both *in vitro* and *in vivo* and supporting thrombosis and haemostasis [12,17]. Indeed, extracellular disulphide exchange reactions between thiol isomerases mentioned above and integrin αIIbβ3 regulate integrin activation [12,17-20].

In this review we will explore the contribution of PDI family proteins to thrombus development in health and disease. We will draw a historical landscape of key discoveries in the field followed by a description of known targets of PDI that regulate thrombosis. Finally, we provide a comprehensive table of thiol isomerase inhibitors and future perspectives.

2. HISTORICAL LANDMARKS

2.1. Discovery of PDI

In 1963, two independent groups made pivotal discoveries of an enzyme that catalysed the reactivation of reduced ribonuclease. The first group, led by Brunó Straub, described that pigeon and chicken pancreas contained a heat-labile 'factor' that induced the reoxidation of reduced RNAse [21], whereas the second, led by Nobel prize-winning Christian B Anfinsen, made similar observations using a microsomal system from rat livers [22]. However, it was only 10 years later that PDI was officially named [23]. PDI was also described as an 'insulin protease' since it catalysed the reduction of insulin, in an assay that has been widely disseminated in the field and still used in contemporary work [24]. The protein sequence and identification of the CGHC active sites of rat PDI were only performed in the 1980s [25]. From hereon, other PDIs were discovered and their relevance to various physiological and pathophysiological processes in cardiovascular cells began to be explored. The historical landmarks of the involvement of PDI in thrombosis are summarized in **Figure 3**.

2.2. PDI is found on the outer surface of cardiovascular cells

Initially thought to be restricted to the ER due to a KDEL sequence, PDI was found to be secreted from activated platelets over 30 years after initial reports by Straub's and Afinsen's groups [14]. Subsequently, Essex et al [26] demonstrated that PDI is localized to the external surface of the platelet plasma membrane. In addition, this group has shown that the

majority of platelet PDI is localized on the platelet surface, while other blood cells showed little PDI when compared to platelets. Further work conducted by this group demonstrated that PDI is recruited to the surface of platelets upon activation [27] and while this process has been shown to be dependent on the reorganisation of the actin cytoskeleton, given its intracellular localisation to the dense tubular system of platelets [28] the exact mechanism of release is unclear. Indeed, work conducted by our group [29] and Raturi et al [30] have identified PDI in plasma extracellular vesicles (EVs). These EVs were capable of potentiating platelet aggregation and displayed reductase activity, both of which were inhibited in the presence of a functional anti-PDI antibody [30].

PDI is also present on the surface of endothelial cells, and the secretion of endothelial cell-derived PDI is able to modulate thrombospondin-1 activity [31]. Upon endothelial lesion, PDI is secreted from endothelial cells to potentiate thrombus formation *in vivo* [13]. PDI has also been detected on the surface of vascular smooth muscle cells (VSMC) [32] and shown to regulate VSMC migration, differentiation and redox homeostasis [33,34]. Therefore, in spite of initial reports describing an ER-resident chaperone with oxidoreductase activity, the relevance of extracellular PDI, also termed peri/epicellular PDI, has been of great interest to the homeostasis of cardiovascular cells. Importantly, Cho et al [35] were the first to demonstrate that peri/epicellular PDI is critical to thrombus formation in a rodent model of thrombosis *in vivo*. Several groups have reiterated this finding [13,36,37]. However, PDI inhibition [27] or genetically deletion [17] does not completely abrogate platelet responses, suggesting that there are other thiol isomerases on the surface of platelets that may also regulate thromboinflammatory responses.

2.3. Other thiol isomerases regulate thromboinflammation

The observation that PDI inhibition was unable to completely inhibit platelet responses has allowed the speculation that other thiol isomerases could be expressed on the platelet surface. Indeed, several additional thiol isomerases have been identified on the platelet membrane [12,38-40]. These include ERp5, which is recruited to the platelet outer membrane where it binds to integrin β3 [18]. Selective inhibition of ERp5 and/or PDI with selective antibodies revealed additive inhibitory effects [18]; findings that were corroborated by Passam et al [41] who showed that ERp5 is required for thrombus formation *in vivo* and directly binds to integrin β3. Other thiol isomerases that have been reported in and on mouse and human platelets and megakaryocytes include: ERp57 [29,38], ERp72 [29,39], ERp44 [29], ERp29 [29], TMX1 [40]

and TMX3 [29], of which only ERP57, ERp72, ERp44 and ERp29 are released by platelets and recruited to the cell surface upon activation [29]. The transmembrane thiol isomerase TMX1 has been identified recently as the first thiol isomerase to negatively regulate platelet function, i.e. inhibition of TMX1 potentiates platelets responses [40].

Similar to ERp5 and PDI, the functions of several other surface thiol isomerases have been characterized in platelets. Selective inhibition of surface ERp57 [38,42] also results in diminished platelet activation, and thrombosis in mice. Using platelet-specific ERp57 KO mice, Wang et al [43] have confirmed the importance of this thiol isomerase for thrombus formation *in vivo*. This work has also demonstrated that the C-terminal, but not the N-terminal, active site of ERp57 is critical for platelet aggregation. More recently, elegant work using trapping mutants has identified the lectin pathway of complement activation as a novel mechanism through which peri/epicellular ERp57 may regulate thromboinflammation [44].

ERp72 has also been shown to regulate platelet function and thrombosis *in vivo* [39,45]. The role of so many similar enzymes with similar function on the platelet surface raises the question as to whether different isomerases are functionally redundant. Important studies by Zhou et al [39] revealed that recovery of platelet aggregation of ERp72-, PDI-, and ERp57-null platelets was only observed when the specific deficient thiol isomerase was reconstituted. This suggests that, although similar in structure and function, thiol isomerases may act at different points to sustain platelet responses and thrombosis, developing specific tasks that cannot be compensated by a different isoform, i.e. thiol isomerases may work in series. It is possible that there is an electron transfer chain between thiol isomerases occurring on the platelet outer membrane, similar to what has been observed in the ER [46]. However, it is still unclear which specific substrates are targeted by each thiol isomerase and in what sequence, fostering many unanswered questions.

2.4. Trafficking and localization of PDIs

It is presently unclear how thiol isomerases translocate to the outer membrane of cells. In eukaryotic cells, secretory proteins often follow a conventional protein secretion route, trafficking from the ER to the Golgi apparatus and subsequently to the plasma membrane [47]. However, we have shown that PDI and ERp57 do not co-localize with secretory vesicles in megakaryocyte or platelets, but are rather concentrated in a subcellular compartment near the inner surface of platelets, corresponding to the sarco/endoplasmic reticulum or dense tubular

system [28]. Moreover, the externalization of PDI and ERp57 were highly dependent on actin polymerization, suggesting cytoskeletal rearrangement is key to the secretion of thiol isomerases in platelets [28]. Similar to platelets, in endothelial cells, PDI translocates to the outer membrane through Golgi-independent routes, although the precise mechanism for this has not been established [48]. In spite of these similarities, the mode of translocation may differ between cells, given that platelet thiol isomerases externalize via actin polymerization, whereas in endothelial cells actin stress fibre disruption enhanced PDI secretion [48].

One feasible alternative yet to be investigated is the possibility that thiol isomerases externalize directly through ER-plasma membrane connections [49]. In fact, these connections were shown to be highly regulated by Ca^{2+} influx [49], which is also key to signalling in platelets and endothelial cells. In summary, although recent efforts have identified mechanisms through which thiol isomerases are trafficked in cardiovascular cells, many questions remain unanswered. Understanding of how thiol isomerases are transferred to the outer membrane of cells may enlighten, for instance, how these proteins become localized in sites of thrombosis, i.e. whether thiol isomerases are actively secreted through secretory pathways or passively diffuse upon cell disruption.

