Dabigatran Attenuates the Binding of Thrombin to Platelets—A Novel Mechanism of Action

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Abstract

Background Thrombin is a multifunctional regulatory enzyme of the haemostasis and has both pro- and anticoagulant roles. It has, therefore, been a main target for drug discovery over many decades. Thrombin is a serine protease and possesses two positively charged regions called exosites, through which it is known to bind to many substrates. Dabigatran is a thrombin inhibitor and is widely used as an oral anticoagulant for the antithrombotic treatment of atrial fibrillation and venous thromboembolism. The mechanism by which dabigatran inhibits thrombin is the blockage of the active site, however, its effect on thrombin binding to its substrates has not been studied thoroughly and is thus poorly understood.

Material and Methods The effect of dabigatran on thrombin binding to platelets was evaluated by flow cytometry using fluorescently labelled thrombin and washed platelets. Further, to confirm the results we utilized modern techniques for biomolecular binding studies, microscale thermophoresis (MST) and surface plasmon resonance (SPR), which validated the results.

Results Dabigatran inhibited thrombin binding to platelets as analysed by flow cytometry. The inhibition was dose dependent with IC50 of 118 nM which was slightly lower than for inhibition of platelet activation and is close to the clinically relevant plasma concentration of dabigatran. MST and SPR also confirmed inhibitory effect of dabigatran on thrombin binding to platelets.

Conclusion Apart from blocking the active site, dabigatran also inhibits thrombin binding to platelets. Since thrombin has numerous functions beyond the cardiovascular system, this finding may have important implications.

Keywords
- thrombin
- dabigatran
- microscale thermophoresis
- platelets

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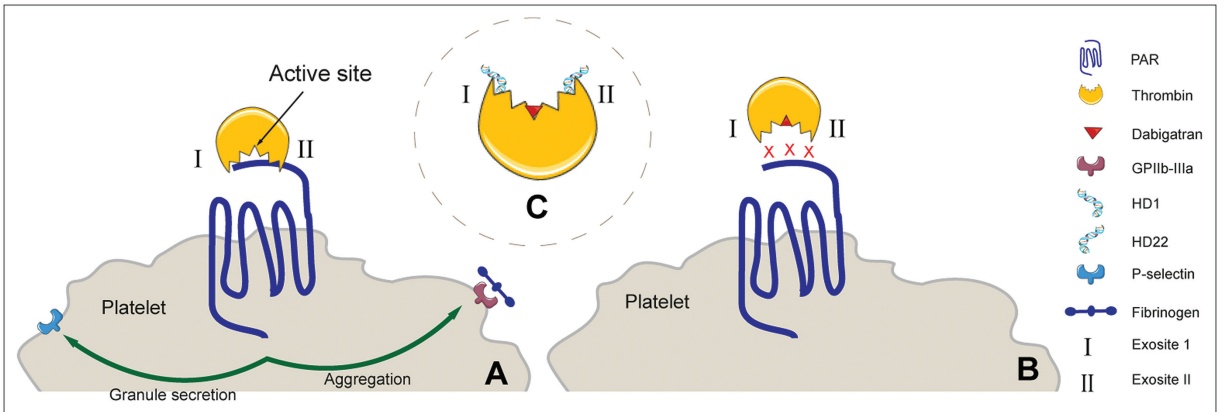
received
May 11, 2024
accepted after revision
October 9, 2024
accepted manuscript online
November 25, 2024

DOI https://doi.org/10.1055/a-2483-0107.
ISSN 0340-6245.

Georg Thieme Verlag KG, Oswald-Hesse-Straße 50, 70469 Stuttgart, Germany



Visual summary. Effect of dabigatran on thrombin binding to its receptor on platelet membrane. Thrombin binds to the protease activated receptor on the platelet membrane and cleaves the N-terminal to activate the receptor which leads to granule secretion and aggregation (A). In the presence of dabigatran, the active site inhibitor, thrombin, is not able to bind to the platelets (B). In an isolated system without platelets, dabigatran does not inhibit binding of thrombin exosite ligands to thrombin (C).

Introduction

Thrombin is not only the prima ballerina in the coagulation cascade but also a strong platelet activator binding to the protease activated receptors (PARs) 1 and 4.[1] The catalytic activity and specificity of thrombin are highly dependent on two intramolecular recognition sites located distant from the active site. These domains, designated as fibrinogen recognition site and heparin binding site, or exosite I and II, respectively, facilitate proteolysis by interacting with anionic surfaces on various substrates (►**Fig. 1**). Exosite II binds to heparin,[2] factors V and VIII,[3] PAR4,[4] and may also bind to glycoprotein Ib (GPIb)[4] on platelets. Exosite I is the binding epitope for bivalirudin, fibrinogen, thrombomodulin, hirudin, and PAR1 and contributes to binding to GPIb.[5] The exosites I and II on thrombin may be blocked by the DNA oligomers HD1 and HD22,[6] respectively. The binding of thrombin exosite I to PAR1 is mediated by a hirudin-like sequence immediately downstream of the tethered ligand domain in PAR1.[7] This sequence does not exist on PAR4. Instead, thrombin has been reported to have low affinity for the thrombin cleavage site of PAR4.[8]

