

Thiol isomerases orchestrate thrombosis and haemostasis

Article

Accepted Version

Simoes Gaspar, R. and Gibbins, J. M. ORCID:
<https://orcid.org/0000-0002-0372-5352> (2021) Thiol
isomerases orchestrate thrombosis and haemostasis.
Antioxidants & Redox Signaling, 35 (13). ISSN 1523-0864 doi:
<https://doi.org/10.1089/ars.2021.0086> Available at
<https://centaur.reading.ac.uk/97598/>

It is advisable to refer to the publisher's version if you intend to cite from the work. See [Guidance on citing](#).

To link to this article DOI: <http://dx.doi.org/10.1089/ars.2021.0086>

Publisher: Mary Ann Leibert Inc

All outputs in CentAUR are protected by Intellectual Property Rights law, including copyright law. Copyright and IPR is retained by the creators or other copyright holders. Terms and conditions for use of this material are defined in the [End User Agreement](#).

www.reading.ac.uk/centaur

CentAUR

Central Archive at the University of Reading

Reading's research outputs online

1 **Thiol isomerases orchestrate thrombosis and haemostasis**

2
3
4 **Running title:** Thiol isomerases regulate platelets

5
6 **Authorship**

7 Renato Simões Gaspar ^{1*}, Jonathan M Gibbins ¹

8
9 **Affiliations**

10 ¹ Institute for Cardiovascular and Metabolic Research, School of Biological Sciences,
11 University of Reading, Reading, UK.

12
13 *** Corresponding author**

14 Renato Simões Gaspar, MD, PhD.

15 Institute for Cardiovascular and Metabolic Research, School of Biological Sciences.

16 University of Reading - Harborne Building, Reading, RG6 6AS, UK.

17 E-mail: renatosgaspar@gmail.com, phone: +44 11 8378 7082

18
19
20 **Conflict of interest statement:** The authors have declared that no conflict of interest exists.

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 α IIb β 3 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 identified including integrin α 2 β 1, Von Willebrand Factor (VWF), GpIb α , 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 $\alpha 2\beta 1$ 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 $\alpha \text{IIb}\beta 3$, 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 $\alpha \text{IIb}\beta 3$ 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 (TxA₂). 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 transnitrosation (**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 $\alpha\text{IIb}\beta 3$ 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 RNase²¹, whereas the second, led by Nobel prize-winning Christian B Anfinsen, made similar observations using a microsomal system from rat livers²². However, it was only 10 years later that PDI was officially named²³. 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 work²⁴. The protein sequence and identification of the CGHC active sites of rat PDI were only performed in the 1980s²⁵. 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 Anfinsen's groups¹⁴. Subsequently, Essex et al²⁶ 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 $\beta 3$ 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 $\beta 3$. 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 TMX3²⁹, of which only ERp57, ERp72, ERp44 and ERp29 are released by platelets and recruited to the cell surface upon activation²⁹. 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 responses⁴⁰.

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 al⁴³ 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 thromboinflammation⁴⁴.

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 al³⁹ 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 ER⁴⁶. 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 membrane⁴⁷. 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

system²⁸. 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 platelets²⁸. 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 established⁴⁸. 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 secretion⁴⁸.

One feasible alternative yet to be investigated is the possibility that thiol isomerases externalize directly through ER-plasma membrane connections⁴⁹. In fact, these connections were shown to be highly regulated by Ca²⁺ influx⁴⁹, 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 PDI⁵⁰ and antioxidant pathways⁵¹. The first trial aims to assess the benefits of administering isoquercetin to patients with hypercoagulable states, after an initial study in healthy volunteers⁵². 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 cancer⁵⁰. Importantly, there were no reports of major hemorrhages in placebo or isoquercetin-treated cohorts⁵⁰. 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 adhesion⁵³. For instance, integrin α IIb β 3 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 α IIb β 3, increasing affinity for its ligands and therefore triggering thrombus formation (reviewed in¹⁰). Indeed, β 3 deficient mice have been shown to display impaired thrombosis⁵⁴. 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. ERp5¹⁸, ERp57⁴³ and PDI^{54,55} have been shown to interact with integrin β 3 and their activities are associated with integrin activation.

In contrast, two thiol isomerases, namely ERp5 and TMX1, were shown to inhibit integrin β 3 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 α IIb β 3⁵⁶. ERp5 was shown to cleave a disulphide bond between Cys177 and Cys184 in the β I domain of integrin β 3, thus leading to fibrinogen release⁵⁶. Meanwhile, the transmembrane thiol isomerase TMX1 was shown to inhibit platelet function through the oxidation of integrin β 3⁴⁰. This reinforces the possibility that several thiol isomerases may orchestrate the redox modulation of integrin β 3 on the surface of cardiovascular cells through oxidizing, reducing and isomerizing cysteines on the cysteine-rich domain of integrin β 3. Interestingly, the association between thiol isomerases and integrin β 3 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 formation⁵⁴. In addition, integrin $\beta 3$ has been reported to possess an endogenous reductase activity through thioredoxin-like domains⁵⁷, 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 al⁵⁸ have demonstrated that inhibition with RL-90, an antibody that targets PDI and to a lesser extent ERp57³⁸, blocked the binding of GFOGER peptide to integrin $\alpha 2\beta 1$. This provided mechanistic evidence as to how PDI and ERp57 modulate adhesion of platelets to collagen surfaces, since integrin $\alpha 2\beta 1$ is an important adhesion receptor for collagen⁵⁹. 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 $\beta 1$ and $\beta 3$ ⁶⁰. Likewise, PDI was shown to interact with integrin $\alpha M\beta 2$ on the surface of neutrophils and regulate the recruitment of these cells during vascular inflammation⁶¹.

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 GPVI⁶³ and secondary activators, such as ADP⁶⁴. It is possible that, while PDI regulates the early activation of integrin $\alpha IIb\beta 3$, it does not affect other molecules required for sustained platelet adhesion to immobilized fibrinogen, as corroborated by data of platelet-specific PDI-deficient mice³⁷. 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. *GpIba*