2.5. Clinical trials with PDI inhibitors

With increasing evidence uncovering the importance of PDI to cardiovascular cells, there has been growing interest to develop PDI inhibitors to treat diseases associated with thrombosis and hypercoagulability. A comprehensive table with known inhibitors of PDI and other thiol isomerases is presented below (Table 1). In spite of this growing interest, clinical trials with truly specific thiol isomerase inhibitors are currently lacking. Two phase II clinical trials are underway using isoquercetin, a flavonoid that targets PDI [50] and antioxidant pathways [51]. The first trial aims to assess the benefits of administering isoquercetin to patients with hypercoagulable states, after an initial study in healthy volunteers [52]. The results from this study (ClinicalTrials.gov Identifier: NCT01722669) have been recently published, showing that daily administration of 1,000 mg isoquercetin for 56 days was able to improve markers of coagulation in patients with advanced cancer [50]. Importantly, there were no reports of major hemorrhages in placebo or isoquercetin-treated cohorts [50]. The second phase II clinical trial using isoquercetin will explore the effects of this flavonoid in thromboinflammatory biomarkers of patients with stable sickle cell disease (ClinicalTrials.gov Identifier: NCT04514510). The primary outcome will measure changes in

the plasma soluble P-selectin levels comparing the baseline to isoquercetin response and the study is due to completion in October 2022.

Therefore, although significant improvements have been made to characterize PDI and other thiol isomerases in cardiovascular cells, the development of more specific inhibitors of thiol isomerases is still an ongoing drug development programme. This issue is currently being tackled with the recent discovery of novel and more selective inhibitors of PDI, as discussed below.

3. PRO-THROMBOTIC TARGETS OF THIOL ISOMERASES

3.1. Integrins

Integrins are heterodimeric transmembrane receptors composed of an α and β subunit essential for cell migration and adhesion [53]. For instance, integrin αIIbβ3 is a platelet specific receptor for fibrinogen and von Willebrand factor and is therefore essential for thrombus formation. Platelet activation culminates in conformational changes in integrin αIIbβ3, increasing affinity for its ligands and therefore triggering thrombus formation (reviewed in [10]). Indeed, β3 deficient mice have been shown to display impaired thrombosis [54]. The observation that several integrins have a cysteine-rich domain, has led to the hypothesis that thiol-disulphide exchange reactions may regulate the activity of these adhesion receptors. Several thiol isomerases have been shown to associate with integrins in platelets and endothelial cells. ERp5 [18], ERp57 [43] and PDI [54,55] have been shown to interact with integrin β3 and their activities are associated with integrin activation.

In contrast, two thiol isomerases, namely ERp5 and TMX1, were shown to inhibit integrin β3 activation. In spite of previous reports suggesting a pro-thrombotic effect of ERp5 [18,41], there is evidence that ERp5 catalyses the release of fibrinogen from activated integrin αIIbβ3 [56]. ERp5 was shown to cleave a disulphide bond between Cys177 and Cys184 in the βI domain of integrin β3, thus leading to fibrinogen release [56]. Meanwhile, the transmembrane thiol isomerase TMX1 was shown to inhibit platelet function through the oxidation of integrin β3 [40]. This reinforces the possibility that several thiol isomerases may orchestrate the redox modulation of integrin β3 on the surface of cardiovascular cells through oxidizing, reducing and isomerizing cysteines on the cysteine-rich domain of integrin β3. Interestingly, the association between thiol isomerases and integrin β3 could also be

perceived as bi-directional, since it has been shown that the genetic deletion of this integrin precludes PDI accumulation on the site of thrombus formation [54]. In addition, integrin β3 has been reported to possess an endogenous reductase activity through thioredoxin-like domains [57], similar to thiol isomerases. Therefore, thiol isomerases may regulate the activity of integrins as well integrins may regulate the exposure of thiol isomerases upon vascular injury. The precise mechanisms governing such interaction are still unclear.

Other integrins have also been reported to be mediated by PDI in vascular cells and leukocytes. Lahav et al [58] have demonstrated that inhibition with RL-90, an antibody that targets PDI and to a lesser extent ERp57 [38], blocked the binding of GFOGER peptide to integrin α2β1. This provided mechanistic evidence as to how PDI and ERp57 modulate adhesion of platelets to collagen surfaces, since integrin α2β1 is an important adhesion receptor for collagen [59]. Integrin regulation is not restricted to platelets only, since in endothelial cells infected with dengue virus, PDI has been shown to co-localize with and regulate the activation of both integrins β1 and β3 [60]. Likewise, PDI was shown to interact with integrin αMβ2 on the surface of neutrophils and regulate the recruitment of these cells during vascular inflammation [61].

In spite of data showing how PDI interact and control integrins, it must be noted that platelet PDI does not affect the adhesion of platelets to fibrinogen [37,62]. Indeed, platelet adhesion to fibrinogen was shown to be mediated by GPVI [63] and secondary activators, such as ADP [64]. It is possible that, while PDI regulates the early activation of integrin αIIbβ3, it does not affect other molecules required for sustained platelet adhesion to immobilized fibrinogen, as corroborated by data of platelet-specific PDI-deficient mice [37]. Therefore, the regulation of several integrins in cardiovascular and circulating cells exerted by PDI and sibling proteins is perceived as a central mechanism through which thiol isomerases regulate thrombus formation, although there are also other targets.

3.2. GpIbα

The adhesion receptor GpIbα, part of the GPIb-IX-V complex, is the main receptor for VWF in platelets, together with the integrin αIIbβ3 [65]. Indeed, the relevance of GpIbα has been indisputably defined since ILR4α/Gp1bα-tg mice, which lack the extracellular domain of this adhesion receptor, were shown to have impaired thrombus formation *in vivo* [65]. Interestingly, the interaction between GPIbα and the A1 domain of VWF was shown to be modulated by the formation of disulphide bonds in GpIbα [66], suggesting that redox processes

may regulate the activation of this receptor. PDI is also capable of targeting Cys2771 and Cys2773 of VWF, influencing the dimerization of VWF, which is necessary for its interaction with GpIbα [67]. Notwithstanding, PDI was demonstrated to be in close proximity with GpIbα on the platelet outer membrane [68], while the inhibition of PDI modulates the exposure of free thiols in GpIbα upon platelet activation, suggesting a functional association between these two proteins [68]. Indeed, Stopa et al [69] have used a kinetic-based trapping approach to show that PDI interacts with GpIbα, while more recently Li et al [70] have reported that PDI directly binds to GpIbα on the platelet surface, catalysing the reduction of disulphide bonds Cys4-Cys17 and Cys209-Cys248. This same study reported that the PDI-GPIbα interaction was relevant to platelet-neutrophil interaction, vascular occlusion under thromboinflammatory conditions and tissue damage in ischemia-reperfusion injury. Therefore, PDI has been proposed as a key regulator of GpIbα on the platelet outer membrane and this interaction seems relevant to various thromboinflammatory diseases.

3.3. Tissue Factor

Tissue factor (TF) is a glycoprotein key to the coagulation system, since it is the cellular receptor of coagulation factors FVII and FVIIa, and the formation of the TF-FVIIa complex triggers signalling events that culminate in the activation of FIX and FX [71]. Importantly, Cys186 and Cys209 of TF are located on the extracellular domain and capable of forming a disulphide bond, which regulates the activation of TF [72]. Initial studies of Ahmed et al have shown that extracellular PDI, but not ERp57, is a negative regulator of TF, suppressing TF coagulant activity through a NO-dependent mechanism [72]. This has been reinforced by evidence showing that inhibition of cell-surface PDI enhances TF procoagulant activity, while addition of exogenous PDI decreases TF activation in endothelial cells [73]. The proposed mechanism of action involved the exposure of phosphatidylserine (PS), since PDI addition led to increased PS exposure [73], which is a known regulator of TF activation (reviewed in [74]).

