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The Intersection of Protein Disulfide Isomerase and Cancer Associated Thrombosis

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Abstract

The mechanisms underlying the hypercoagulability of cancer are complex and include the upregulation coagulation factors or procoagulant proteins, shedding of microparticles, and direct activation of vascular cells. Protein disulfide isomerase (PDI) is a thiol isomerase secreted from activated platelets and endothelial cells and plays a critical role in both platelet aggregation and fibrin generation. A number of potential intravascular targets of PDI have been identified including cell surface receptors (e.g. β-integrins and glycoprotein Ib), receptor ligands (e.g. fibrinogen and von Willebrand factor), serine proteases (e.g. cathepsin G and kallekrein-14), and coagulation factors (e.g. factor XI and factor V). Recent clinical studies demonstrated that a small molecule inhibitor of PDI, isoquercetin, decreases platelet-dependent thrombin generation and PDI activity in plasma following oral administration. This review explores the mechanistic overlap between the molecular drivers of cancer associated thrombosis and the potential roles PDI plays in mediating thrombosis. These molecular insights provide rationale for clinical trials targeting PDI to prevent thrombosis in cancer patients.

Introduction

Cancer is associated with an increase in both venous and arterial thrombosis (1–4). Proposed mechanisms underlying the hypercoagulability of cancer include modulation of coagulation factor activity, increased adhesion of platelets, and elaboration of prothrombotic proteins or microparticles. Protein disulfide isomerase (PDI) is a thiol isomerase secreted by platelets and endothelial cells and plays a critical role in thrombus formation in vivo (5–7). A number of extracellular substrates of PDI have been identified (8–16). The mechanisms underlying the regulatory role of PDI in thrombus formation appear to intersect with the dysregulated

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thrombotic pathways of the malignant state. Clinical studies are underway to determine whether targeting extracellular PDI will prevent thrombosis in advanced cancer populations.

PDI and thrombus formation

PDI is the most critical, and most extensively studied, thiol isomerase (17–26). PDI is the head of the PDI superfamily, which currently includes 21 different thiol isomerases, all of which contain at least one thioredoxin-like domain (17–19, 25, 26). PDI is a 57 kD protein, structured in an a-b-b'-x-a'-c confirmation, displayed in Figure 1. PDI contains two active sites in the a and a' domains, and two protein-substrate binding sites in the b and b' domains (19, 26). The x linker region serves allows flexibility of the a' domain, motion which is critical for catalysis (27, 28). Both active site domains consist of the classical Cys-X-X-Cys motif, and the intervening sequences vary between the family members (17, 18, 20). In PDI, both active sites have a Cys-Gly-His-Cys motif, which is common among several thiol isomerases expressed in humans (17).

PDI acts as a necessary protein folding catalyst, with classic chaperone activity toward nascent peptides (20, 21, 29). The removal of PDI at the transcriptional or translational levels is lethal because of its importance in protein folding in the endoplasmic reticulum (ER) (21). While PDI is crucial for protein folding, it also is capable of carrying out multiple enzymatic functions. PDI can also serve as a thiol oxidoreductase and isomerase, and is capable of further post-translational cysteine modifications such as S-nitrosylation or S-glutathionylation (20, 22, 29–31). For oxidoreductase activity, the CXXC motif will cycle between a reduced state containing two free thiols (-SH), and an oxidized state in which both cysteines are linked via a disulfide bond (S-S) as shown in Figure 2. During reduction, the N-terminal cysteine (N-Cys) acts as the nucleophile to begin reducing a disulfide bond on another protein (22, 23). This is possible because the local pK_a of the active site causes the N-Cys to be a stable thiolate anion (S-), instead of a true free thiol (-SH). The disulfide-linked PDI-substrate complex shuffles its electrons among the four cysteine residues, until the C-terminal free thiol of PDI (C-Cys) forms a disulfide bond with its active site partner. This results in an effective disulfide bond transfer between substrate and PDI. The oxidation reaction takes place similarly but in reverse, effectively transferring a disulfide bond from PDI to a substrate protein which requires oxidation (22, 23, 29).