Binding of thrombin to GPIb via exosite II facilitates the binding to PAR1,[4,9] whereas exosite I binds directly to the hirudin-like domain of PAR1.[10] Thrombin's strong affinity for PAR1 allows transient PAR1 signaling sufficient to elicit significant α-granule release and platelet aggregation in presence of therapeutic heparin concentration at physiological antithrombin levels. In contrast, inhibition of thrombin-induced PAR4-mediated platelet activation was complete and sustained.[11]

Oral anticoagulants are used worldwide to prevent thrombosis, for example, in patients with atrial fibrillation, venous thrombosis, and prosthetic heart valves. In recent years oral reversible direct thrombin and factor Xa inhibitors have substituted vitamin K antagonists to a large extent for most indications, with the exceptions of prosthetic heart valves and patients with thromboembolism and being triple positive for lupus anticoagulant. The first drug of this class in clinical use at present, approved in 2010, was dabigatran etexilate from Boehringer-Ingelheim which is a prodrug metabolized to its active form dabigatran by endogenous esterases.[12,13] Dabigatran is a direct, reversible thrombin inhibitor with half-maximal inhibitory concentration (IC50) of 9.3 nmol/L,[14] K_i 4.5 nmol/L.[15] Dabigatran inhibits both free and fibrin-bound thrombin and has no effect on thrombin binding to a glycoprotein Ibα peptide.[16] In this study we report that along with blocking the active site, dabigatran also inhibits binding of thrombin to platelets.

Materials and Methods

Reagents and Antibodies

H-D-Phe-Pro-Arg-chloromethylketone (PPACK) dihydrochloride, apyrase, PGI_2, 14-azido-3,6,9,12-tetraoxatetradecan-1-amine and disposable PD 10 desalting columns were from Merck, Darmstadt, Germany. Human α-thrombin was purchased from Haematologic Technologies, Vermont, USA. The DNA aptamers HD1 and HD22 and dibensocyclooctyne (DBCO) functionalized clickable aptamers were from Biomers.net (Ulm, Germany). Dabigatran was purchased from Selleckchem (Munich, Germany). Argatroban (Novastan) was purchased from FrostPharma, Danderyd, Sweden. Melagatran was purchased from MedChemTronica, Sollentuna, Sweden. Blood collection tubes were from Greiner Bio-One GmbH, Frickenhausen, Germany. Amine coupling kit, HBS-EP buffer, and acetate buffer were from Cytiva Europe GmbH, Uppsala, Sweden. Antibodies, anti-CD62P (P-selectin)-phycoerythrin (PE) or allophycocyanin (APC) with corresponding isotype antibodies, were from BD Biosciences, thrombin monoclonal antibody (MA1–43019) and Alexa Fluor™ 555 Antibody labeling kit were from Life Technologies Europe BV, and fluorescein isothiocyanate (FITC)-conjugated chicken antibodies directed toward human fibrinogen was provided by Diapensia AB (Linköping, Sweden, www.diapensia.se). Protein labeling kit RED-NHS 2nd generation and Monolith Premium Capillaries were from NanoTemper Technologies, Munich, Germany.