However, several groups have contested the negative regulation of TF by PDI and have described opposite results. First PDI was able to enhance the procoagulant activity of TF in microvesicles in a process regulated by the chaperone activity of PDI [75]. These findings have been explored *in vivo*, showing that PDI directly promotes TF-dependent fibrin generation in a murine model of thrombosis, although the proposed mechanism involves the isomerisation of disulphide bonds in TF by reduced PDI [32]. In line with these observations, PDI stimulated

the coagulant activity of TF present in extracellular vesicles secreted by endothelial cells [75], which could be related to TF-dependent fibrin deposition *in vivo*. More recently, Chen et al [76] have shown that PDI enhances TF-dependent thrombin generation in human peripheral blood mononuclear cells, which could also propagate thrombus development upon vascular damage and fibrin deposition. Indeed, it has been proposed that the two main regulators of cell-surface TF are PS exposure and cell-surface PDI, while there are PDI-dependent and independent pathways that fine-tune the activation of TF and signalling of the coagulation cascade [74]. Since thiol isomerases have a role in fibrin deposition and TF is rapidly recruited to the site of intravascular damage [77], one could speculate that the stimulation of TF by PDI could represent an initial coagulation step during thrombus development.

Altogether, it is patent that PDI is able to interact with TF and regulate its activity, although we still fail to fully understand how such regulation occurs. It is also unclear if other thiol isomerases facilitate TF activation, which could potentially explain contrasting results obtained by different groups, since most PDI inhibitors used in previous studies were later shown inhibit other thiol isomerases, e.g. PACMA31 used by Chen et al [76] is able to inhibit ERp5, ERp46, ERp57 and ERp72 [78]. Additional studies should be performed in platelet-specific PDI-deficient mice to assess if TF deposition and function are affected, or if other thiol isomerases are able to overcome PDI deficiency. Likewise, it is yet unclear if platelet TF (compared to other sources of TF) is relevant for thrombus development. Platelet-specific double PDI/TF KO mice could also be generated to investigate if PDI and TF are complementary to one another. These experiments can generate evidence to the role of the PDI-TF interaction in the propagation of thrombosis and regulation of fibrin deposition *in vivo*.

3.4. NADPH Oxidases (Noxes)

The Nox enzymatic system has been recently implicated as a positive regulator of platelet function [79]. This enzyme complex, which was first described in phagocytes, has been identified in endothelial cells [80], vascular smooth muscular cells (VSMC) [81] and platelets [82]. Currently, seven isoforms of Nox have been described: Nox-1, Nox-2, Nox-3, Nox-4, Nox-5, Duox-1 and Duox-2 [83]. However, only Nox-1, Nox-2 and Nox-4 have been found in platelets [79] although the presence of Nox4 remains a matter of debate [84] and the potential presence of other Duox proteins has not been tested. The Nox complex system consists of transmembrane (gp91-phox (Nox-2) and p22-phox) as well as cytosolic subunits ($p22^{phox}$, $p40^{phox}$, $p47^{phox}$,

p67^{phox}, Noxo1, Noxa1, Rac1 and Rac2) that assemble and regulate Nox activity [85]. Upon phosphorylation, the cytosolic subunits bind to the transmembrane subunit, for instance p47^{phox} to p22^{phox}, through different mechanisms, depending on the subunits involved (for review, see [86]). Recently, Vara et al [87] have shown using knockout mouse models of both Nox-1 and 2, that Nox-1 was the primary source of platelet superoxide downstream of GPVI, whereas Nox-2 was key for responses to thrombin. Moreover, recent data suggest that platelets secrete Nox-1 in platelet-derived extracellular vesicles produced upon platelet activation with TRAP-6 [88].

In this regard, PDI has been proposed as an important modulator of Nox-1 activity, through a redox interaction with p47^{phox} in leukocytes [89] and by increasing Nox-1 activation in VSMC [90] to cite two examples. In VSMC, PDI was shown to co-localize with Rac1 [91], which is an essential molecule that positively regulates Nox activity (especially Nox-2) in various cells [92]. More recently, it was described that Cys400 of PDI, which is situated on the C-terminal active site of PDI [93], forms a disulphide bond with Cys196 of p47^{phox} to regulate Nox-1 assembly [94]. This is of particular relevance to the platelet, given that others and we proposed the inhibition of the C-terminal active site of PDI as a new antithrombotic strategy [93,95]. Indeed, we have recently shown that PDI and Nox-1 translocate to a closer proximity in CRP-activated platelets and that the expression levels of these enzymes are increased in platelets of individuals presenting cardiometabolic risk factors, such as obesity and high blood pressure [96].

Importantly, Rac1 was shown to modulate platelet hyperaggregation and endothelial dysfunction in diabetes [97], whilst p47phox knockout mice presented limited thrombus formation [98], indicating the Nox-regulatory proteins Rac1 and p47phox to be central in thromboinflammatory conditions. Indeed, in both studies, Nox activity was shown to be a relevant mechanism for the effects observed. Therefore it is possible that PDI may also be involved in the Rac-1-Noxes and/or p47phox-Noxes axis and alters thrombotic conditions due to the modulation of Nox activity – an alternative that is yet to be explored.

3.5. Vitronectin

Vitronectin (from Latin: *vitreous*, 'of glass') is a glycoprotein known to be relevant for intercellular adhesion in several biological systems, including in thrombus formation (reviewed in [99]). This glycoprotein is abundantly present in plasma (200 to 500 μg/mL) and within platelet α-granules [100], while two different groups have shown that vitronectin-defficient mice have impaired thrombus formation *in vivo* [36,101]. Relevant to thrombosis,

vitronectin was shown to bind to integrins, fibrinogen, collagens, PKC, plasminogen and to form a complex with thrombin and antithrombin III [99]. Importantly, there is evidence that PDI catalyses the formation of the vitronectin-thrombin-antithrombin III complex *in vitro* [102]. Indeed, Bowley et al [36] have shown *in vitro* that PDI can reduce disulphide bonds between Cys137-Cys161 and Cys274-Cys453 of vitronectin, which would enable the binding of vitronectin to integrins αIIbβ3 and αVβ3, thus sustaining thrombus formation. However, it is still unclear if this process occurs on the site of vascular injury *in vivo*. It is also unknown if vitronectin regulate thrombosis through other mechanisms, given that it is able to bind to other pro-thrombotic substances, such as collagens and fibrinogen.

3.5.Other targets

The characterization of kinetic substrate-trapping techniques to identify proteins capable of a physical interaction with PDI was a significant achievement in the field. In a seminal paper, Stopa et al [69] demonstrated that kinetic trapping oxidized PDI variants released by platelets were able to bind to GpIbα, cathepsin G, glutaredoxin-1 and thioredoxin, while reduced PDI variants were associated with annexin V, collagen VI, tetranectin, heparanase, serpin B6, kallekrein-14 and ERp57. Through a different approach, Moretti et al [103] described an evolutionary conserved gene pairing between genes of the PDI and Rho guanine-dissociation inhibitors (GDI) family of proteins. These authors have also reported a physical interaction between PDI and Rho-GDI *in vitro.* Indeed, platelets express RhoGDI, which is involved in cytoskeleton rearrangement of several eukaryotic cells, however its function in thrombosis and haemostasis are still unexplored [104]. Finally, it was shown that PDI binds to Cys374 of β-actin and that activation of integrin αIIbβ3 in the megakaryocytic cell line MEG-01 was essential for the PDI-β-actin interaction [105]. Therefore, there are several potential pro-thrombotic molecules that have been shown to interact with PDI. Nevertheless, it remains unclear if the interaction of PDI with these molecules is relevant to the process of thrombosis and haemostasis.

A summary of the main pro-thrombotic targets of thiol isomerases is presented in **Figure 4**.