The adhesion receptor GpIba, part of the GPIb-IX-V complex, is the main receptor for VWF in platelets, together with the integrin $\alpha IIb\beta 3$ ⁶⁵. Indeed, the relevance of GpIba has been indisputably defined since ILR4 α /Gp1b α -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 GPIb α and the A1 domain of VWF was shown to be modulated by the formation of disulphide bonds in GpIba⁶⁶, 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 GpIb α ⁶⁷. Notwithstanding, PDI was demonstrated to be in close proximity with GpIb α on the platelet outer membrane ⁶⁸, while the inhibition of PDI modulates the exposure of free thiols in GpIb α 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 GpIb α , while more recently Li et al ⁷⁰ have reported that PDI directly binds to GpIb α on the platelet surface, catalysing the reduction of disulphide bonds Cys4-Cys17 and Cys209-Cys248. This same study reported that the PDI-GpIb α 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 GpIb α 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}, Nox1, Nox2, 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 p47^{phox} knockout mice presented limited thrombus formation ⁹⁸, indicating the Nox-regulatory proteins Rac1 and p47^{phox} 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 p47^{phox}-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-deficient 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 α IIb β 3 and α V β 3, 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 GpIba, 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 α IIb β 3 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) levels¹⁰⁸. This induces vasodilator-stimulated phosphoprotein (VASP) phosphorylation, which inactivates $\alpha\text{IIb}\beta 3$ 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 radicals¹¹⁴. 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 isomerases¹¹⁷. 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 TMX4¹². 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 proteins¹¹⁸. Elegant work performed by Zhao et al⁴⁰ using TMX1-deficient platelets as well as recombinant TMX1 addition have demonstrated that TMX1 decreases platelet and thrombotic responses through the oxidation of integrin $\alpha\text{Ib}\beta 3$. Moreover, addition of an anti-TMX1 antibody potentiated platelet aggregation, while addition of recombinant TMX1 inhibited platelet aggregation exerted by different agonists⁴⁰. 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 al¹¹⁹ 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 PDI¹²⁰. LOC14, a reversible inhibitor of PDI, was also identified after screening for potent rescue of a Huntington's disease cell based model¹²⁰. In a similar approach, Vatolin et al¹²¹ 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 IC₅₀ for CCF642 in the insulin turbidimetry assay against several thiol isomerases⁷⁸. 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 al¹²² 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 IC₅₀ 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 IC₅₀ 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 IC₅₀ 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 IC₅₀ 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 rutinosyl 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 concentrations⁶². 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 inhibition⁶², 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 (*Salvia miltiorrhiza*) was shown to inhibit ERp57 and promote platelet inhibition at low micromolar concentrations, depending on the agonist used¹⁴². 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 neuroblastoma cells, suggesting ERp57 to be the main target of punicalagin in cellular systems¹⁴³. Similarly, tannic acid was demonstrated to bind to PDI with high affinity, after a directed *in silico* screening of over 60 natural compounds¹⁴⁴. 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 Akt¹⁴⁵. 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 platelets¹⁴⁶. 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 platelets¹⁴⁷. Likewise, quercetin, catechin and other structurally related flavonoids were shown to inhibit platelet aggregation, and act as competitive of the thromboxane A₂ (TxA₂) receptor¹⁴⁸. 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 β 3 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 bepristat 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.

ACKNOWLEDGEMENTS

This study was funded by the British Heart Foundation (RG/15/2/31224), Medical Research Council (MR/J002666/1). This work is part of the PhD thesis of RSG. All figures were made using BioRender.com.

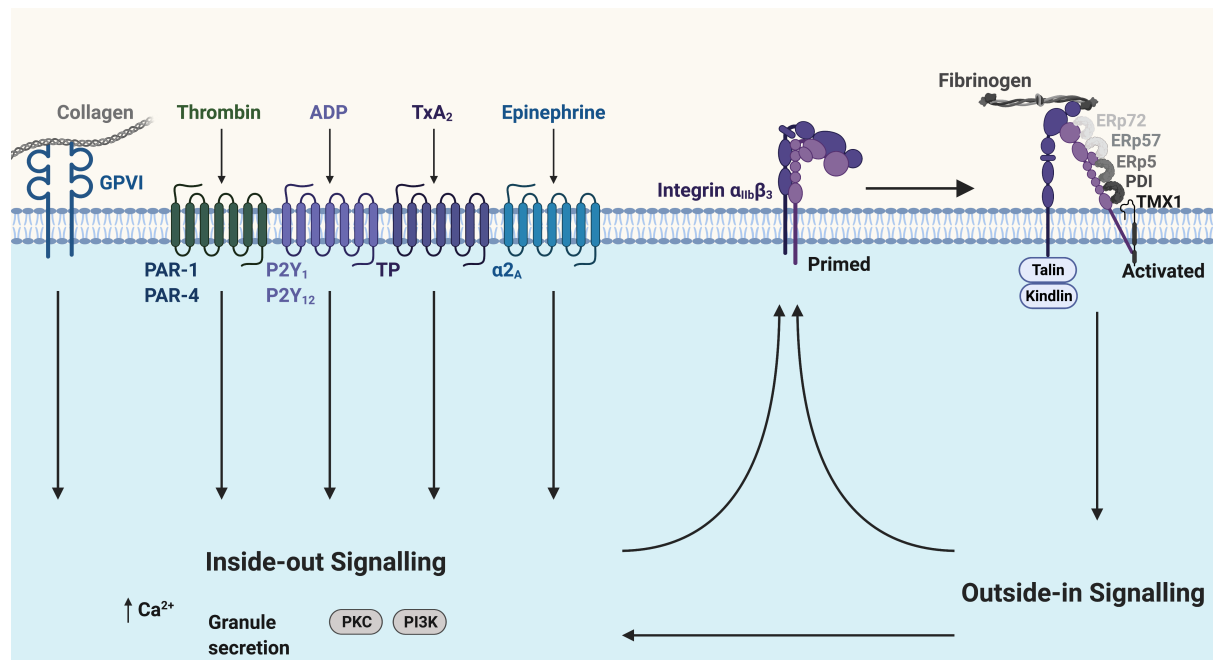


Figure 1. Inside-out and outside-in signalling in platelets. Platelet activation can be didactically divided in two pathways: inside-out and outside-in signalling. Inside-out signalling refers to binding of agonists to their respective receptors on the platelet membrane (e.g. collagen binding to GPVI). This initial binding will lead to specific pathways of each receptor that will culminate in a common pathway that involves increased intracellular Ca²⁺ 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 α_{IIb}β₃ in a process that requires protein disulphide isomerase (PDI) as well as other thiol isomerases. Upon binding to fibrinogen, the integrin α_{IIb}β₃ 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. TxA₂: thromboxane A₂.