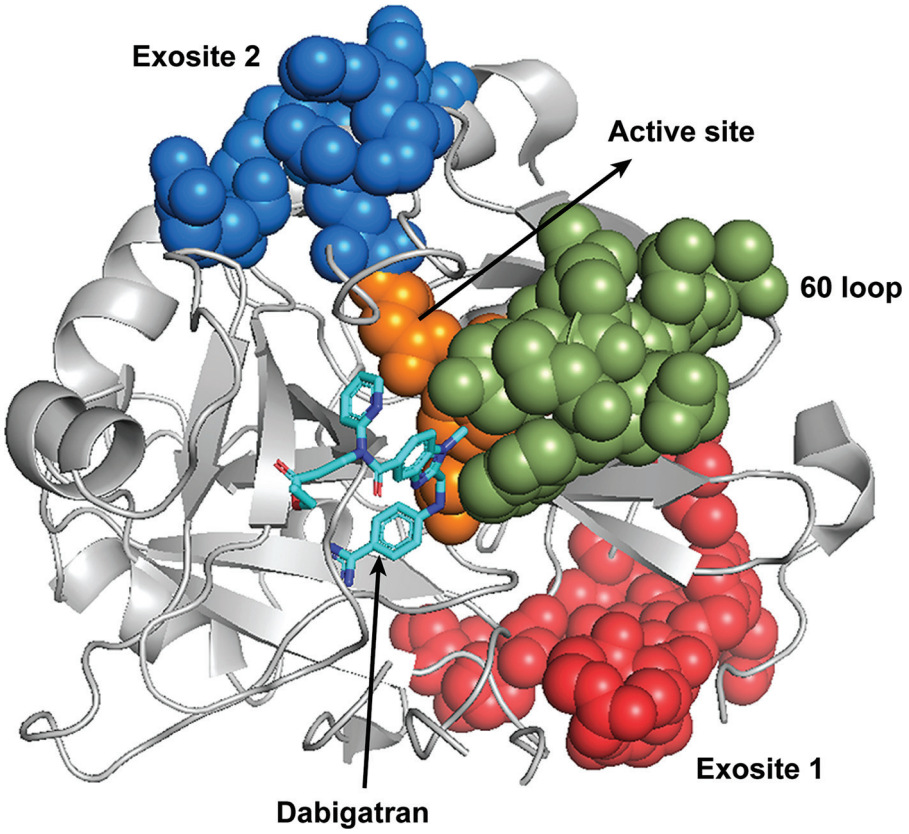


Fig. 1 Important structural components of human α-thrombin. Human α-thrombin bound to dabigatran in the active site pocket has been shown. The secondary structural components of thrombin are represented as cartoons and dabigatran as sticks. The structural units of thrombin like exosite 1 (red), exosite 2 (blue), active site (orange), and the 60 loop (green), which are considered important for binding, catalytic activity, and substrate specificity, are shown as spheres.

Blood Collection and Sample Preparation

Blood from healthy human volunteers was collected, after informed consent, in Vacuette®, 9 mL ACD-A tubes to prepare platelets rich plasma (PRP) and washed as reported previously.[17] Briefly, the blood was centrifuged at 150 × g for 15 minutes at room temperature (RT) to get PRP and then again at 480 × g after addition of 1 U/mL apyrase and 100 nM PGI2 to pellet the platelets. The plasma phase was carefully replaced by Krebs Ringer glucose (KRG; 20 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 1.7 mM KH_2PO_4, 8.3 mM Na_2HPO_4, 10 mM glucose, pH 7.3) buffer supplemented with apyrase and PGI2 and platelet count was adjusted after recalcification. The platelet samples were incubated for 30 minutes at RT after isolation before usage in any experiments. The collection of blood samples from healthy donors was approved by the local ethics committee in Linköping, Sweden, decision No. 2012/382–31.

Chromogenic Thrombin Substrate Assay

Thrombin activity was measured using the Chromogenix S-2238™ substrate (DiaPharma, West Chester, USA), according to the manufacturer's instructions using Enspire multimode plate reader (Perkin Elmer, Sollentuna, Sweden) to evaluate and compare the effect of labeling of thrombin and the treatment of PPACK (covalent inhibitor of active site). Thrombin was labeled using protein labeling kit RED-NHS 2nd generation according to the manufacturer's instructions. For PPACK treatment, thrombin was first incubated with double molar concentration of PPACK dihydrochloride for 30 minutes and unbound PPACK was removed using PD10 chromatography column. Briefly, unlabeled, labeled, and PPACK thrombin were diluted in 50 µL buffer (50 mM Tris, with 0.2% bovine serum albumin (BSA) pH 8.3) and incubated at 37°C for 4 minutes. The reaction was started by mixing 50 µL of S-2238 substrate and incubated at 37°C for 3 minutes. The final concentration of thrombin and PPACK in the reaction mixture was 0.16 µM. The reaction was stopped with the addition of 25 µL of 20% acetic acid, and the absorbance was measured at 405 nm. The data was calculated using one-way ANOVA followed by Bartlett's test using GraphPad Prism version 10.0.2 for Windows, GraphPad Software, Boston, Massachusetts, USA, www.graphpad.com.

Flow Cytometry

Binding of thrombin to platelets was analyzed by flow cytometry using washed platelets (300×10^9/L). RED-NHS-labeled thrombin (0.5 µM) was used to determine the binding of thrombin to platelets in the presence or absence of various inhibitors. To study the contribution of the thrombin exosites, DNA aptamers HD1 (exosite 1 inhibitor) or HD22 (exosite 2 inhibitor) was used at 1 µM concentration as previously reported.[4] To study the role and contribution of the thrombin active site, four different