4. ANTITHROMBOTIC EFFECTS OF THIOL ISOMERASES

In spite of substantial evidence pointing towards pro-thrombotic effects of PDI [12,17,19,20,106,107], this enzyme also exerts paradoxical inhibition of platelet aggregation through

NO transference in a process named transnitrosation. NO is an important platelet inhibitor that acts through activating guanylate cyclase and increasing cyclic guanosine monophosphate (cGMP) levels [108]. This induces vasodilator-stimulated phosphoprotein (VASP) phosphorylation, which inactivates αIIbβ3 [109-111]. The discovery that PDI has denitrosation activity was first reported by the Mutus laboratory that showed that S-nitrosothiols (RSNOs) inhibit platelets through a dual mechanism: first through denitrosation of RSNOs by PDI, thus releasing NO and secondly due to a direct RSNO reaction with PDI, rendering it unable to perform disulphide exchange on the platelet membrane [112,113]. Indeed it was recently demonstrated in a cell-free environment that 57% of peroxynitrite, which is the product of the reaction between NO and superoxide, oxidizes PDI through a 2-electron mechanism while 43% is converted to nitrate and other radicals [114]. This was further studied by other groups that showed that different NO donors attenuate platelet function through PDI-mediated denitrosation [115,116]. More recently, Bekendam et al have proposed that the S-nitrosylation of vascular thiol isomerases PDI, ERp5 and ERp57 by NO is able to at least partially mediate vascular quiescence through the inhibition of these thiol isomerases [117]. Altogether, the pro-thrombotic activity of PDI seems to overcome its inhibitory effect in physiological scenarios – whether this would hold true in the context of disease is yet to be defined.

In addition to S-transnitrosation of thiol isomerases, it has been recently described that some thiol isomerases can exert a negative regulation of platelet function, i.e. inhibit platelet activation. The only thiol isomerase protein described to exert such inhibitory effect thus far is the transmembrane TMX1. This protein was first detected in megakaryocytes over 10 years ago, together with other isoforms, namely: TMX2, TMX3 and TMX4 [12]. TMX1 helps with protein folding in the ER through a CPAC-active site (in contrast to the CGHC active site of PDI, ERp5, ERp57 and ERp72) through the formation of disulphide bonds in newly formed proteins [118]. Elegant work performed by Zhao et al [40] using TMX1-deficient platelets as well as recombinant TMX1 addition have demonstrated that TMX1 decreases platelet and thrombotic responses through the oxidation of integrin α_IIbβ3. Moreover, addition of an anti-TMX1 antibody potentiated platelet aggregation, while addition of recombinant TMX1 inhibited platelet aggregation exerted by different agonists [40]. Therefore, it is possible that other transmembrane thiol isomerases found on the platelet membrane are also able to negatively regulate platelet responses. The investigation of the effects and possible inter-regulation of different thiol isomerases present on the platelet outer surface will allow for a more comprehensive understanding of how this family of proteins may modulate

thromboinflammatory conditions. In this regard, the discovery of selective inhibitors of thiol isomerases will greatly advance the field.

5. INHIBITORS OF THIOL ISOMERASES

5.1 *Small molecule inhibitors*

Considering the deleterious effects of PDI and other thiol isomerases to the cardiovascular system [14,16,36,42,44], there has been great scientific effort to identify novel, non-toxic and selective inhibitors of thiol isomerases. Indeed, many compounds have been identified over the last decades (an up to date summary of PDI inhibitors is presented in **Table 1**). These small molecule inhibitors were often characterized through high throughput screening of chemical libraries. Frequently, PDI inhibitors were tentatively identified from compounds screened for neurodegenerative diseases or cancer. For instance, Hoffstrom et al [119] screened 68,887 compounds against a cell based model for Huntington's disease and found that PDI was the molecular target for the top 5 hits, which included 16F16, a compound that was later shown to bind covalently to Cys36 and Cys39 of the N-terminal active site of PDI [120]. LOC14, a reversible inhibitor of PDI, was also identified after screening for potent rescue of a Huntington's disease cell based model [120]. In a similar approach, Vatolin et al [121] screened 30,335 compounds for activity against *in vivo* and *in vitro* models of multiple myeloma and have identified CCF642 as a lead compound. This same report used the di-eosin reductase assay to show that CCF642 was able to inhibit PDI and other thiol isomerases at low micromolar concentrations, although a recent report has shown a much higher IC_{50} for CCF642 in the insulin turbidimetry assay against several thiol isomerases [78]. It is possible that discrepant results using CCF642 were due to different assays being employed to characterize the anti-PDI activity. This notion is corroborated by Bekendam et al [122] who have elegantly shown that reversible PDI inhibitors bepristat 1a and bepristat 2a, which were identified after a high-throughput screening of 348,505 compounds, were able to inhibit PDI activity only when this was assessed using the insulin turbidimetry assay.

In parallel to PDI inhibitors identified after screening for neurodegenerative or cancer diseases, several groups have conducted high throughput screening in which the primary screen consisted of PDI reductase assay. This is the case for AS15, an aminobenzylphenol compound which covalently binds and inhibits PDI at nanomolar concentrations and

decreases cell proliferation of Glioblastoma cell lines [123]. In a similar approach, this same group has identified 35G8, which is another nanomolar inhibitor of PDI that also inhibits proliferation of Glioblastoma cell lines [124]. Importantly, 35G8 was shown to covalently bind to Cys397 of the C-terminal active site of PDI [124], which has been shown by us [125] and others [126] to be a relevant target site to limit the pro-thrombotic actions of PDI in platelets. In contrast, KSC-34 has been described as the only inhibitor to be ~30 times more selective towards the N-terminal over the C-terminal active site of PDI [127]. One could hypothesize that different inhibitors that target different parts of PDI could exert opposing effects in thrombosis and haemostasis, however at present neither KSC-34 nor 35G8 have been tested in platelets or other cardiovascular cells. Indeed, the majority of small molecule inhibitors described thus far have not been tested for their effects in thrombosis and haemostasis. These include: 16F16 [119], 35G8 [124], AS15 [123], BAP1 and BAP2 [128], CCF642 [121], E64FC26 [78], KSC-34 [129], LOC14 [120], Origamicin [130], Securinine [131], SK053 [132] and STK076545 [133]. Recent data from our lab suggests that LOC14 exerts anti-platelet effects, while CCF642 and 16F16 do not alter platelet function (data not shown). It would be important to assess the anti-platelet potential of other PDI inhibitors.

Only five small molecule inhibitors have been assessed for their effects in thrombosis and haemostasis. Bepristats are selective and reversible inhibitors of PDI with an IC_{50} ranging from 0.7 to 1.2 μM against the reductase activity of PDI measured through insulin turbidimetry [122], while PACMA-31 is an irreversible micromolar inhibitor of PDI [122,134]. In spite of the low IC_{50} for PDI reductase activity, Bekendam et al have shown that 30 μM of bepristats or PACMA-31 was able to abrogate platelet aggregation, while bepristats exerted no effect in P-selectin exposure [122]. Unpublished data from our lab suggest that concentrations as low as 7.5 μM of bepristat 2a can inhibit platelet aggregation, activation and calcium mobilization, depending on the agonist used. Such discrepancy between the concentration needed to inhibit PDI in a cell-free system and the one needed to inhibit platelets was also found for ML359. This inhibitor was able to marginally inhibit thrombin-induced platelet aggregation (25%) at 30 μM [135] in spite of an IC_{50} over 100 times lower for PDI reductase activity, suggesting poor biochemical properties or off-target effects [136]. On the other hand, HPW-RX40 has shown similar low micromolar IC_{50} for both PDI reductase assay measured through the di-eosin assay and platelet aggregation induced by several agonists [137]. Finally, we have recently shown that the cysteinyl LT receptor antagonist zafirlukast is a pan inhibitor of thiol isomerases, decreasing the reductase activity of PDI, ERp5, ERp57, ERp72 and TRX at micromolar concentrations [138]. Similar concentrations of zafirlukast were able to

inhibit platelet aggregation, activation, calcium mobilization and *in vivo* thrombosis with no effect on bleeding time [138]. Therefore, there are currently few small molecule inhibitors of PDI and other thiol isomerases with well-described effects in thrombosis and haemostasis.