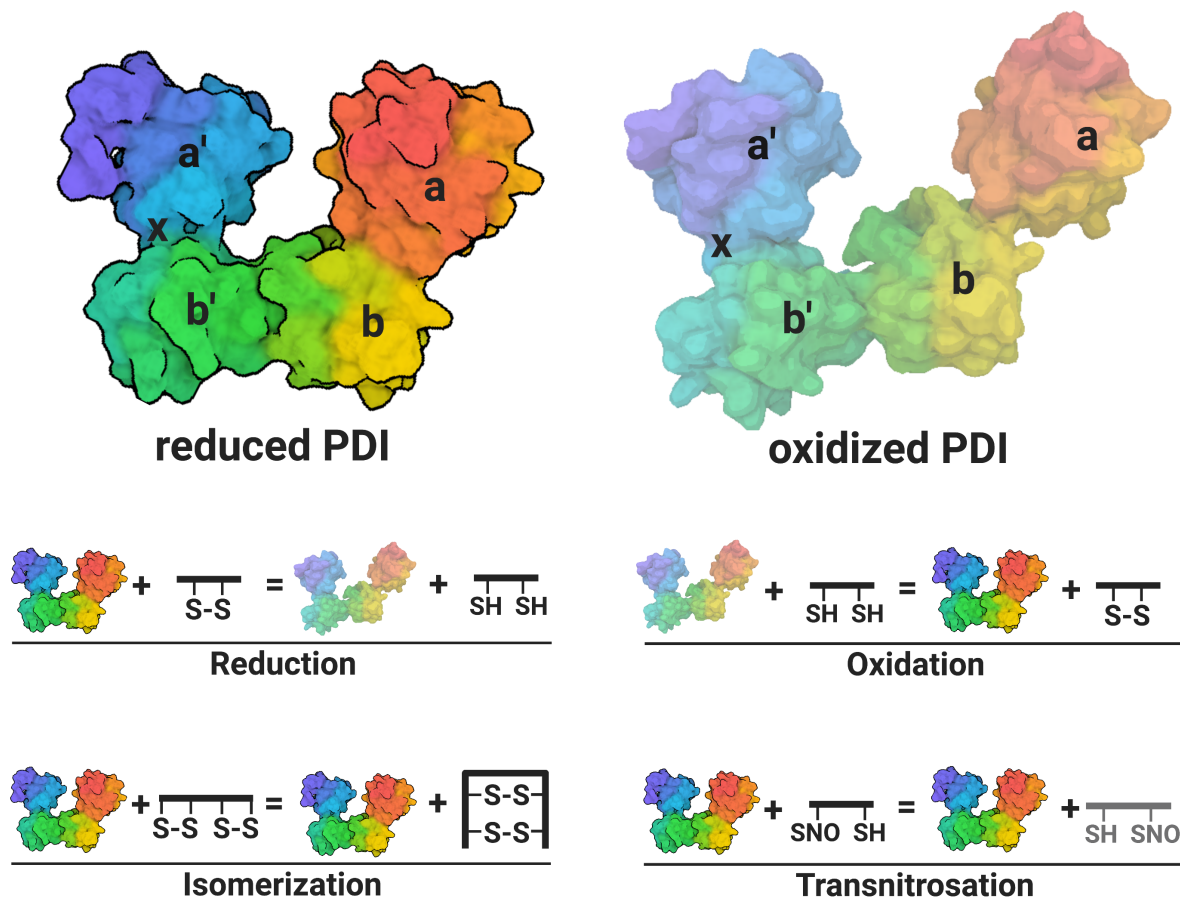


Figure 2. Protein disulphide isomerase catalyses redox reactions. The 3D structures of protein disulphide isomerase (PDI) were obtained from the PDB database (PDB ID: 4EL1 for oxidized and PDB ID: 4EKZ for reduced). PDI has 4 domains and an x-linker to promote flexibility. Its catalytic sites are located 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.

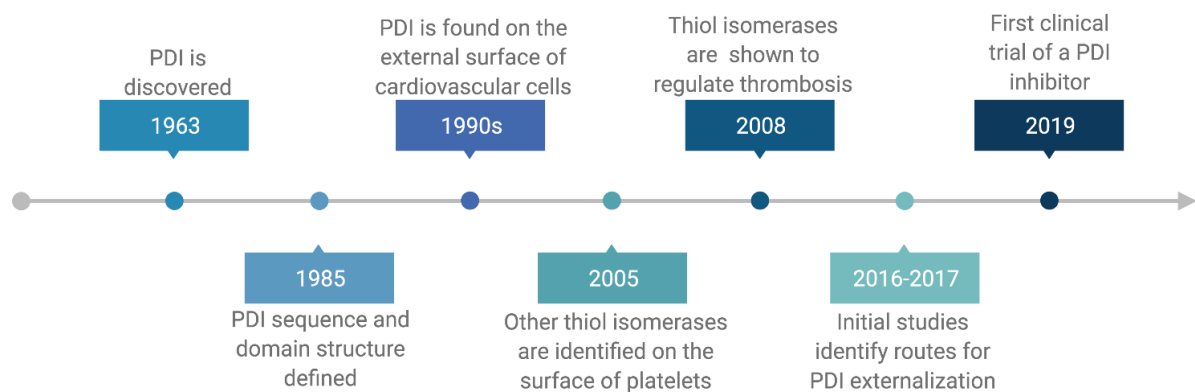


Figure 3. Historical landmarks of the PDI field. Decades of intensive research output led to many discoveries linking PDI and sibling proteins to thrombosis and haemostasis. From its initial characterisation in the 1960s-1980s, PDI was later found on the outer surface of cardiovascular cells. Other thiol 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.

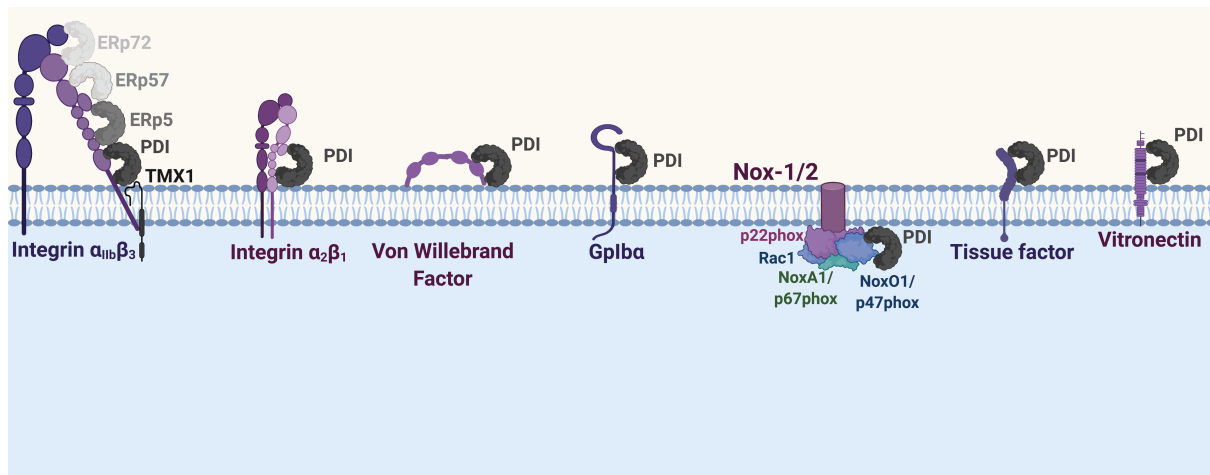


Figure 4. Pro-thrombotic targets of thiol isomerases. PDI, ERp5, ERp57 and ERp72 are known regulators of platelet function through their interaction with integrin $\alpha\text{IIb}\beta_3$. In contrast, TMX1 has been shown to inhibit integrin $\alpha\text{IIb}\beta_3$, being the first thiol isomerase described to negatively regulate thrombosis. PDI is also able to interact with integrin $\alpha_2\beta_1$, Von Willebrand Factor (VWF), GpIba, 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.