active site inhibitors dabigatran (318 nM), argatroban (318 nM), melagatran (1 μM) or PPACK was used. For experiments with PPACK, it was prepared as described in above section and 0.5 μM PPACK thrombin labeled with RED-NHS 2nd generation was compared with RED-NHS 2nd generation labeled thrombin. HD1 and HD22 (1 μM) were also used with PPACK thrombin. Platelet activation was assessed by using anti-human-CD62P (P-selectin, PE or APC labeled and 1.25 μg/mL) and FITC-conjugated anti-human fibrinogen chicken antibody (1 μg/mL). Corresponding isotype antibodies were used as a control. Briefly, washed platelets were incubated with various combinations of thrombin, antibodies, and inhibitors in HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1 mg/mL bovine serum albumin, and 20 mM HEPES, pH 7.40) and dimethyl sulfoxide (DMSO) (final concentration 0.5% v/v) in the dark at RT for 10 minutes. Samples were then diluted 1:10 with HEPES buffer and analyzed by flow cytometry on a Gallios™ flow cytometer (Beckman Coulter Inc., Fullerton, CA, USA). The median fluorescence intensity (MFI) was used for analysis. MFI of thrombin without any inhibitors was set to 100% and other results were normalized against it and presented as relative fluorescence intensity (RFI). These experiments were also performed with unlabeled thrombin using and Alexa Fluor™ 555 labeled thrombin monoclonal antibody which resulted in similar outcome. The data was calculated using one-way ANOVA followed by Brown-Forsythe test using GraphPad Prism. For IC50 analysis, washed platelets were "spiked" with dabigatran to the final concentrations of 2 to 600 nM and 0.5% (v/v) DMSO in all samples in HEPES and analyzed as described above. The logarithm of the dabigatran concentration was plotted against the response (expressed in MFI) and the IC50 values were calculated using the GraphPad Prism's built-in equation "Dose-Response - Inhibition" and "log(inhibitor) vs. response - Variable slope (four parameters)." The curves are fit using nonlinear regression with the drug concentrations transformed to a logarithmic scale.

Microscale Thermophoresis

Microscale thermophoresis (MST) was used to analyze the interaction of thrombin with platelets. Thrombin was labeled using NHS-Red 2nd generation and the concentration of thrombin to be used was determined by the degree of fluorescence using pretest analysis of the Monolith NT.115 i software (NanoTemper) and the average concentration used was 55 nM. The platelet concentration used was approximately $1{,}800 \times 10^9$ /mL and 1,276 PAR1,[18] 539 PAR4,[19] and 25,000 GPIb[20] receptors per platelet were used for the calculation of receptor concentration. Thus, the highest ligand concentration of platelets in the MST experiment was 80 nM which was diluted 16 times serially and evaluated for binding to constant thrombin concentration to get the binding curve. Briefly, washed human platelets (highest concentration $1{,}800 \times 10^9$ /mL) were diluted serially 16 times using PBS + 0.005% NP40 buffer, pH 7.4 and mixed with equal amount (v/v) of RED-NHS-labeled thrombin (55 nM) diluted in the same buffer in the presence or absence of various inhibitors like HD1 (1 μM), HD22 (1 μM), dabigatran (318 nM), or argatroban (318 nM) and transferred to the Monolith Premium Capillaries. The capillaries were placed in the Monolith instrument and thermophoresis was measured at RT with medium MST power and 80% LED intensity. The data was analyzed using Monolith NT.115 i software. PPACK thrombin was used instead of regular thrombin for the experiments of PPACK. To investigate the effect of dabigatran (318 nM) on thrombin exosites, binding of DNA aptamers HD1 (highest concentration 1 μM) or HD22 (highest concentration 1 μM) or heparin (highest concentration 1 U/mL) to thrombin in a buffer without platelets was evaluated. As described above the ligand (HD1/HD22/heparin) was diluted 16 times serially and mixed with equal amount (v/v) of RED-NHS-labeled thrombin (55 nM) in the presence or absence of dabigatran 318 nM or 3,180 nM and binding was evaluated using MST. Data analyses were performed using the MO. Affinity analysis software (NanoTemper) and normalized display of fraction bound versus ligand concentration, or ΔFnorm (%) versus ligand concentration were used. For plots with fraction bound, ΔFnorm values of the curve are divided by curve amplitude resulting in range of values between 0 and 1. To compute change in normalized fluorescence ΔFnorm (%) the baseline Fnorm values were subtracted from all data points. For the statistical calculations shown in ►**Fig. 2**, the binding curves from the MST experiments are fitted using the one phase decay equation in the exponential model using GraphPad Prism.

Surface Plasmon Resonance

Surface plasmon resonance (SPR) was used to evaluate the effect of dabigatran on the binding of thrombin to DNA aptamers HD1 and HD22 using a Biacore 3000 system. HD1 and HD22 were immobilized in multiple steps in separate channels on a CM5 sensor chip (Biacore, Uppsala, Sweden). Briefly, 75 μL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimid (NHS) were injected at a flowrate of 5 μL/min; then 90 μL 10 mM 14-azido-3,6,9,12-tetraoxatetradecan-1-amine in pH 11 potassium dihydrogen phosphate/NaOH buffer was injected at a flowrate of 1 μL/min. This was followed by an injection of 60 μL ethanol amine at 10 μL/min. Finally, 90 μL DBCO functionalized aptamer at a concentration of 42 μg/mL in pH 4 acetate buffer was injected at 1 μL/min. The DBCO moiety on the aptamer will bind to the azide via click chemistry. Thrombin was used at 100 nM as maximum response was obtained at this concentration. It was mixed with three molar equivalents of dabigatran dissolved in DMSO or the corresponding volume of DMSO and injections of 30 μL were performed at 10 μL/min, repeated three times. The data was analyzed using MATLAB (R2023b). The data was filtered for noise using the smooth function with a 5-point moving average over areas where the needle movement of the Biacore induced additional noise. Spike artifacts induced by the large jump in refractive index upon start and finish of injection