In addition to the five small molecule inhibitors that have been tested in platelets, bacitracin was initially perceived as a selective PDI inhibitor. However, this was challenged over 10 years ago, when Karala and Ruddock definitively showed that bacitracin is not a selective inhibitor of PDI, neither does it exert its cellular effects through the inhibition of thiol isomerases [139]. Therefore, the identification of novel inhibitors of PDI faces several challenges. First, it is possible that current molecules perceived as PDI inhibitors do not exert their anti-platelet effect through targeting this enzyme, similar to bacitracin. This is corroborated by literature exposed above that show that bepristats and PACMA-31 need a much higher concentration to inhibit platelets than to inhibit thiol isomerases in cell-free environments [122]. Definitive proof of the specificity of inhibitors could be achieved through the use of platelet PDI-deficient models. Secondly, it is yet unknown if small molecule inhibitors described as anti-cancer agents have effects on thrombosis and haemostasis. Lastly, although there are a few PDI-selective inhibitors, such as bepristats, there are no selective inhibitors for other thiol isomerases. The identification of such compounds would forward the field as it has been shown that different thiol isomerases may have distinct modes of action in platelets [39]. In conclusion, it would be beneficial if future studies: 1) prove that current and future PDI inhibitors act through targeting a specific thiol isomerase, 2) investigate if the anti-cancer and anti-platelet properties of thiol isomerase inhibitors overlap and 3) identify inhibitors that target specific protein activities (reductase, oxidase, isomerase and chaperone activity).

5.2 Flavonoids and natural compounds

Similar to small molecule inhibitors, flavonoids and other natural compounds have been extensively studied as potential inhibitors of thiol isomerases. The most prominent and clinically advanced natural compound is isoquercetin, which is a derivative of the flavonoid quercetin currently being employed in phase II clinical trial as a potential anti-thrombotic drug as described above [50]. However, rutin, which is a quercetin derivative containing a rutinose lateral chain in carbon 3, was the first derivative proposed as a promising PDI inhibitor [140]. Jasuja et al [140] demonstrated the ability of rutin to inhibit thrombosis in mice at concentrations as low as 0.1 mg/kg. This same group have reported that rutin binds reversibly

to the b'x domain of PDI, similar to bepristats [122,141]. Recently, we have reported that myricetin, which is a flavonoid of similar structure to quercetin, is also able to inhibit platelets at low micromolar concentrations [62]. This was attributed to the inhibition of PDI and ERp5, since this flavonoid was shown to inhibit these enzymes at similar concentrations needed to achieve platelet inhibition [62], although definitive proof is still needed.

Other natural compounds have also been proposed as novel inhibitors of thiol isomerases. For instance, rosmarinic acid, commonly found in Danshen (*Salva miltiorrhiza*) was shown to inhibit ERp57 and promote platelet inhibition at low micromolar concentrations, depending on the agonist used [142]. Punicalagin was also shown to inhibit ERp57 at low micromolar concentrations in a cell-free environment, while the biological actions of this polyphenolic compound were lost in ERp57-silenced neuroblatoma cells, suggesting ERp57 to be the main target of punicalagin in cellular systems [143]. Similarly, tannic acid was demonstrated to bind to PDI with high affinity, after a directed *in silico* screening of over 60 natural compounds [144]. This study demonstrated that tannic acid inhibits several thiol isomerases and prevents thrombus formation in the cremaster laser-induced model of thrombosis *in vivo* [144]. Therefore, several natural compounds have been identified as potent inhibitors of PDI and other thiol isomerases, with implications to thrombosis and haemostasis. However, it is still unclear if thiol isomerase inhibition is indeed the mechanism of action of these compounds in biological systems.

Juglone, which is an allelopathic compound present in the roots of walnut trees, has been shown to inhibit platelet aggregation, possibly through the inhibition of both PDI and Akt [145]. Indeed, other flavonoids, such as quercetin, apigenin and catechin have been shown to act as kinase inhibitors and to inhibit the activity of Src family kinases in platelets [146]. A previous study of our lab showed that quercetin and other structurally related flavonoids were able to interact with fibrinogen and collagen, to prevent Syk phosphorylation and to be internalized by megakaryocytes and platelets [147]. Likewise, quercetin, catechin and other structurally related flavonoids were shown to inhibit platelet aggregation, and act as competitive of the thromboxane A_2 (TxA_2) receptor [148]. Therefore, similar to small molecule inhibitors, it is still unclear if the biological activity of natural compounds described as PDI inhibitors is indeed due to thiol isomerase inhibition. A thorough analysis of which compounds exert their effect through thiol isomerase inhibition and which thiol isomerases are involved would greatly benefit the development of more effective compounds to treat and prevent thrombosis.

5.3 Peptide inhibitors

Peptides have been used to treat diseases for nearly 100 years, since insulin was first isolated and commercialized (for review, see [149]). However, there is currently only one peptide inhibitor described to inhibit thiol isomerases. The peptide CxxCpep was first synthesized by de A. Paes et al [150] as the 12 amino-acid sequence of the CGHC-redox active site of PDI (VEFYAPWCGHCK). These authors have shown that CxxCpep was able to inhibit PDI in neutrophils, thus decreasing the assembly of NADPH oxidase complexes [150]. We have expanded these studies and shown that CxxCpep inhibits platelets and binds to Cys397 and Cys400 of the C-terminal active site [125] – supporting the notion that the pro-thrombotic effects of PDI are orchestrated by the C-terminal redox active site [37,126]. In addition, we have evidence that this peptide is membrane impermeable (unpublished), reiterating that the extracellular pool of thiol isomerases is important to regulate platelet function. However, definitive proof is still lacking to ascertain the specificity of CxxCpep towards PDI. A summary of PDI inhibitors with known binding sites is presented in **Figure 5**. Future research should design peptides to selectively inhibit other thiol isomerases. These inhibitors could serve as templates for the development of stable, selective and non-toxic peptide inhibitors.

6. FUTURE PERSPECTIVES

There has been great scientific advancement since PDI was first identified in 1963 [21] and its protein sequence determined in 1985 [25]. Several decades later, it is now undisputed that PDI and other thiol isomerases control platelet function, acting as an orchestrating force in the complex and dynamic process of thrombosis and haemostasis. In parallel, there has been a surge of novel inhibitors of thiol isomerases discovered through high-throughput screening of small molecules, such as bepristasts [122] and repurposing of drugs currently used in other settings, such as zafirlukast [138]. However, there are still pressing questions left unanswered in order to translate basic findings to the clinic.

First, it would be important to understand which molecules are targeted by thiol isomerases and how these interactions occur. It is widely accepted that PDI, ERp5, ERp57 and ERp72 regulate integrin β3 activation, while TMX1 acts as a negative regulator, as exposed above in **subheading 3.1**. Interestingly, platelet aggregation in ERp72-, PDI-, and ERp57-null platelets was only recovered when the deleted thiol isomerase was added back [39],

suggesting that each enzyme acts in series, targeting different molecules or different parts of the same molecules. This also reinforces the notion that thiol isomerases are not redundant in platelets. Another feasible alternative is that thiol isomerases interact amongst themselves on the outer surface of cardiovascular cells. Moreover, it is unclear if ERp44, ERp29 and TMX3, which were found in platelets [29], are also able to influence thrombosis and haemostasis.