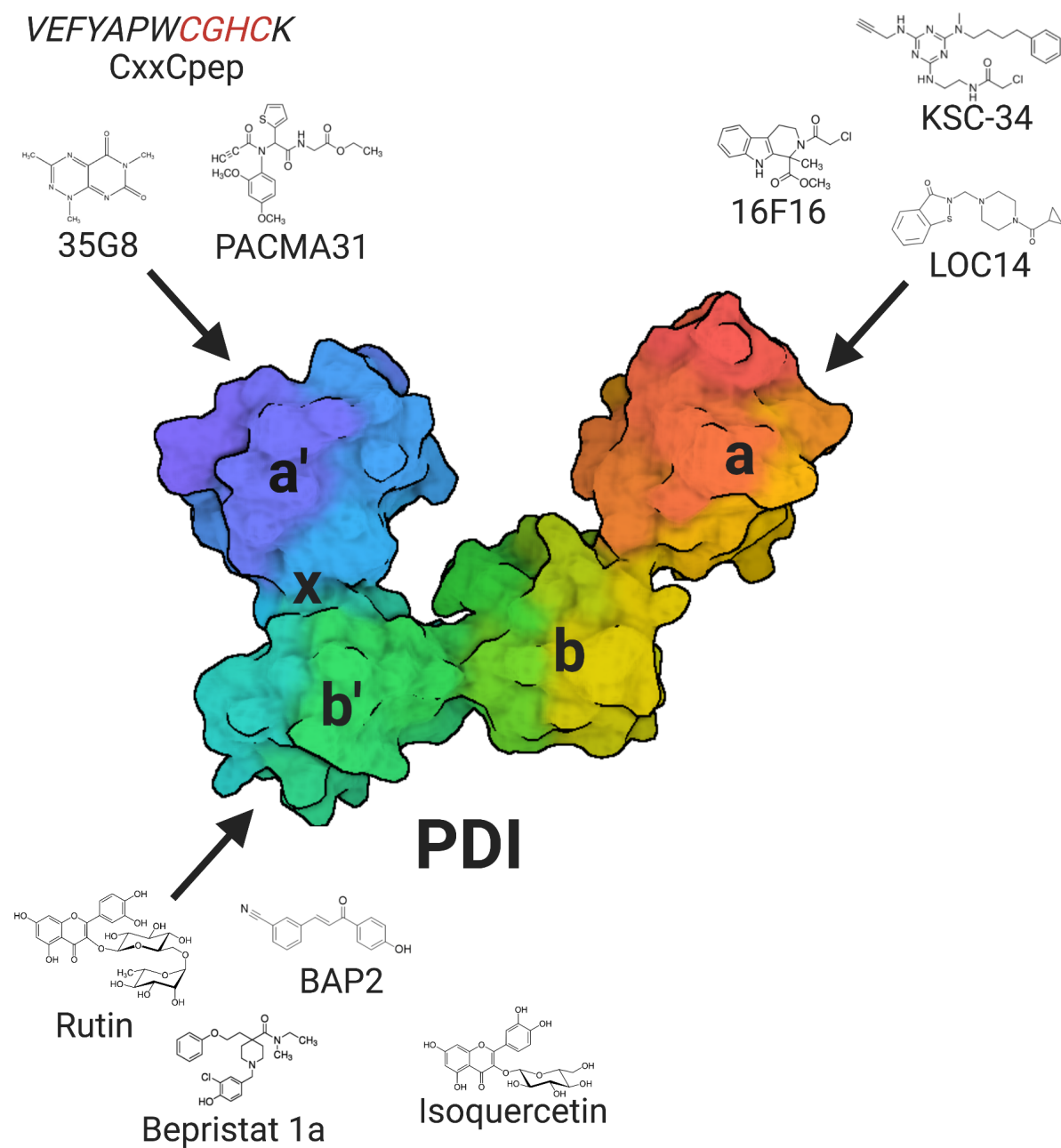


Figure 5. PDI inhibitors with known binding sites. Several PDI inhibitors have been characterized, however only a few of these have a known binding site. 16F16, KSC-34 and LOC14 bind close to the a'-active site; CxxCpep, 35G8 and PACMA-31 bind close to the a-active site, while rutin, bepristats, BAPs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

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

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

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

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

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

	Mastoparan	PDI	Not tested	Not tested	Platelet function □	158,159
--	-------------------	-----	------------	------------	------------------------	---------

696 TI: Thiol isomerase. *Preliminary data from our lab. **: Disputed. □:Decreased.

697 □:Increased. -:Unaffected. #:Predicted.

698

- 1 Bizzozero, J. Ueber einen neuen Formbestandtheil des Blutes und dessen Rolle bei der Thrombose und der Blutgerinnung. *Archiv für pathologische Anatomie und Physiologie und für klinische Medizin* **90**, 261-332 (1882).
- 2 Quick, A. J. *Bleeding problems in clinical medicine*. (Saunders, 1970).
- 3 Roskam, J., Hugues, J. & Bounameaux, Y. LHEMOSTASE SPONTANEE ETUDE SYNTHETIQUE ET ANALYTIQUE. *JOURNAL DE PHYSIOLOGIE* **53**, 175-& (1961).
- 4 Bettex-Galland, M. & Lüscher, E. Thrombosthenin—a contractile protein from thrombocytes. Its extraction from human blood platelets and some of its properties. *Biochimica et biophysica acta* **49**, 536-547 (1961).
- 5 Zahn, F. W. Untersuchungen über thrombose. *Archiv für pathologische Anatomie und Physiologie und für klinische Medizin* **62**, 81-124 (1874).
- 6 de Gaetano, G. Historical overview of the role of platelets in hemostasis and thrombosis. *Haematologica* **86**, 349-356 (2001).
- 7 Bye, A. P., Unsworth, A. J. & Gibbins, J. M. Platelet signaling: a complex interplay between inhibitory and activatory networks. *Journal of Thrombosis and Haemostasis* **14**, 918-930 (2016).
- 8 Savage, B., Saldivar, E. & Ruggeri, Z. M. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell* **84**, 289-297 (1996).
- 9 Massberg, S. *et al.* A crucial role of glycoprotein VI for platelet recruitment to the injured arterial wall in vivo. *J Exp Med* **197**, 41-49 (2003).
- 10 Durrant, T. N., van den Bosch, M. T. & Hers, I. Integrin $\alpha\text{IIb}\beta 3$ outside-in signaling. *Blood* **130**, 1607-1619 (2017).
- 11 Mancuso, M. E. & Santagostino, E. Platelets: much more than bricks in a breached wall. *Br J Haematol* **178**, 209-219, doi:10.1111/bjh.14653 (2017).
- 12 Holbrook, L. M. *et al.* Platelets release novel thiol isomerase enzymes which are recruited to the cell surface following activation. *Br J Haematol* **148**, 627-637, doi:10.1111/j.1365-2141.2009.07994.x (2010).
- 13 Jasuja, R., Furie, B. & Furie, B. C. Endothelium-derived but not platelet-derived protein disulfide isomerase is required for thrombus formation in vivo. *Blood, The Journal of the American Society of Hematology* **116**, 4665-4674 (2010).
- 14 Chen, K., Lin, Y. & Detwiler, T. C. Protein disulfide isomerase activity is released by activated platelets. (1992).
- 15 Wu, Y. & Essex, D. W. Vascular thiol isomerases in thrombosis: The Yin and Yang. *Journal of Thrombosis and Haemostasis* (2020).
- 16 Gaspar, R. S., Trostchansky, A. & Paes, A. M. Potential Role of Protein Disulfide Isomerase in Metabolic Syndrome-Derived Platelet Hyperactivity. *Oxid Med Cell Longev* **2016**, 2423547, doi:10.1155/2016/2423547 (2016).
- 17 Kim, K. *et al.* Platelet protein disulfide isomerase is required for thrombus formation but not for hemostasis in mice. *Blood* **122**, 1052-1061, doi:10.1182/blood-2013-03-492504 (2013).
- 18 Jordan, P. A. *et al.* A role for the thiol isomerase protein ERP5 in platelet function. *Blood* **105**, 1500-1507, doi:10.1182/blood-2004-02-0608 (2005).
- 19 Holbrook, L. M. *et al.* The platelet-surface thiol isomerase enzyme ERp57 modulates platelet function. *J Thromb Haemost* **10**, 278-288, doi:10.1111/j.1538-7836.2011.04593.x (2012).
- 20 Holbrook, L. M. *et al.* A humanized monoclonal antibody that inhibits platelet-surface ERp72 reveals a role for ERp72 in thrombosis. *J Thromb Haemost* **16**, 367-377, doi:10.1111/jth.13878 (2018).