The activities of PDI are known to extend beyond the ER as PDI is required for thrombus formation in vivo. Following a laser-induced vascular injury in an mouse model of thrombosis, PDI will accumulate rapidly at the site of injury in a growing thrombus (7). The enzymatic activity of PDI, and not just its localization, is essential, because blocking PDI activity with inhibitory antibody (RL90) or small molecule inhibitors like bacitracin, diminishes platelet accumulation and fibrin formation (7, 15, 32). PDI is released from platelets, leukocytes, and endothelial cells upon activation and is capable of "binding-back" to the extracellular membrane through β_3 integrin complexes such as $\alpha_{IIb}\beta_3$ on platelets and $\alpha_V\beta_3$ on endothelial cells (14). On the endothelium, PDI also associates with the β_1 integrin (33). While the exact mechanism of PDI action is still controversial, multiple studies have shown interaction between a myriad of proteins known to be important for blood cell activation and thrombosis (8, 10, 11, 34, 35). Several other thiol isomerase family members

such as ERp5, ERp57, and ERp72 have also been shown to affect thrombus formation when inhibited (5, 6)

To identify proteins substrates of PDI or other thiol isomerases, a technique called kinetic substrate trapping has proven useful (8, 11, 24, 25). The majority of these studies utilized PDI variants which lack the second active site cysteine (CGHA) which forms stable covalent disulfide-linked intermediates between PDI and substrate proteins to enable isolation and identification *via* mass spectroscopy. One limitation of this approach is the requirement for substrates to undergo PDI-mediated disulfide reduction and not oxidation or rearrangement. Our group recently developed PDI variants capable of performing kinetic substrate trapping in both the oxidation and reduction directions (8). These PDI variants with amino acid substitutions in the intervening sequences between the cysteines, alter the pK_a and spacial geometry of the active site to slow catalysis enough to trap the disulfide-linked intermediate complexes. This study identified several proteins as PDI substrates, from multiple classes of proteins involved with thrombosis, including coagulation factors, extracellular scaffolds, activating proteases, and cell surface receptors.

The binding substrates of extracellular PDI and how PDI regulates thrombus formation are not clearly defined. PDI binds to cell surface receptors such β-integrins, annexin V, and GP1β (8, 35) as well as cell surface receptor protein ligands such as fibrinogen, fibrin, and collagen VI (8, 36, 37). Previous studies identified von Willebrand's factor and thrombospondin-1 as substrates for thiol isomerases (10, 12, 38). Cell surface receptors can be activated via binding, but also through cleavage by proteases; kallekrein-14 and cathepsin G are PDI substrates and are known to cleave and activate members of the PAR receptor family (8, 39, 40). PDI can modulate phosphatidylserine (PS) exposure (16, 41) and may regulate coagulation through disulfide regulation of tissue factor, activation of factor XI, maturation of platelet factor V, and attenuating factor XIII cross-linking activity toward fibrin (8, 9, 13, 42, 43). More globally, PDI is believed to sense and regulate the redox state of the extracellular membrane, by interacting with small molecule effectors like glutathione and nitric oxide (NO), and modulating the redox state of other extracellular thiol isomerases (5, 6, 44). Recently identified was glutaredoxin-1, a regulator of NO synthetase, and also the other thiol isomerases thioredoxin and ERp57 (8, 45).

Hypercoagulability of cancer and potential interface with PDI

Certain coagulation factors and other procoagulant proteins are upregulated in expression and secretion in cancer. For example, many cancers are capable of constitutively expressing tissue factor (TF) at an elevated level (46, 47). Tissue factor initiates the extrinsic pathway of blood coagulation forming a complex with activated factor VII (VIIa), which then activates factor X. Tumor cells not only express TF on their cell surface but also generate circulating microparticles which express TF (47, 48). These tissue factor-bearing microparticles are associated with a heightened risk of venous thrombeombolism (49, 50). The regulation of tissue factor activation is complex and incompletely understood (42, 51, 41, 52). The reduction of an allosteric disulfide bond converting "cryptic" TF to the active form may serve as a key regulatory step (51). This disulfide bond reduction may be mediated by PDI, which is also overexpressed and secreted by cancer cells (34, 44), but this hypothesis has not

been proven and remains controversial (51, 53, 54). PDI can alter TF activity indirectly through PS exposure (16, 41, 55) or through the enzyme heparanase which increases the procoagulant activity of tissue factor by preventing the binding of tissue factor pathway inhibitor (TFPI) (52, 56). Heparanase requires disulfide bond oxidation for activation and was identified as a potential substrate for extracellular PDI (8, 57). Taken together, elevated levels of tissue factor and PDI in cancer may lead to hypercoagulability of cancer through increased TF activity and down-regulation of TFPI through heparanase activity.