Second, novel research should address the possible overlap between the anticancer, neuroprotective and antiplatelet properties of thiol isomerase inhibitors. Specifically, it would be interesting to know if inhibitors that target different parts of PDI are able to modulate different aspects regulated by PDI. For instance, the C-terminal, but not the N-terminal, active site of PDI is required to modulate platelets [17,93]. In parallel, the neuroprotective inhibitor 16F16 targets the N-terminal active site [120], while the anticancer inhibitor 35G8 targets the C-terminal active site of PDI [124], therefore, it would be important to understand how these and other inhibitors regulate platelet function. Such characterization would deepen our knowledge on possible side effects of thiol isomerase inhibitors and propose a template for the development of more selective compounds.

Finally, the characterization of selective inhibitors for each thiol isomerase and translation of these inhibitors to the clinic are of great interest to the field. Currently there are a few inhibitors that are selective to PDI over sibling proteins, such as 16F16 [119,120] and bepristats [122], however there is no such equivalent to ERp5, ERp57 or ERp72. In addition, full characterization of off-target effects of these inhibitors *in vivo* is still lacking. It is also unclear how thiol isomerases in platelets are correlated to thrombosis in pro-thrombotic conditions, such as metabolic syndrome and cancer. Promising findings of Zwicker et al [50] have shown a potential benefit of using isoquercetin to prevent cancer-associated thrombosis and it is expected that this positive outcome will bring interest to the development of selective inhibitors of other thiol isomerases.

In conclusion, thiol isomerases are central to many biological systems and could be perceived as a driving force that dictates thrombus development. This complex interplay involves redox reactions with key adhesion receptors occurring at the platelet outer membrane. Ultimately, it becomes increasingly evident that platelets are highly regulated by redox processes, while novel techniques, inhibitors and other tools are fostering exciting discoveries in this rapid-growing field.

AUTHOR CONTRIBUTIONS

R.S. Gaspar wrote the manuscript and drafted the figures, while J.M. Gibbins wrote and revised the manuscript. All authors approved the final version submitted.

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DISPLAY ITEMS

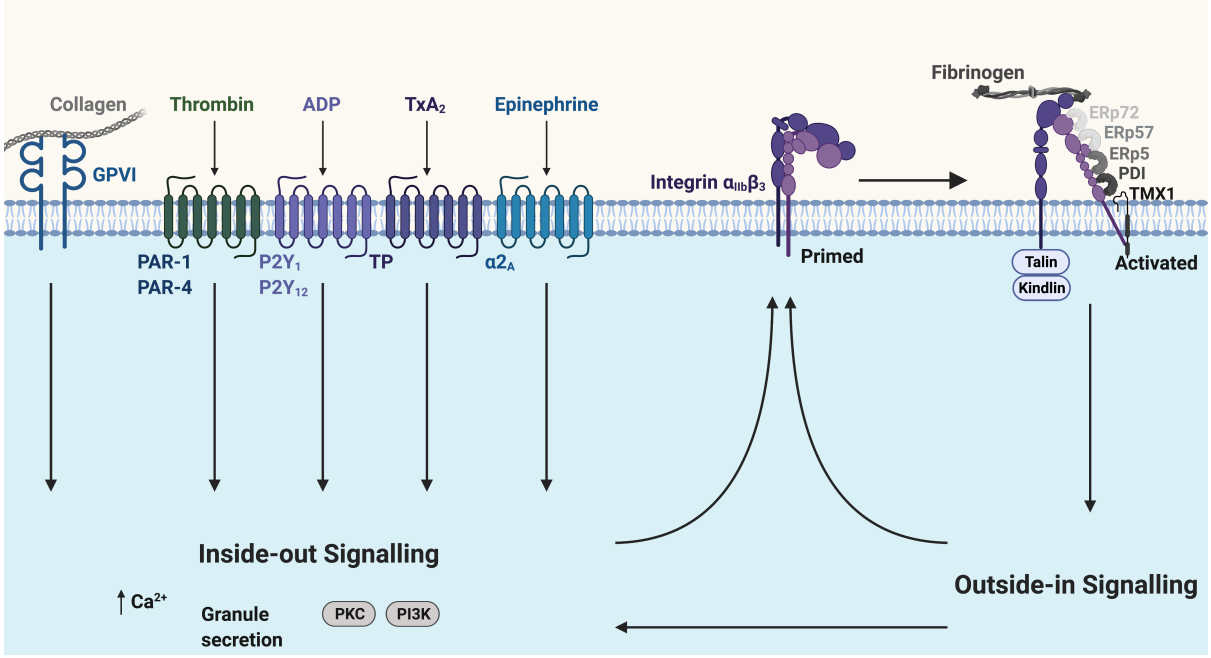


Figure 1. Inside-out and outside-in signalling in platelets. Platelet activation can be didactically divided in two pathways: inside-out and outside-in signalling. Inside-out signalling refers to binding of agonists to their respective receptors on the platelet membrane (e.g. collagen binding to GPVI). This initial binding will lead to specific pathways of each receptor that will culminate in a common pathway that involves increased intracellular Ca^{2+} mobilisation, granule secretion, activation of protein kinase C (PKC), phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinases (MAPKs). These molecules and signalling events will then activate integrin αIIbβ3 in a process that requires protein disulphide isomerase (PDI) as well as other thiol isomerases. Upon binding to fibrinogen, the integrin αIIbβ3 will cause a series of intracellular signalling events, termed outside-in signalling, that will potentiate initial response by agonists. PAR: protease-activated receptor. ADP: adenosine diphosphate. TP: thromboxane receptor. TxA_2: thromboxane A_2.