- 21 Venetianer, P. & Straub, F. The enzymic reactivation of reduced ribonuclease. *Biochimica et Biophysica Acta (BBA)-Specialized Section on Enzymological Subjects* **67**, 166-168 (1963).
- 22 Goldberger, R. F., Epstein, C. J. & Anfinsen, C. B. Acceleration of reactivation of reduced bovine pancreatic ribonuclease by a microsomal system from rat liver. *Journal of biological chemistry* **238**, 628-635 (1963).
- 23 Hawkins, H. C. & Freedman, R. B. Randomly reoxidised soybean trypsin inhibitor and the possibility of conformational barriers to disulphide isomerization in proteins. *FEBS letters* **58**, 7-11 (1975).
- 24 Tomizawa, H. H. Mode of action of an insulin-degrading enzyme from beef liver. *Journal of Biological Chemistry* **237**, 428-431 (1962).
- 25 Edman, J. C., Ellis, L., Blacher, R. W., Roth, R. A. & Rutter, W. J. Sequence of protein disulphide isomerase and implications of its relationship to thioredoxin. *Nature* **317**, 267-270 (1985).
- 26 Essex, D. W., Chen, K. & Swiatkowska, M. Localization of protein disulfide isomerase to the external surface of the platelet plasma membrane. (1995).
- 27 Manickam, N., Sun, X., Li, M., Gazitt, Y. & Essex, D. W. Protein disulphide isomerase in platelet function. *British journal of haematology* **140**, 223-229 (2008).
- 28 Crescente, M. *et al.* Intracellular trafficking, localization, and mobilization of platelet-borne thiol isomerases. *Arteriosclerosis, thrombosis, and vascular biology* **36**, 1164-1173 (2016).
- 29 Holbrook, L. M. *et al.* Platelets release novel thiol isomerase enzymes which are recruited to the cell surface following activation. *British journal of haematology* **148**, 627-637 (2010).
- 30 Raturi, A., Miersch, S., Hudson, J. W. & Mutus, B. Platelet microparticle-associated protein disulfide isomerase promotes platelet aggregation and inactivates insulin. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1778**, 2790-2796 (2008).
- 31 Hotchkiss, K. A., Matthias, L. J. & Hogg, P. J. Exposure of the cryptic Arg-Gly-Asp sequence in thrombospondin-1 by protein disulfide isomerase. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology* **1388**, 478-488 (1998).
- 32 Reinhardt, C. *et al.* Protein disulfide isomerase acts as an injury response signal that enhances fibrin generation via tissue factor activation. *The Journal of clinical investigation* **118**, 1110-1122 (2008).
- 33 Tanaka, L. Y. *et al.* Peri/epicellular protein disulfide isomerase-A1 acts as an upstream organizer of cytoskeletal mechanoadaptation in vascular smooth muscle cells. *American Journal of Physiology-Heart and Circulatory Physiology* **316**, H566-H579 (2019).
- 34 Tanaka, L. Y. *et al.* Peri/epicellular protein disulfide isomerase sustains vascular lumen caliber through an anticonstrictive remodeling effect. *Hypertension* **67**, 613-622 (2016).
- 35 Cho, J., Furie, B. C., Coughlin, S. R. & Furie, B. A critical role for extracellular protein disulfide isomerase during thrombus formation in mice. *The Journal of clinical investigation* **118**, 1123-1131 (2008).
- 36 Bowley, S. R., Fang, C., Merrill-Skoloff, G., Furie, B. C. & Furie, B. Protein disulfide isomerase secretion following vascular injury initiates a regulatory pathway for thrombus formation. *Nature communications* **8**, 1-13 (2017).
- 37 Kim, K. *et al.* Platelet protein disulfide isomerase is required for thrombus formation but not for hemostasis in mice. *Blood, The Journal of the American Society of Hematology* **122**, 1052-1061 (2013).

- 38 Wu, Y. *et al.* The disulfide isomerase ERp57 mediates platelet aggregation, hemostasis, and thrombosis. *Blood, The Journal of the American Society of Hematology* **119**, 1737-1746 (2012).
- 39 Zhou, J. *et al.* The disulfide isomerase ERp72 supports arterial thrombosis in mice. *Blood* **130**, 817-828 (2017).
- 40 Zhao, Z. *et al.* The transmembrane protein disulfide isomerase TMX1 negatively regulates platelet responses. *blood* **133**, 246-251 (2019).
- 41 Passam, F. H. *et al.* Both platelet-and endothelial cell-derived ERp5 support thrombus formation in a laser-induced mouse model of thrombosis. *Blood, The Journal of the American Society of Hematology* **125**, 2276-2285 (2015).
- 42 HOLBROOK, L. M. *et al.* The platelet-surface thiol isomerase enzyme ERp57 modulates platelet function. *Journal of Thrombosis and Haemostasis* **10**, 278-288 (2012).
- 43 Wang, L. *et al.* Platelet-derived ERp57 mediates platelet incorporation into a growing thrombus by regulation of the α IIb β 3 integrin. *Blood* **122**, 3642-3650 (2013).
- 44 Eriksson, O. *et al.* Thiol isomerase ERp57 targets and modulates the lectin pathway of complement activation. *Journal of Biological Chemistry* **294**, 4878-4888 (2019).
- 45 Holbrook, L. M. *et al.* A humanized monoclonal antibody that inhibits platelet-surface ER p72 reveals a role for ER p72 in thrombosis. *Journal of Thrombosis and Haemostasis* **16**, 367-377 (2018).
- 46 Araki, K. *et al.* Ero1- α and PDIs constitute a hierarchical electron transfer network of endoplasmic reticulum oxidoreductases. *Journal of Cell Biology* **202**, 861-874 (2013).
- 47 Viotti, C. in *Unconventional Protein Secretion* 3-29 (Springer, 2016).
- 48 Araujo, T. L. *et al.* Protein disulfide isomerase externalization in endothelial cells follows classical and unconventional routes. *Free Radical Biology and Medicine* **103**, 199-208 (2017).
- 49 Giordano, F. *et al.* PI (4, 5) P2-dependent and Ca²⁺-regulated ER-PM interactions mediated by the extended synaptotagmins. *Cell* **153**, 1494-1509 (2013).
- 50 Zwicker, J. I. *et al.* Targeting protein disulfide isomerase with the flavonoid isoquercetin to improve hypercoagulability in advanced cancer. *JCI insight* **4** (2019).
- 51 Jayachandran, M. *et al.* Isoquercetin upregulates antioxidant genes, suppresses inflammatory cytokines and regulates AMPK pathway in streptozotocin-induced diabetic rats. *Chemico-biological interactions* **303**, 62-69 (2019).
- 52 Stopa, J. D. *et al.* Protein disulfide isomerase inhibition blocks thrombin generation in humans by interfering with platelet factor V activation. *JCI insight* **2** (2017).
- 53 Hynes, R. O. Integrins: bidirectional, allosteric signaling machines. *cell* **110**, 673-687 (2002).
- 54 Cho, J. *et al.* Protein disulfide isomerase capture during thrombus formation in vivo depends on the presence of β 3 integrins. *Blood, The Journal of the American Society of Hematology* **120**, 647-655 (2012).
- 55 Swiatkowska, M., Szymański, J., Padula, G. & Cierniewski, C. S. Interaction and functional association of protein disulfide isomerase with α V β 3 integrin on endothelial cells. *The FEBS journal* **275**, 1813-1823 (2008).
- 56 Passam, F. *et al.* Mechano-redox control of integrin de-adhesion. *Elife* **7**, e34843 (2018).
- 57 O'Neill, S. *et al.* The platelet integrin α IIb β 3 has an endogenous thiol isomerase activity. *Journal of Biological Chemistry* **275**, 36984-36990 (2000).
- 58 Lahav, J. *et al.* Enzymatically catalyzed disulfide exchange is required for platelet adhesion to collagen via integrin α 2 β 1. *Blood* **102**, 2085-2092 (2003).
- 59 Inoue, O., Suzuki-Inoue, K., Dean, W. L., Frampton, J. & Watson, S. P. Integrin α 2 β 1