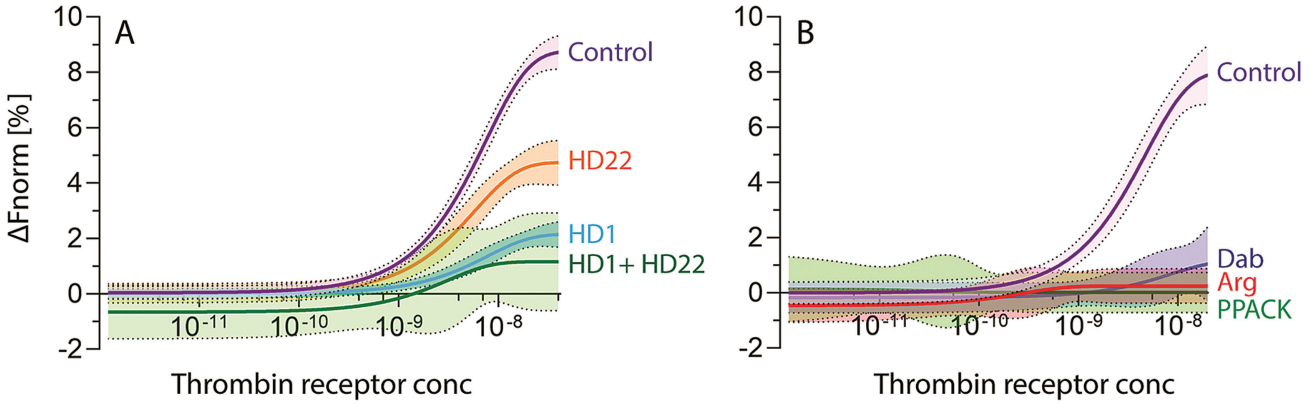


Fig. 2 Inhibition of thrombin binding to platelets as measured by microscale thermophoresis (MST). Binding of thrombin to the platelets and the effect of inhibitors were evaluated using MST. In this normalized display, the change in normalized fluorescence (ΔFnorm) of thrombin was plotted against the concentration (conc) of the thrombin receptors on platelets in log scale. Binding curve of thrombin to platelets without any inhibitors (control) is compared with the binding of thrombin (55 nM) to platelets in the presence of (**A**) thrombin exosite specific inhibitors HD1 (1 μM) or HD22 (1 μM) or both and (**B**) thrombin active site inhibitors dabigatran (318 nM), argatroban (318 nM), or PPACK (55 nM). The data was calculated using the one phase decay equation in the exponential model using GraphPad Prism. The results shown are mean with standard deviation (SD) represented as shadow outlines around the binding curves, where number of blood donors (n) ≥5.

were removed using the Hampel function. Baseline correction was done using a straight line and an asymmetric truncated quadratic function using script by Mazet.[21] The used MATLAB scripts and raw data are available upon request.

Results

Binding of Thrombin to Platelets and Effect of Inhibitors

The binding of thrombin to platelets was determined using fluorescently labeled thrombin (0.5 μM) and washed platelets, measured by flow cytometry. The labeling of thrombin had no significant effect on the enzymatic activity as confirmed by the chromogenic substrate assay. Thus, compared with unlabeled thrombin (0.16 μM), RED-NHS-labeled thrombin (0.16 μM) retained 94% activity whereas PPACK-treated thrombin (0.16 μM) showed 0.5% activity (►**Supplementary Fig. S1**, available in the online version). When considering total thrombin binding to platelets in the absence of any inhibitors as 100%, HD1 (1 μM) and HD22 (1 μM) inhibited thrombin binding to platelets by 43 and 32%, respectively (►**Fig. 3**). The combination of both aptamers inhibited binding completely, as expected. In these experiments, dabigatran (318 nM), a thrombin active site inhibitor, was used as a positive control that inhibits enzymatic activity. Surprisingly, along with inhibiting the enzymatic activity, dabigatran also blocked thrombin binding to the platelets. To confirm the efficacy of the inhibitors and the viability or reactivity of the platelets used for the experiments, platelet activation/function markers like P-selectin exposure and fibrinogen binding to platelet were also measured, which showed similar trends, complementing the thrombin binding results (►**Fig. 3**). The result on binding indicated that the blockage of the active site also blocks the binding of thrombin exosites I and II. Thus, the active site, along with the exosites, plays an important role in binding thrombin to platelets. To confirm this hypothesis, we used argatroban (318 nM) and melagatran (1 μM) inhibitors, similar in size, nature, and function to thrombin, which showed similar characteristics, i.e., inhibition of thrombin binding to platelets along with blocking the active site