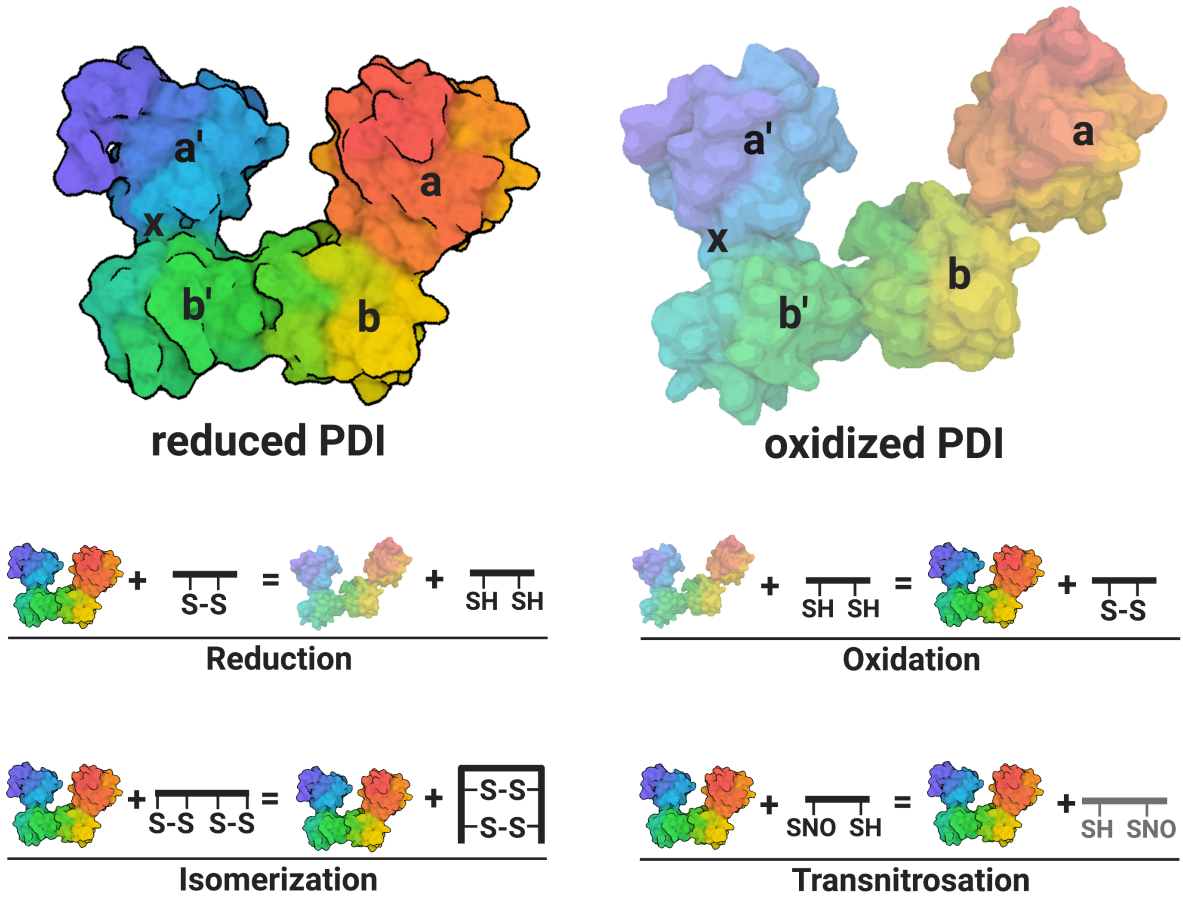


Figure 2. Protein disulphide isomerase catalyses redox reactions. The 3D structures of protein disulphide isomerase (PDI) were obtained from the PDB database (PDB ID: 4EL1 for oxidized and PDB ID: 4EKZ for reduced). PDI has 4 domains and an x-linker to promote flexibility. Its catalytic sites are located in a and a'-domains. Due to its particular structure, PDI can catalyse the reduction of disulphide bonds into free thiols, oxidation of thiols into disulphide bonds or isomerisation of disulphide bonds, leading to a different protein conformation. Alternatively, it can also transfer nitric oxide (NO) from nitrosothiols (SNO) between proteins in a process named transnitrosation.

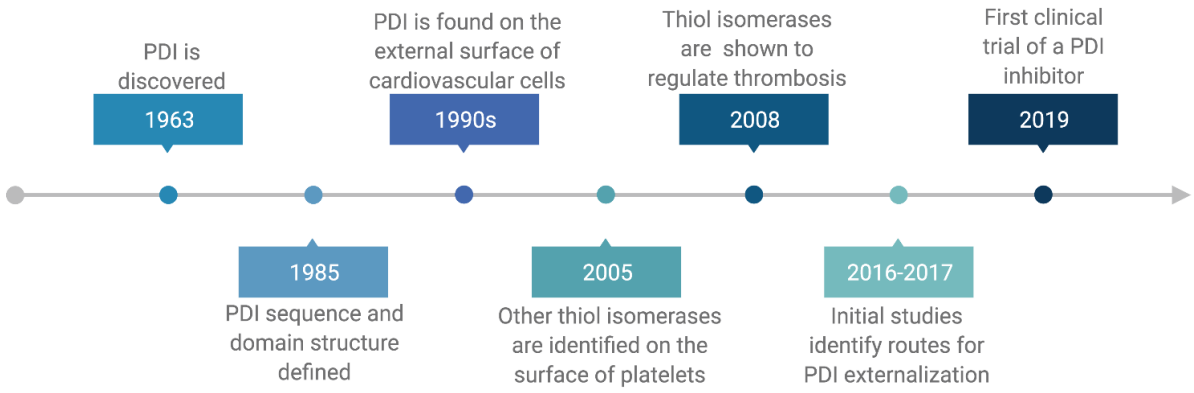


Figure 3. Historical landmarks of the PDI field. Decades of intensive research output led to many discoveries linking PDI and sibling proteins to thrombosis and haemostasis. From its initial characterisation in the 1960s-1980s, PDI was later found on the outer surface of cardiovascular cells. Other thiol isomerases were also identified and collectively shown to regulate thrombosis. More recently, we started to uncover how these proteins become externalized while a feasible candidate for drug development is currently being tested in clinical trials.

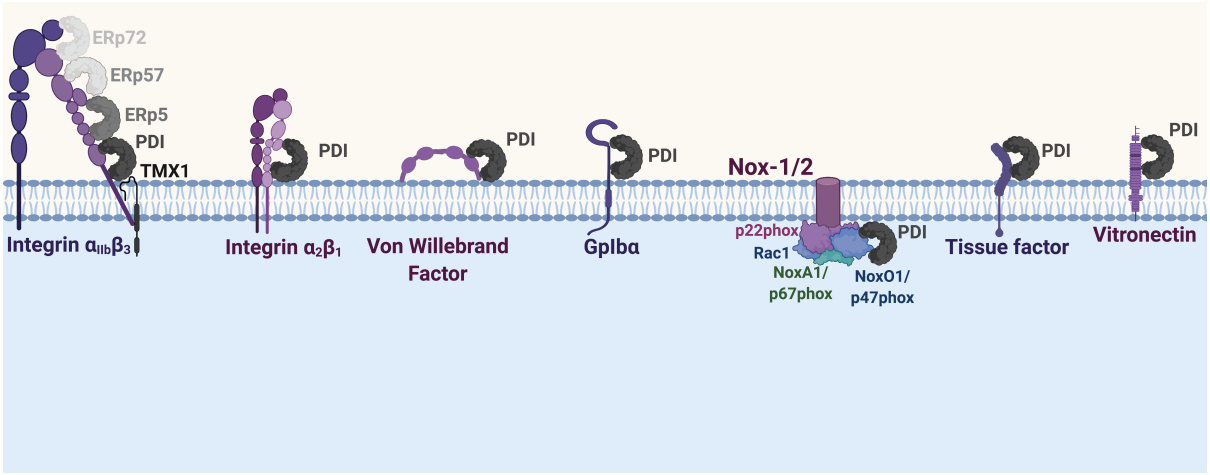
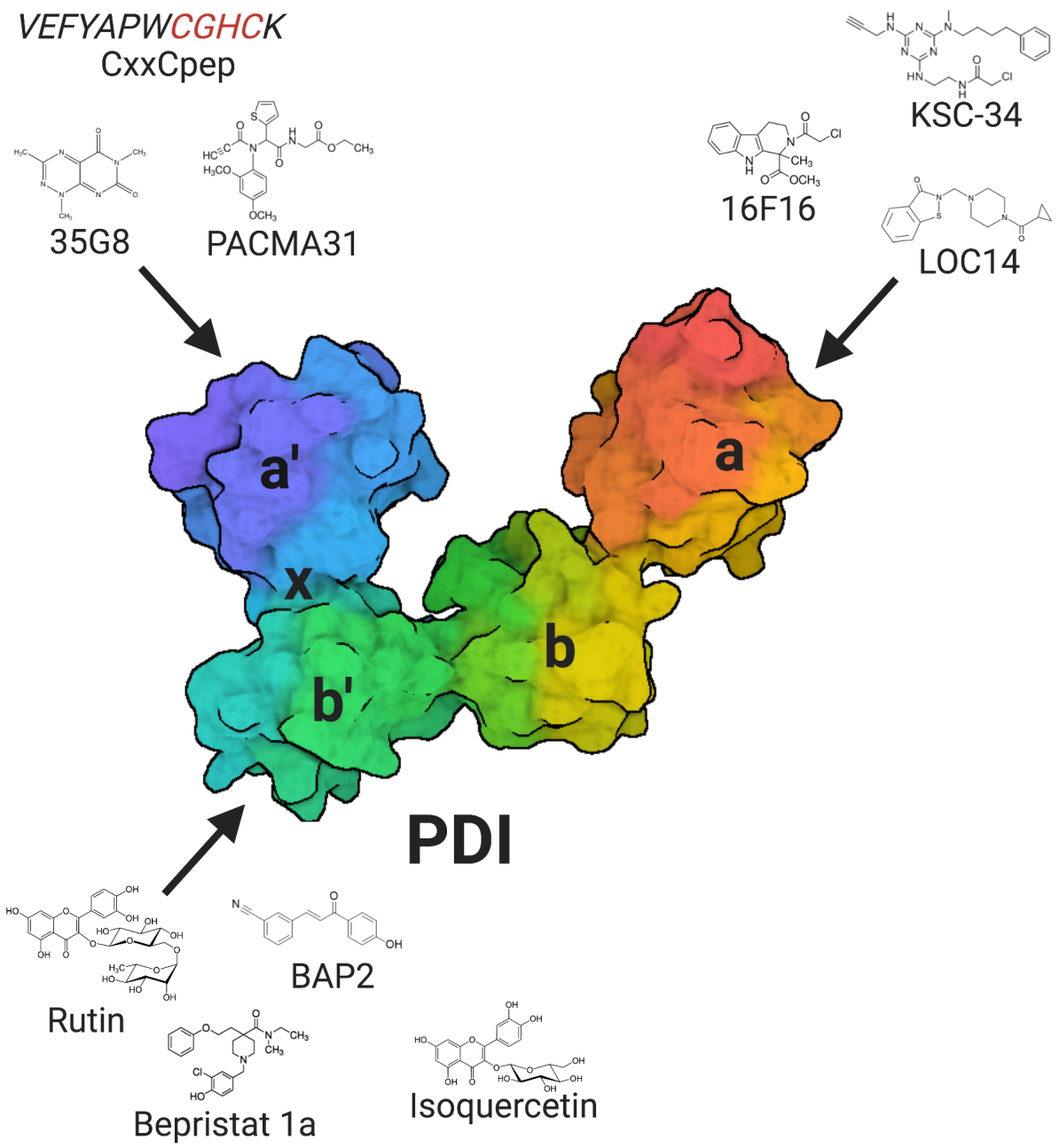