- mediates outside-in regulation of platelet spreading on collagen through activation of Src kinases and PLC γ 2. *The Journal of cell biology* **160**, 769-780 (2003).
- 60 Wan, S. W. *et al.* Endothelial cell surface expression of protein disulfide isomerase activates β 1 and β 3 integrins and facilitates dengue virus infection. *Journal of cellular biochemistry* **113**, 1681-1691 (2012).
- 61 Hahm, E. *et al.* Extracellular protein disulfide isomerase regulates ligand-binding activity of α M β 2 integrin and neutrophil recruitment during vascular inflammation. *Blood, The Journal of the American Society of Hematology* **121**, 3789-3800 (2013).
- 62 Gaspar, R. S. *et al.* Myricetin, the main flavonoid in *Syzygium cumini* leaf, is a novel inhibitor of platelet thiol isomerases PDI and ERp5. *Frontiers in pharmacology* **10**, 1678 (2020).
- 63 Mangin, P. H. *et al.* Immobilized fibrinogen activates human platelets through glycoprotein VI. *haematologica* **103**, 898 (2018).
- 64 Remijn, J. A. *et al.* Role of ADP receptor P2Y₁₂ in platelet adhesion and thrombus formation in flowing blood. *Arteriosclerosis, thrombosis, and vascular biology* **22**, 686-691 (2002).
- 65 Bergmeier, W., Chauhan, A. K. & Wagner, D. D. Glycoprotein Iba and von Willebrand factor in primary platelet adhesion and thrombus formation: lessons from mutant mice. *Thrombosis and haemostasis* **99**, 264-270 (2008).
- 66 Blenner, M. A., Dong, X. & Springer, T. A. Structural basis of regulation of von Willebrand factor binding to glycoprotein Ib. *Journal of Biological Chemistry* **289**, 5565-5579 (2014).
- 67 Lippok, S. *et al.* von Willebrand factor is dimerized by protein disulfide isomerase. *Blood, The Journal of the American Society of Hematology* **127**, 1183-1191 (2016).
- 68 Burgess, J. K. *et al.* Physical proximity and functional association of glycoprotein Iba and protein-disulfide isomerase on the platelet plasma membrane. *Journal of Biological Chemistry* **275**, 9758-9766 (2000).
- 69 Stopa, J. D., Baker, K. M., Grover, S. P., Flaumenhaft, R. & Furie, B. Kinetic-based trapping by intervening sequence variants of the active sites of protein-disulfide isomerase identifies platelet protein substrates. *Journal of Biological Chemistry* **292**, 9063-9074 (2017).
- 70 Li, J. *et al.* Platelet protein disulfide isomerase promotes glycoprotein Iba-mediated platelet-neutrophil interactions under thromboinflammatory conditions. *Circulation* **139**, 1300-1319 (2019).
- 71 Rapaport, S. I. & Rao, L. V. M. The tissue factor pathway: how it has become a “prima ballerina”. *Thrombosis and haemostasis* **73**, 007-017 (1995).
- 72 Ahamed, J. *et al.* Disulfide isomerization switches tissue factor from coagulation to cell signaling. *Proceedings of the National Academy of Sciences* **103**, 13932-13937 (2006).
- 73 Popescu, N. I., Lupu, C. & Lupu, F. Extracellular protein disulfide isomerase regulates coagulation on endothelial cells through modulation of phosphatidylserine exposure. *Blood* **116**, 993-1001 (2010).
- 74 Ansari, S. A., Pendurthi, U. R. & Rao, L. V. M. Role of cell surface lipids and thiol-disulphide exchange pathways in regulating the encryption and decryption of tissue factor. *Thrombosis and haemostasis* **119**, 860-870 (2019).
- 75 Versteeg, H. H. & Ruf, W. Tissue factor coagulant function is enhanced by protein-disulfide isomerase independent of oxidoreductase activity. *Journal of Biological Chemistry* **282**, 25416-25424 (2007).
- 76 Chen, F. *et al.* Protein disulfide isomerase enhances tissue factor-dependent thrombin generation. *Biochemical and biophysical research communications* **501**, 172-177

- (2018).
- 77 Falati, S., Gross, P., Merrill-Skoloff, G., Furie, B. C. & Furie, B. Real-time in vivo imaging of platelets, tissue factor and fibrin during arterial thrombus formation in the mouse. *Nature medicine* **8**, 1175-1180 (2002).
 - 78 Robinson, R. M. *et al.* Inhibitors of the protein disulfide isomerase family for the treatment of multiple myeloma. *Leukemia* **33**, 1011-1022 (2019).
 - 79 Delaney, M. K. *et al.* Differential Roles of the NADPH-Oxidase 1 and 2 in Platelet Activation and Thrombosis. *Arterioscler Thromb Vasc Biol* **36**, 846-854, doi:10.1161/ATVBAHA.116.307308 (2016).
 - 80 Bayraktutan, U., Blayney, L. & Shah, A. M. Molecular characterization and localization of the NAD (P) H oxidase components gp91-phox and p22-phox in endothelial cells. *Arteriosclerosis, thrombosis, and vascular biology* **20**, 1903-1911 (2000).
 - 81 Griendling, K. K., Minieri, C. A., Ollerenshaw, J. D. & Alexander, R. W. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circulation research* **74**, 1141-1148 (1994).
 - 82 Seno, T. *et al.* Involvement of NADH/NADPH oxidase in human platelet ROS production. *Thrombosis research* **103**, 399-409 (2001).
 - 83 Rastogi, R., Geng, X., Li, F. & Ding, Y. NOX activation by subunit interaction and underlying mechanisms in disease. *Frontiers in cellular neuroscience* **10**, 301 (2017).
 - 84 Vara, D., Campanella, M. & Pula, G. The novel NOX inhibitor 2-acetylphenothiazine impairs collagen-dependent thrombus formation in a GPVI-dependent manner. *Br J Pharmacol* **168**, 212-224, doi:10.1111/j.1476-5381.2012.02130.x (2013).
 - 85 Lambeth, J. D., Kawahara, T. & Diebold, B. Regulation of Nox and Duox enzymatic activity and expression. *Free Radical Biology and Medicine* **43**, 319-331 (2007).
 - 86 Schroder, K., Weissmann, N. & Brandes, R. P. Organizers and activators: Cytosolic Nox proteins impacting on vascular function. *Free Radic Biol Med* **109**, 22-32, doi:10.1016/j.freeradbiomed.2017.03.017 (2017).
 - 87 Vara, D., Cifuentes-Pagano, E., Pagano, P. J. & Pula, G. A novel combinatorial technique for simultaneous quantification of oxygen radicals and aggregation reveals unexpected redox patterns in the activation of platelets by different physiopathological stimuli. *Haematologica* **104**, 1879-1891, doi:10.3324/haematol.2018.208819 (2019).
 - 88 Gaspar, R. S., Ferreira, P. M., Mitchell, J. L., Pula, G. & Gibbins, J. M. Platelet-derived extracellular vesicles express NADPH oxidase-1 (Nox-1), generate superoxide and modulate platelet function. *Free Radical Biology and Medicine* **165**, 395-400 (2021).
 - 89 de, A. P. A. M. *et al.* Protein disulfide isomerase redox-dependent association with p47(phox): evidence for an organizer role in leukocyte NADPH oxidase activation. *J Leukoc Biol* **90**, 799-810, doi:10.1189/jlb.0610324 (2011).
 - 90 Fernandes, D. C., Manoel, A. H., Wosniak, J., Jr. & Laurindo, F. R. Protein disulfide isomerase overexpression in vascular smooth muscle cells induces spontaneous preemptive NADPH oxidase activation and Nox1 mRNA expression: effects of nitrosothiol exposure. *Arch Biochem Biophys* **484**, 197-204, doi:10.1016/j.abb.2009.01.022 (2009).
 - 91 Pescatore, L. A. *et al.* Protein disulfide isomerase is required for platelet-derived growth factor-induced vascular smooth muscle cell migration, Nox1 NADPH oxidase expression, and RhoGTPase activation. *Journal of Biological Chemistry* **287**, 29290-29300 (2012).
 - 92 Hordijk, P. L. Regulation of NADPH oxidases: the role of Rac proteins. *Circulation*