Tumor cells also exert their procoagulant state by interacting with vascular cells such as endothelial cells and platelets through direct activation (46, 58–60). These platelet-tumor cell interactions are mediated through multiple receptors, most notably the P-selectin receptor and β_3 integrin (3, 58, 60, 61). Interaction with either of these receptors leads to platelet activation which can enhance the metastatic potential of the tumor through shielding from an anti-tumor immune response (59, 60, 62). Several cell surface receptors, receptor agonist proteases, and large scaffolding proteins which bind to these receptors across multiple cells and strengthen the thrombus have all been identified as PDI substrates (9, 12, 14, 33, 35, 38, 43).

Cancer cells elaborate growth factors and cytokines to promote tumor growth and aggressiveness (46, 60, 63). These growth factors will activate other cells to begin to remodel the extracellular matrix. In cancer, this increase in metalloproteinases and circulating growth factors leads to extracellular remodeling but also to vascular permeability and metastasis (64). One of the more important metalloproteinases is ADAM17, which is responsible for the cleavage/shedding of multiple EGFR ligands and TNF-α, has been shown to be regulated by extracellular PDI (65). Following disulfide isomerization catalyzed by PDI, ADAM17 is able to adopt an active conformation facilitating tumor growth and metastasis thus potentially contributing to the hypercoagulable state caused by interaction of tumors and platelets.

Targeting PDI with quercetin flavonoids

In light of the central role PDI plays in thrombosis, our group and others have investigated targeting PDI as an antithrombotic therapeutic (32, 64, 66–68). In a screen of compounds for PDI inhibitors, the bioactive small molecule quercetin-3-rutinoside (rutin) was identified (32). Rutin is a member of the quercetin family of compounds, which are naturally occurring in many fruits and vegetables. Epidemiologic studies have shown that populations with an increased amount of quercetin-rich foods in their diets show a decreased prevalence of thrombosis-related death, such as stroke (69). Rutin, and other similarly structured quercetin family members with a glycosyl moiety at the 3-position, inhibit PDI with a half maximal inhibitory concentration (IC_{50}) in the micromolar range (32). Rutin binds to PDI in the x-linker region between the b’ and a’ domains, preventing any flexibility between the two domains (27, 28). This “locking” of PDI inhibits catalysis, and the binding to the x-linker region gives these quercetins increased specificity to PDI over the other thiol isomerase family members. In a laser-injury model of mouse thrombosis, rutin administered either intravenously or orally decreased thrombus formation in a dose-dependent manner (32).

Notably, in a tail bleeding model, rutin did not affect the bleeding time indicating that PDI may be required for thrombosis but not hemostasis (6, 32, 70).

Quercetin-3-glucoside (isoquercetin) has better bioavailability than rutin when administered orally (71) and inhibits PDI in vitro with the same low micromolar IC_{50} affinity as rutin (32). To evaluate whether isoquercetin could be a viable antithrombotic in humans, we conducted a pharmacokinetic-pharmacodynamic study in healthy volunteers. The results are summarized in Table 1. Quercetin aglycone (500 mg), which lacks the necessary glycosyl group did not inhibit PDI. Notably, significant PDI inhibition was only observed in the volunteers which received 1000 mg of isoquercetin two hours after ingestion, whereas the quercetin aglycone or isoquercetin at 500 mg did not inhibit PDI relative to the pre-ingestion sample.

Platelet-dependent thrombin generation, where thrombin generation is propagated through the activation of platelets, is increased in hypercoagulable states (72, 73). Treatment with isoquercetin (1000 mg) decreased the platelet-dependent thrombin generation after isoquercetin ingestion by 51% compared to the pre-ingestion time zero sample. The observed effect on platelet-dependent thrombin generation was reversed with the addition of exogenous PDI, indicating that the effect of isoquercetin is a PDI-dependent process. Isoquercetin also did not affect coagulation times, thrombin activity, prothrombin cleavage, or Xa activity using isoquercetin-spiked plasma at the peak concentration observed in the volunteer samples.

Platelet factor V was identified as a potential substrate of PDI, and factor V is a necessary cofactor in the prothrombinase complex, where factors Xa and Va convert prothrombin to thrombin (55, 74). Platelet factor V is associated with multimerin-1 in the platelet alpha-granule, where they are linked through disulfide interactions (75). This interaction is thought to inhibit platelet factor V from being activated by thrombin, and the complex must be dissolved before full factor V activation is possible (76). Multimerin-1 was also identified as a PDI substrate using the same kinetic-substrate trapping technique, and like PDI, multimerin-1 also binds back to β_3 integrins on the cell surface (8, 9, 14, 77). We hypothesize that PDI and the multimerin-factor V complex are released during platelet activation and bind back on the cell surface via interaction with β_3 integrins thus permitting PDI to release the disulfide linkage between multimerin-1 and factor V to provide additional factor V for thrombin generation (Figure 3).