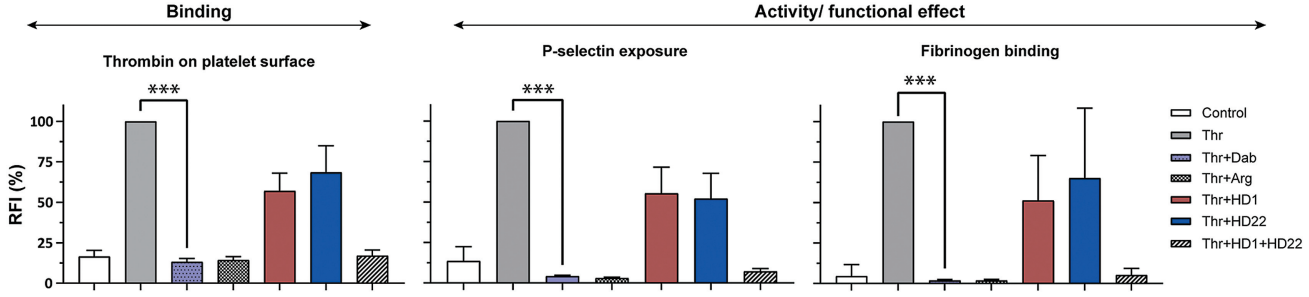


Fig. 3 Inhibition of thrombin binding to platelets and effects on platelet activation by flow cytometry. Thrombin binding to platelets and its effect on platelet activation have been shown in the presence and absence of various inhibitors of the thrombin exosites and the active site. Platelet activation markers P-selectin exposure and fibrinogen binding were measured to confirm inhibitors' efficacy and platelet reactivity. Negative control test without thrombin has been compared and shown here as control. Median fluorescence intensity of thrombin without any inhibitor has been considered as 100% and the relative fluorescence intensity (%) to thrombin without any inhibitor has been shown as bar graph. The data was calculated using a one-way ANOVA test and results shown are the mean with standard error of mean (SEM) where number of blood donors (n) ≥5. RFI, relative fluorescence intensity.

(►**Fig. 3** and ►**Supplementary Fig. S2**, available in the online version). PPACK, another active site inhibitor, also blocked thrombin binding to platelets, albeit not completely like dabigatran (►**Supplementary Fig. S3**, available in the online version). PPACK, however, is structurally different from dabigatran and is a covalent inhibitor of the active site. To rule out any interference of fluorescence labeling on thrombin binding to platelets, the results obtained with labeled thrombin were confirmed by unlabeled thrombin and an antithrombin monoclonal antibody (►**Supplementary Fig. S4**, available in the online version), which showed a similar trend and strengthened our findings. Similarly, the effect of pre-incubation of dabigatran to thrombin was also evaluated, which showed that dabigatran-inhibited thrombin binds to the platelets irrespective of pre-incubation status (►**Supplementary Fig. S5**, available in the online version).

Binding Analysis Using Microscale Thermophoresis

We used MST to validate the dabigatran effect on thrombin binding to platelets. Complementing the findings observed with flow cytometry, HD1 (1 µM) and HD22 (1 µM) inhibited thrombin (55 nM) binding to platelets, but not completely (►**Fig. 2A**). However, dabigatran (318 nM) showed complete inhibition of binding using MST (►**Fig. 2B**). To further confirm the effect of blockage of active site on overall binding, we used argatroban (318 nM) as well as the covalent inhibitor of active site, PPACK (55 nM), both of which inhibited binding of thrombin to the platelets.

Effect of Dabigatran on Thrombin Exosites

To further investigate the mechanism by which dabigatran block thrombin binding to platelets, we assessed the effect of dabigatran binding to thrombin active site on the binding of HD1 and HD22 to the exosites. This was achieved by evaluating the binding of thrombin to HD1 and HD22 in the presence and absence of dabigatran in a pure system without platelets using two biophysical methods, microscale thermophoresis[22,23] and SPR.[24]

MST did not show any effect of the presence or absence of the dabigatran (318 nM) on thrombin binding to HD1 (1 µM; ►**Fig. 4A**) or HD22 (1 µM; ►**Fig. 4B**) or heparin (1 U/mL) (►**Fig. 4C**). Additionally, increasing the concentration of the dabigatran 10 times (3,180 nM) also had no effect on exosite ligands binding (►**Supplementary Fig. S6**, available in the online version).

SPR also did not indicate any effect of dabigatran binding to thrombin on interaction with either HD1 (►**Fig. 5A**) or HD22 (►**Fig. 5B**). The shape and intensities (response signal) of the sensograms with and without dabigatran were very similar. HD22 initially showed a minor trend where the thrombin–dabigatran complex might have a slightly higher affinity to HD22; however, increased injection volume (60 µL) showed no differences in the sensograms (►**Fig. 5C**). The signal intensity of the SPR experiments were on a relatively low level, which is consistent with other published data on thrombin in SPR.[25,26] To allow closer inspection of the obtained sensograms individual plots can be found in ►**Supplementary Fig. S7** (available in the online version).