- research **98**, 453-462 (2006).
- 93 Sousa, H. R. *et al.* Novel antiplatelet role for a protein disulfide isomerase-targeted peptide: evidence of covalent binding to the C-terminal CGHC redox motif. *J Thromb Haemost* **15**, 774-784, doi:10.1111/jth.13633 (2017).
 - 94 Gimenez, M. *et al.* Redox Activation of Nox1 (NADPH Oxidase 1) Involves an Intermolecular Disulfide Bond Between Protein Disulfide Isomerase and p47(phox) in Vascular Smooth Muscle Cells. *Arterioscler Thromb Vasc Biol* **39**, 224-236, doi:10.1161/ATVBAHA.118.311038 (2019).
 - 95 Zhou, J. *et al.* The C-terminal CGHC motif of protein disulfide isomerase supports thrombosis. *J Clin Invest* **125**, 4391-4406, doi:10.1172/JCI80319 (2015).
 - 96 Gaspar, R. S. *et al.* Protein Disulphide Isomerase and NADPH Oxidase 1 Cooperate to Control Platelet Function and Are Associated with Cardiometabolic Disease Risk Factors. *Antioxidants* **10**, 497 (2021).
 - 97 Schiattarella, G. G. *et al.* Rac1 modulates endothelial function and platelet aggregation in diabetes mellitus. *Journal of the American Heart Association* **7**, e007322 (2018).
 - 98 Wang, X. *et al.* p47phox deficiency impairs platelet function and protects mice against arterial and venous thrombosis. *Redox Biology*, 101569 (2020).
 - 99 Leavesley, D. I. *et al.* Vitronectin—master controller or micromanager? *IUBMB life* **65**, 807-818 (2013).
 - 100 Preissner, K. T. & Seiffert, D. Role of vitronectin and its receptors in haemostasis and vascular remodeling. *Thrombosis research* **89**, 1-21 (1998).
 - 101 Reheman, A. *et al.* Vitronectin stabilizes thrombi and vessel occlusion but plays a dual role in platelet aggregation. *Journal of Thrombosis and Haemostasis* **3**, 875-883 (2005).
 - 102 Essex, D. W., Miller, A., Swiatkowska, M. & Feinman, R. D. Protein Disulfide Isomerase Catalyzes the Formation of Disulfide-Linked Complexes of Vitronectin with Thrombin– Antithrombin. *Biochemistry* **38**, 10398-10405 (1999).
 - 103 Moretti, A. I. *et al.* Conserved gene microsynteny unveils functional interaction between protein disulfide isomerase and rho guanine-dissociation inhibitor families. *Scientific reports* **7**, 1-18 (2017).
 - 104 Aslan, J. E. & McCarty, O. J. Rho GTPases in platelet function. *Journal of Thrombosis and Haemostasis* **11**, 35-46 (2013).
 - 105 Sobierajska, K. *et al.* Protein disulfide isomerase directly interacts with β -actin Cys374 and regulates cytoskeleton reorganization. *Journal of Biological Chemistry* **289**, 5758-5773 (2014).
 - 106 Versteeg, H. H. & Ruf, W. Tissue factor coagulant function is enhanced by protein-disulfide isomerase independent of oxidoreductase activity. *J Biol Chem* **282**, 25416-25424, doi:10.1074/jbc.M702410200 (2007).
 - 107 Chen, K., Detwiler, T. C. & Essex, D. W. Characterization of protein disulphide isomerase released from activated platelets. *Br J Haematol* **90**, 425-431 (1995).
 - 108 Radomski, M., Palmer, R. & Moncada, S. An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proceedings of the National Academy of Sciences* **87**, 5193-5197 (1990).
 - 109 Russo, I. *et al.* The activity of constitutive nitric oxide synthase is increased by the pathway cAMP/cAMP-activated protein kinase in human platelets. New insights into the antiaggregating effects of cAMP-elevating agents. *Thromb Res* **114**, 265-273, doi:10.1016/j.thromres.2004.06.036 (2004).
 - 110 Kwon, H. W., Shin, J. H., Cho, H. J., Rhee, M. H. & Park, H. J. Total saponin from Korean Red Ginseng inhibits binding of adhesive proteins to glycoprotein IIb/IIIa via

- phosphorylation of VASP (Ser(157)) and dephosphorylation of PI3K and Akt. *J Ginseng Res* **40**, 76-85, doi:10.1016/j.jgr.2015.05.004 (2016).
- 111 Fuentes, E. & Palomo, I. Role of oxidative stress on platelet hyperreactivity during aging. *Life Sci* **148**, 17-23, doi:10.1016/j.lfs.2016.02.026 (2016).
- 112 Ramachandran, N., Root, P., Jiang, X. M., Hogg, P. J. & Mutus, B. Mechanism of transfer of NO from extracellular S-nitrosothiols into the cytosol by cell-surface protein disulfide isomerase. *Proc Natl Acad Sci U S A* **98**, 9539-9544, doi:10.1073/pnas.171180998 (2001).
- 113 Root, P., Sliskovic, I. & Mutus, B. Platelet cell-surface protein disulphide-isomerase mediated S-nitrosoglutathione consumption. *Biochem J* **382**, 575-580, doi:10.1042/BJ20040759 (2004).
- 114 Peixoto, A. S. *et al.* Peroxynitrite preferentially oxidizes the dithiol redox motifs of protein-disulfide isomerase. *J Biol Chem* **293**, 1450-1465, doi:10.1074/jbc.M117.807016 (2018).
- 115 Shah, C. M., Bell, S. E., Locke, I. C., Chowdrey, H. S. & Gordge, M. P. Interactions between cell surface protein disulphide isomerase and S-nitrosoglutathione during nitric oxide delivery. *Nitric Oxide* **16**, 135-142, doi:10.1016/j.niox.2006.08.001 (2007).
- 116 Bell, S. E., Shah, C. M. & Gordge, M. P. Protein disulfide-isomerase mediates delivery of nitric oxide redox derivatives into platelets. *Biochem J* **403**, 283-288, doi:10.1042/BJ20061146 (2007).
- 117 Bekendam, R. H. *et al.* Protein disulfide isomerase regulation by nitric oxide maintains vascular quiescence and controls thrombus formation. *Journal of Thrombosis and Haemostasis* **16**, 2322-2335 (2018).
- 118 Matsuo, Y. *et al.* Identification of a novel thioredoxin-related transmembrane protein. *Journal of Biological Chemistry* **276**, 10032-10038 (2001).
- 119 Hoffstrom, B. G. *et al.* Inhibitors of protein disulfide isomerase suppress apoptosis induced by misfolded proteins. *Nature chemical biology* **6**, 900-906 (2010).
- 120 Kaplan, A. *et al.* Small molecule-induced oxidation of protein disulfide isomerase is neuroprotective. *Proceedings of the National Academy of Sciences* **112**, E2245-E2252 (2015).
- 121 Vatolin, S. *et al.* Novel protein disulfide isomerase inhibitor with anticancer activity in multiple myeloma. *Cancer research* **76**, 3340-3350 (2016).
- 122 Bekendam, R. H. *et al.* A substrate-driven allosteric switch that enhances PDI catalytic activity. *Nature communications* **7**, 1-11 (2016).
- 123 Shergalis, A. *et al.* Characterization of aminobenzylphenols as protein disulfide isomerase inhibitors in glioblastoma cell lines. *Journal of medicinal chemistry* **63**, 10263-10286 (2020).
- 124 Kyani, A. *et al.* Discovery and mechanistic elucidation of a class of protein disulfide isomerase inhibitors for the treatment of glioblastoma. *ChemMedChem* **13**, 164-177 (2018).
- 125 Sousa, H. *et al.* Novel antiplatelet role for a protein disulfide isomerase-targeted peptide: evidence of covalent binding to the C-terminal CGHC redox motif. *Journal of Thrombosis and Haemostasis* **15**, 774-784 (2017).
- 126 Zhou, J. *et al.* The C-terminal CGHC motif of protein disulfide isomerase supports thrombosis. *The Journal of clinical investigation* **125**, 4391-4406 (2015).
- 127 Cole, K. S. *et al.* Characterization of an A-site selective protein disulfide isomerase A1 inhibitor. *Biochemistry* **57**, 2035-2043 (2018).
- 128 Xu, S. *et al.* Inhibition of protein disulfide isomerase in glioblastoma causes marked downregulation of DNA repair and DNA damage response genes. *Theranostics* **9**,