To test this hypothesis, factor Va levels were measured from washed platelets activated in the presence of increasing concentrations of isoquercetin (9). As isoquercetin concentration increased, factor Va levels decreased, correlating with levels of PDI inhibition and platelet-dependent thrombin generation. Factor Va levels were also measured from platelets in samples before and after oral administration of isoquercetin (1000 mg). Plasma samples from both time points were obtained and immunodepleted of plasma factor V. Platelet rich plasma was then reconstituted with healthy donor platelets, using both the FV-depleted and FV-replete plasma samples, and FVa levels were measured after activation. While isoquercetin inhibited factor Va generation in the FV-replete samples (26% reduction), the decrease in levels was enhanced in the FV-depleted samples (55% reduction), indicating a

greater effect of isoquercetin on factor Va generation from platelets. The addition of factor Va was able to fully restore thrombin generation after isoquercetin treatment. In summary, these data indicate that the inhibition of PDI with isoquercetin decreases platelet-dependent thrombin generation by decreasing the amount of available factor Va generated from platelets. Platelet factor V accounts for approximately 20% of the total factor V in circulation, but the absence of plasma factor V does not significantly reduce endogenous thrombin potential (78, 79). In contrast, patients which lack platelet factor V have a higher risk of bleeding, suggesting platelet factor V plays a more critical role in supporting hemostasis (80).

Clinical trial of isoquercetin to prevent venous thromboembolism in cancer

Due to the fortuitous discovery that the small molecule flavonoid, isoquercetin, inhibited PDI activity and thrombus formation in animal models, we are able to transition to later stage clinical trials in humans. In light of the potential intersection between PDI and cancer associated thrombosis, we initiated a multi-center clinical trial (CATIQ) to evaluate the potential efficacy of isoquercetin to prevent thrombosis in advanced cancer patients (NCT02195232). Eligible patients are required to have locally advanced or metastatic adenocarcinoma of the pancreas, metastatic colorectal or unresectable or metastatic non-small lung cancer. As the greatest risk for venous thromboembolism is the within the first few months following the initiation of chemotherapy (1), we limited enrollment only to those patients newly initiated first or second line chemotherapy. All patients are monitored for the development of venous thromboembolism that includes a bilaterally lower extremity duplex ultrasound at two months. These inclusion and exclusion criteria are similar to our prior phase II thromboprophylaxis study where the observed rate of venous thromboembolism in the non-anticoagulated arm exceeded 25% at two months (49). We anticipate completion of the phase II study in 2018.

Summary

The mechanisms contributing to the hypercoagulability of cancer are multi-faceted. PDI has been shown to play a key role in thrombosis and recent advances in our understanding of the extracellular substrates of PDI suggests considerable mechanistic overlap with the molecular drivers of cancer associated thrombosis. Clinical trials with anti-PDI small molecules are currently underway which may lead to novel therapeutics for the prevention and treatment of thrombosis in cancer patients.