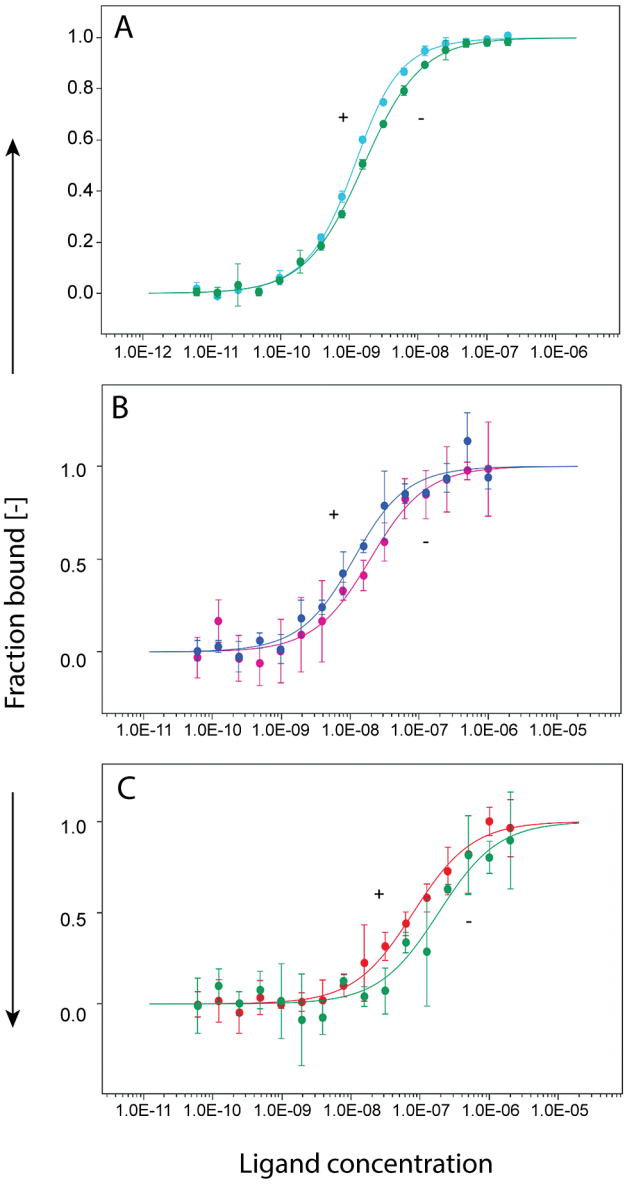


Fig. 4 Effect of dabigatran on thrombin binding to exosite ligands by microscale thermophoresis (MST). The effect of dabigatran on exosite ligands binding to thrombin without platelets was analyzed by MST. Binding of NHS-labeled thrombin (55 nM) to exosite 1 ligand HD1 (**A**; highest concentration 1 µM), exosite 2 ligand HD22 (**B**; highest concentration 1 µM), or another exosite 2 ligand heparin (**C**; highest concentration 1 U/mL) in the presence (+) or absence (−) of dabigatran (318 nM) was measured by MST. In this normalized display, the change in normalized fluorescence (fraction bound) is plotted against the concentration of the ligand in log scale. All the results shown are mean with standard deviation (SD) where $n \geq 3$.

Dabigatran-Mediated Inhibition of Binding is Dose Dependent

To find out whether the dabigatran effect on thrombin binding is dose dependent or not, IC50 analysis was performed. Dabigatran concentrations from 4 to 600 nM were used for these experiments and their effect on thrombin binding and its