- 2282 (2019).
- 129 Cole, K. S. *et al.* Characterization of an A-Site Selective Protein Disulfide Isomerase A1 Inhibitor. *Biochemistry* **57**, 2035-2043, doi:10.1021/acs.biochem.8b00178 (2018).
 - 130 Watashi, K. *et al.* Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Molecular cell* **19**, 111-122 (2005).
 - 131 Kaplan, A. & Stockwell, B. R. Structural elucidation of a small molecule inhibitor of protein disulfide isomerase. *ACS medicinal chemistry letters* **6**, 966-971 (2015).
 - 132 Chlebowska-Tuz, J. *et al.* Inhibition of protein disulfide isomerase induces differentiation of acute myeloid leukemia cells. *haematologica* **103**, 1843-1852 (2018).
 - 133 Greve, E. *et al.* Route exploration and synthesis of the reported pyridone-based PDI inhibitor STK076545. *Organic & Biomolecular Chemistry* **18**, 6665-6681, doi:10.1039/D0OB01205J (2020).
 - 134 Xu, S. *et al.* Discovery of an orally active small-molecule irreversible inhibitor of protein disulfide isomerase for ovarian cancer treatment. *Proceedings of the National Academy of Sciences* **109**, 16348-16353 (2012).
 - 135 Khodier, C. *et al.* in *Probe Reports from the NIH Molecular Libraries Program [Internet]* (National Center for Biotechnology Information (US), (2014).
 - 136 Xiong, B., Jha, V., Min, J.-K. & Cho, J. Protein disulfide isomerase in cardiovascular disease. *Experimental & Molecular Medicine*, 1-10 (2020).
 - 137 Kung, P.-H. *et al.* HPW-RX40 prevents human platelet activation by attenuating cell surface protein disulfide isomerases. *Redox Biology* **13**, 266-277 (2017).
 - 138 Holbrook, L. M. *et al.* Zafirlukast is a broad-spectrum thiol isomerase inhibitor that inhibits thrombosis without altering bleeding times. *British Journal of Pharmacology* (2020).
 - 139 Karala, A. R. & Ruddock, L. W. Bacitracin is not a specific inhibitor of protein disulfide isomerase. *The FEBS journal* **277**, 2454-2462 (2010).
 - 140 Jasuja, R. *et al.* Protein disulfide isomerase inhibitors constitute a new class of antithrombotic agents. *The Journal of clinical investigation* **122**, 2104-2113 (2012).
 - 141 Lin, L. *et al.* Quercetin-3-rutinoside inhibits protein disulfide isomerase by binding to its b' x domain. *Journal of Biological Chemistry* **290**, 23543-23552 (2015).
 - 142 Zou, J. *et al.* Discovery of a novel ERp57 inhibitor as antiplatelet agent from danshen (*Salvia miltiorrhiza*). *Evidence-Based Complementary and Alternative Medicine* **2018** (2018).
 - 143 Giamogante, F. *et al.* Punicalagin, an active pomegranate component, is a new inhibitor of PDIA3 reductase activity. *Biochimie* **147**, 122-129 (2018).
 - 144 Ren, L. *et al.* Molecular docking-assisted screening reveals tannic acid as a natural protein disulphide isomerase inhibitor with antiplatelet and antithrombotic activities. *Journal of Cellular and Molecular Medicine* (2020).
 - 145 Kao, C.-C. *et al.* Juglone prevents human platelet aggregation through inhibiting Akt and protein disulfide isomerase. *Phytomedicine*, 153449 (2020).
 - 146 Wright, B., Watson, K. A., McGuffin, L. J., Lovegrove, J. A. & Gibbins, J. M. GRID and docking analyses reveal a molecular basis for flavonoid inhibition of Src family kinase activity. *The Journal of nutritional biochemistry* **26**, 1156-1165 (2015).
 - 147 Wright, B. *et al.* A structural basis for the inhibition of collagen-stimulated platelet function by quercetin and structurally related flavonoids. *British journal of pharmacology* **159**, 1312-1325 (2010).
 - 148 Guerrero, J. *et al.* Flavonoids inhibit platelet function through binding to the thromboxane A2 receptor. *Journal of Thrombosis and Haemostasis* **3**, 369-376 (2005).

- 149 Lee, A. C.-L., Harris, J. L., Khanna, K. K. & Hong, J.-H. A comprehensive review on
current advances in peptide drug development and design. *International journal of
molecular sciences* **20**, 2383 (2019).
- 150 de A. Paes, A. M. *et al.* Protein disulfide isomerase redox-dependent association with
p47phox: evidence for an organizer role in leukocyte NADPH oxidase activation.
Journal of leukocyte biology **90**, 799-810 (2011).
- 151 Chamberlain, N. *et al.* Lung epithelial protein disulfide isomerase A3 (PDIA3) plays
an important role in influenza infection, inflammation, and airway mechanics. *Redox
biology* **22**, 101129 (2019).
- 152 ZHAO, Z.-z., CHEN, F.-w., ZHOU, J.-s. & Cyrus, T. H. C. Effect of Small-molecule
Inhibitor of Protein Disulfide Isomerase-PACMA31 in Platelet Activation and
Thrombosis. *Chinese Journal of Thrombosis and Hemostasis*, 2 (2016).
- 153 Cui, G. *et al.* Novel anti-thrombotic agent for modulation of protein disulfide
isomerase family member ERp57 for prophylactic therapy. *Scientific Reports* **5**,
10353 (2015).
- 154 Trnková, L., Ricci, D., Grillo, C., Colotti, G. & Altieri, F. Green tea catechins can
bind and modify ERp57/PDIA3 activity. *Biochimica et Biophysica Acta (BBA)-
General Subjects* **1830**, 2671-2682 (2013).
- 155 Mosawy, S., Gaiz, A., Karaksha, A. & Singh, I. The green tea extract epigallocatechin
gallate inhibits human platelet function but not plasma coagulation. *International
Journal of Prevention and Treatment* **5**, 17-21 (2016).
- 156 Khan, M. M. *et al.* Discovery of a small molecule PDI inhibitor that inhibits reduction
of HIV-1 envelope glycoprotein gp120. *ACS chemical biology* **6**, 245-251 (2011).
- 157 Sachetto, A. T. A., Rosa, J. G. & Santoro, M. L. Rutin (quercetin-3-rutinoside)
modulates the hemostatic disturbances and redox imbalance induced by Bothrops
jararaca snake venom in mice. *PLoS neglected tropical diseases* **12**, e0006774 (2018).
- 158 Klappa, P., Hawkins, H. C. & Freedman, R. B. Interactions between protein
disulphide isomerase and peptides. *European journal of biochemistry* **248**, 37-42
(1997).
- 159 Ozaki, Y. *et al.* Mastoparan, a wasp venom, activates platelets via pertussis toxin-
sensitive GTP-binding proteins. *Biochemical and biophysical research
communications* **170**, 779-785 (1990).