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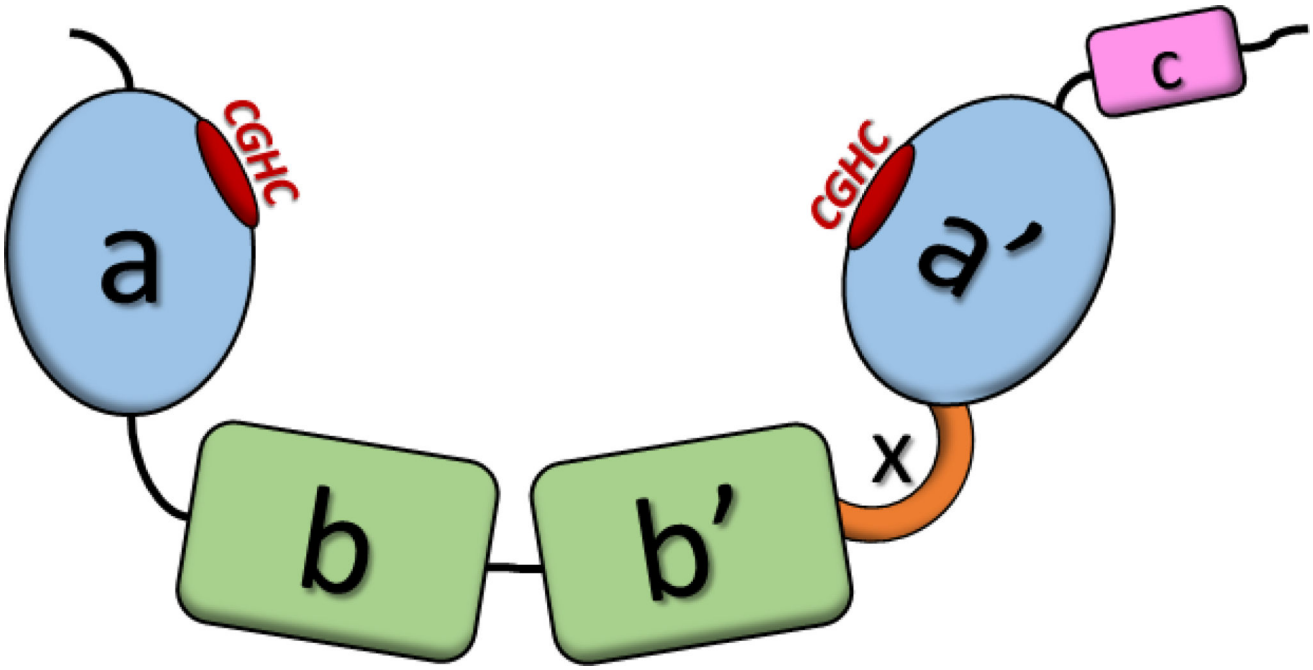

Figure 1. Schematic Representation of Protein Disulfide Isomerase

The a-b-b'-x-a'-c structure of PDI, with the CGHC active sites displayed in red.

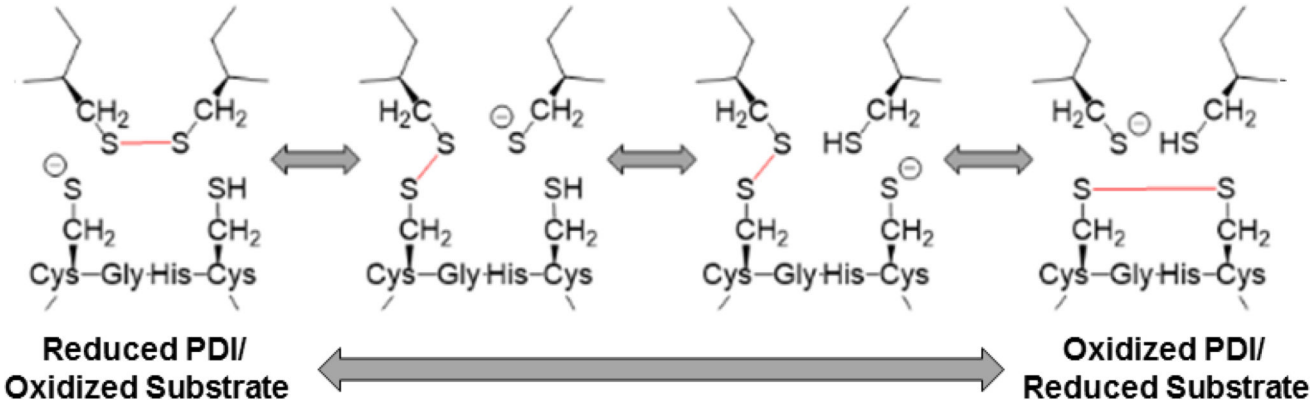

Figure 2. Reduction/Oxidation Mechanism of Protein Disulfide Isomerase

Shown is a reaction scheme diagramming the PDI active site transitioning between a reduced (*left*) and oxidized (*right*) state performing disulfide bond reduction (*left* to *right*) or oxidation (*right* to *left*) on a protein substrate. PDI active site (*lower*) and protein substrate (*upper*) are shown. Modified from (8)