Figure 4. Pro-thrombotic targets of thiol isomerases. PDI, ERp5, ERp57 and ERp72 are known regulators of platelet function through their interaction with integrin αIIbβ3. In contrast, TMX1 has been shown to inhibit integrin αIIbβ3, being the first thiol isomerase described to negatively regulate thrombosis. PDI is also able to interact with integrin α2β1, Von Willebrand Factor (VWF), GpIbα, Nox-1, Nox-2, tissue factor and vitronectin all of which are pro-thrombotic. Therefore, the interaction of thiol isomerases with these pro-thrombotic molecules is a feasible mechanism through which thiol isomerases can control platelet function. Other proteins, such as β-actin and RhoGDI were also shown to interact with PDI, however the relevance of this interaction to thrombosis and haemostasis is still unclear. It is also unclear if there are other targets that could contribute to the pro-thrombotic (or anti-thrombotic for TMX1) effect of thiol isomerase proteins.



687

688 **Figure 5. PDI inhibitors with known binding sites.** Several PDI inhibitors have been
689 characterized, however only a few of these have a known binding site.16F16, KSC-34 and
690 LOC14 bind close to the a'-active site; CxxCpep, 35G8 and PACMA-31 bind close to the a-
691 active site, while rutin, bepristats, BAPs and isoquercetin bind near the b' and x active sites.
692 There are currently no inhibitors that selectively target the b-domain.

693

Table 1. List of known thiol isomerase inhibitors and their effects on thrombosis and haemostasis.

Inhibitor	TI targeted	IC_{50} for TI inhibition	Binding site	Effects on thrombosis and haemostasis	ref
Small molecule inhibitors					
16F16	PDI ERp5 ERp57 ERp72	6 μM for PDI 50 μM for ERp72 30 μM for ERp5	Cys36 and Cys39 of the N-terminal active site of PDI	Platelet function - *	120 119
35G8	PDI	0.17 μM for PDI	Cys397 of the C-terminal active site of PDI	Not tested	124
AS15	PDI	0.3 μM	Not tested	Not tested	123
BAP1 **BAP2**	PDI	0.83 μM 0.93 μM	His256 b'domain	Not tested	128
Bepristat 1a **Bepristat 2a**	PDI	0.7 μM 1.2 μM	b'x domain	Platelet function □ *In vivo* thrombosis□	122
CCF642	PDI ERp5	100 μM for PDI** 100 μM for ERp5**	Near C-terminal active site of PDI[#]	Platelet function - *	121 78
E64FC26	PDI ERp5 ERp57	2 μM for PDI 25 μM for ERp5	Not tested	Not tested	78

	ERp72	20 μM for ERp57 25 μM for ERp72			
HPW-RX40	PDI ERp5 ERp57 ERp72	1.45 μM for PDI 2.6 μM for ERp5 4.3 μM for ERp57 18.8 μM for ERp72	Near C-terminal active site of PDI[#]	Platelet function □ *In vitro* thrombosis□	137
KSC-34	PDI	3.5 μM for PDI	Cys53 of the N-terminal active site of PDI	Not tested	129
LOC14	PDI ERp5 ERp57 ERp72	150 μM for PDI 45 μM for ERp5 4.97 μM for ERp57 100 μM for ERp72	Near the N-terminal active site of PDI	Platelet function □*	120 151 78
ML359	PDI	0.25 μM for PDI	Not tested	Platelet function □	135
Origamicin	PDI ERp5 ERp57 ERp72	Not tested	Not tested	Not tested	130
PACMA31	PDI	7 μM for PDI	Cys397 and	Platelet	78

	ERp5 ERp46 ERp57 ERp72	5 μM for ERp5 20 μM for ERp72 Not tested for ERp57 and ERp46	Cys400 of the C-terminal active site of PDI	function □ *In vitro* thrombosis□ Haemostasis □	[134] [152] [122]
Securinine	PDI	Not tested	Near the N-terminal active site of PDI[#]	Not tested	[131]
SK053	PDI	10 μM for PDI	Near the C-terminal active site of PDI[#]	Not tested	[132]
STK076545	PDI	2.16 μM for PDI	Not tested	Not tested	[133]
Zafirlukast	PDI ERp5 ERp57 ERp72 TRX	Not tested	Not tested	Platelet function □ *In vivo* thrombosis□ Haemostasis -	[138]
Flavonoids and natural compounds					
ADTM	PDI ERp5 ERp57 ERp72	Not tested	Not tested	Platelet function □ *In vivo* thrombosis□	[153]
Galloylated catechins	ERp57	Not tested	Near the active sites of ERp57[#]	Platelet function □	[154,155]

Juglone	PDI	1.61 μM	Not tested	Platelet function □ *In vitro* thrombosis□	145
Juniferdin epoxide	PDI ERp5 Thioredoxin	0.15 μM for PDI 5 μM for ERp5 3 M for Thioredoxin	Not tested	Platelet function □	156 135
Myricetin	PDI ERp5	Not tested	Near the active sites of PDI and ERp5[#]	Platelet function □ *In vitro* thrombosis□ Haemostasis -	62
Punicalagin	ERp57	1 μM	Not tested	Not tested	143
Rosmarinic acid	ERp57	176 μM	Near the active sites of ERp57[#]	Platelet function □	142
Rutin	PDI	7-10 μM	b'x domain	Platelet function □ *In vivo* thrombosis□ Haemostasis -	78,140,141,157
Tannic acid	PDI	Not tested	Near the C-terminal active site of PDI[#]	Platelet function □ *In vivo* thrombosis□ Haemostasis -	144
Peptide inhibitors					
CxxCpep	PDI	Not tested	Cys400 of reduced PDI	Platelet function □	125

	Mastoparan	PDI	Not tested	Not tested	Platelet function □	[158,159]

TI: Thiol isomerase. *Preliminary data from our lab. **: Disputed. □:Decreased. □:Increased. -:Unaffected. #:Predicted.

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