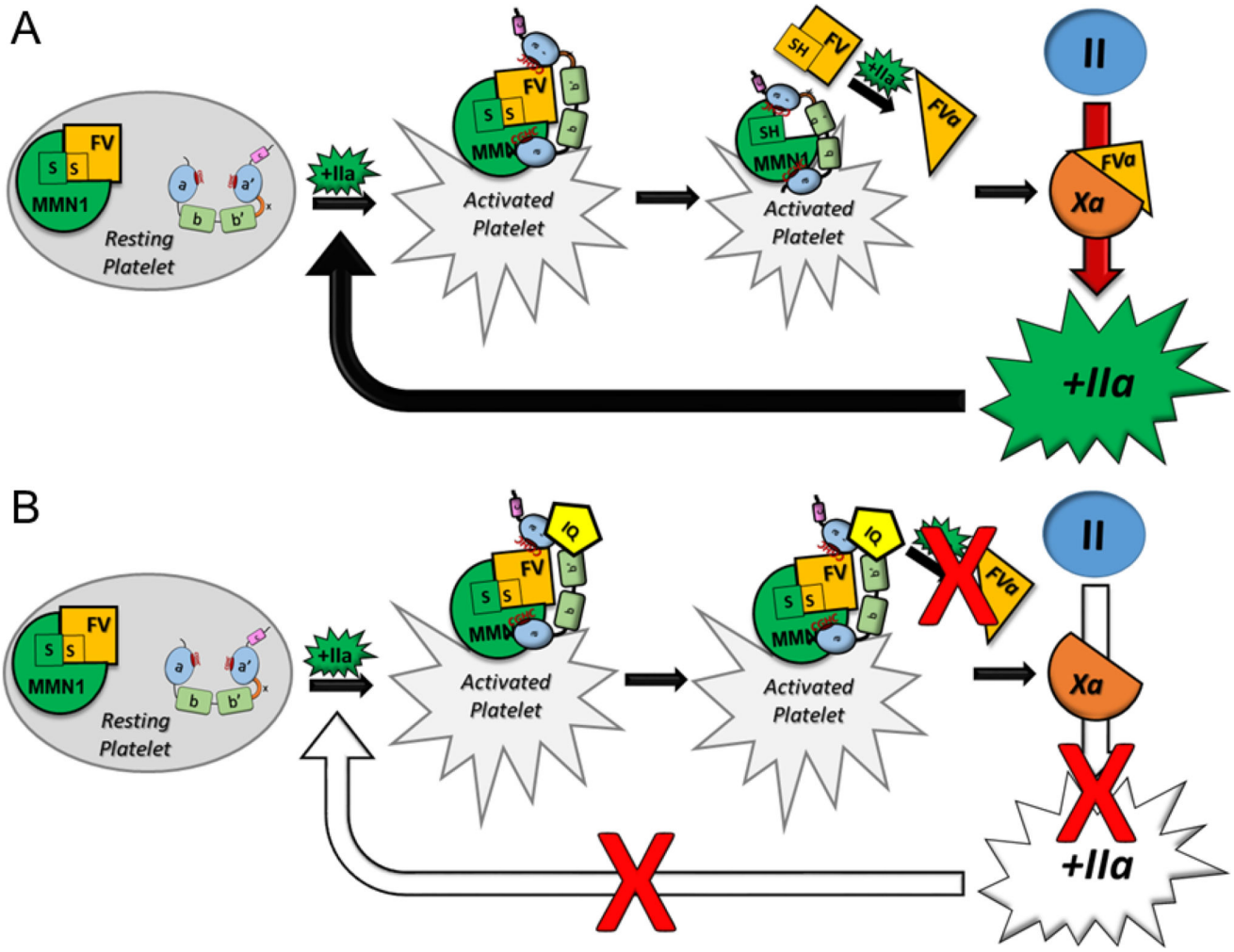

Figure 3. Inhibition of PDI with isoquercetin decreases thrombin-induced thrombin generation through FVa

Diagram of the proposed role of PDI in the activation of platelet factor V and the downstream generation of thrombin in the absence (Panel A) and presence (Panel B) of isoquercetin (9) MMN1: Multimerin-1; FV: Platelet Factor V; IIa: Thrombin; FVa: Factor Va; Xa: Factor Xa; II: Prothrombin; IQ: Isoquercetin; a-b-b'-a': PDI.

Table 1

Pharmacokinetics and PDI inhibition following ingestion of quercetin flavonoids (9)

Quercetin Flavonoid	Median Peak Conc. (μM)	AUC (μM hr/l)	PDI Inhibition (%)	Thrombin Gen. (%)
Quercetin Aglycone (500 mg)	0.8	3.8	N.S.	N.D.
Isoquercetin (500 mg)	4.22	18.3	N.S.	N.S.
Isoquercetin (1000 mg)	9.2	39.4	38% (p = 0.03)	51% (p = 0.0004)

N.S. = Not Significant (P>0.05)
N.D. = Not Determined