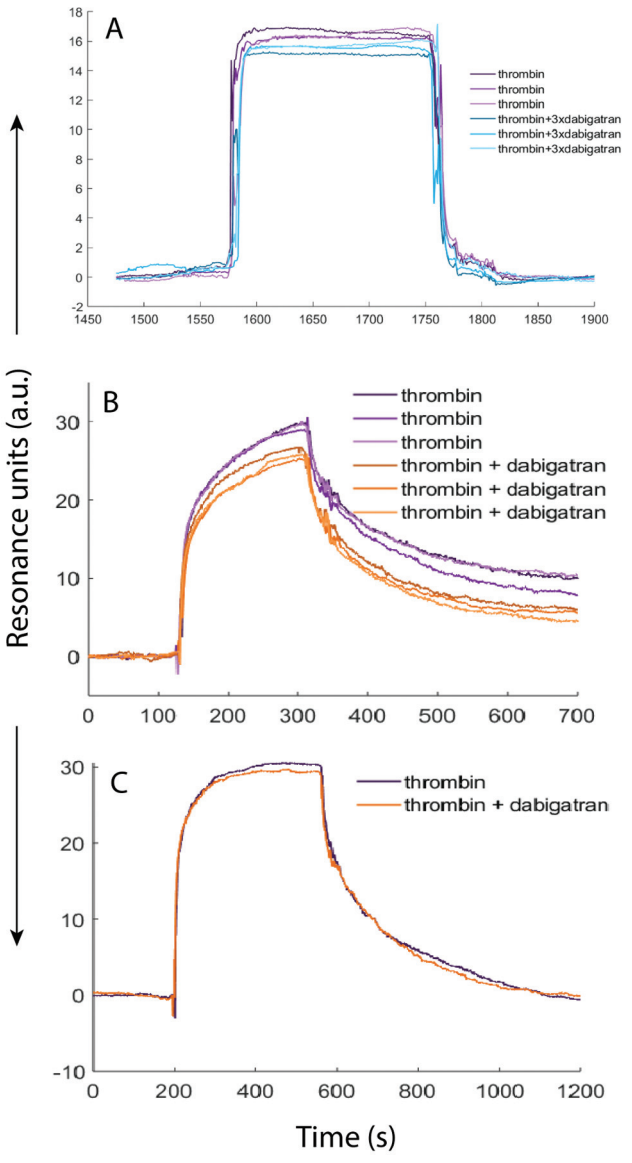


Fig. 5 Surface plasmon resonance (SPR) sensogram of thrombin injected onto an SPR chip with a carboxylated dextran with immobilized aptamers. The interactions between thrombin exosites and DNA aptamers were analyzed. The aptamers HD1 and HD22 are immobilized in separate flow channels to an increase in resonance units (RU) of 971 and 1,395, respectively. Thrombin was injected at a concentration of 100 nM in the presence and absence of 300 nM dabigatran. (**A**, **B**) Sensograms of thrombin in the presence and absence of dabigatran injected over immobilized HD1 and HD22, respectively. To further evaluate the interaction between thrombin and HD22 the injection volume was doubled to 60 uL (**C**). Overall, no significant differences between thrombin in the presence and absence of dabigatran can be observed. Although response curves are of different shapes for the two aptamers, the addition of dabigatran does not alter the response.

functional activity indicators P-selectin exposure and fibrinogen binding was evaluated using flow cytometry and fluorescently labeled thrombin and the respective antibodies. We found the effect of dabigatran to be dose dependent and that the IC50 for inhibition of binding was 118 nM. The IC50 for inhibition of P-selectin exposure and fibrinogen binding was 126 and 185 nM, respectively (►**Fig. 6**).

Discussion

In this study we have shown that the thrombin active site inhibitor dabigatran effectively inhibits thrombin binding to all platelet thrombin receptors. The binding was analyzed using flow cytometry and further characterized by microscale thermophoresis and surface plasmon resonance.

Most studies analyzing affinity or binding of thrombin to platelets used radiolabeled thrombin and were performed more than 30 years ago; at that time the platelet thrombin receptors PAR1 and 4 were not yet identified.[27,28] Although the use of radiolabeled proteins to analyze the interaction between two biomolecules has been a classic method, it is not practical due to obvious health hazards and strict regulations. Thus, to confirm our findings, we used two modern biophysical techniques specialized on the measurement of interactions between biomolecules, MST and SPR. Both the techniques are safe and have been successfully applied to measure the affinity between biomolecules. MST can analyze biomolecular interactions in an immobilization-free system where one of the binding partners is fluorescently labeled and its motion in a micro temperature gradient is measured. This technique is particularly useful for this study as live platelets can be used as one of the partners to analyze the binding characteristics. On the other hand, SPR is a standard and valuable method specialized in analyzing interactions between molecules with respect to both affinity and kinetics.[29,30]

It is a possibility that the labeling of thrombin with a fluorescent dye might affect its ability to bind or function properly; however, labeled thrombin's unaffected ability to cleave S-2238 chromogenic substrate (►**Supplementary Fig. S1**, available in the online version), eliciting P-selectin exposure, fibrinogen binding, and similar results obtained when using antithrombin antibody (►**Supplementary Fig. S4**, available in the online version) rule out this suspicion. Further, dabigatran quenching the fluorescence signal or affecting exosite affinity can also be excluded by unaffected binding of exosite ligands to the thrombin in the presence of dabigatran which is demonstrated with both MST and SPR (►**Figs. 4** and **5**). Although, PPACK is a potent inhibitor of the active site, it showed only 50% reduction in the binding when evaluated by flow cytometry (►**Supplementary Fig. S3**, available in the online version), which has also been observed before.[31] Further, the remaining approximately 50% binding was not only due to the exosites, as blockage of the exosites by aptamers could not abolish the binding to platelets completely (►**Supplementary Fig. S3**, available in the online version). However, when analyzed by MST, no binding could be detected to platelets which points toward the differences in techniques but strengthened the finding of this study as dabigatran showed inhibition irrespectively. The inhibition of thrombin by dabigatran has been shown to be the active site blockage with a reported K_d of 10 nM[15] for thrombin-induced aggregation of gel-filtered platelets; however, to the best of our knowledge, the effect of dabigatran on binding of thrombin exosites has not been reported. The IC50 values were obtained using flow cytometry and washed platelets. IC50 for platelet activation markers P-selectin exposure

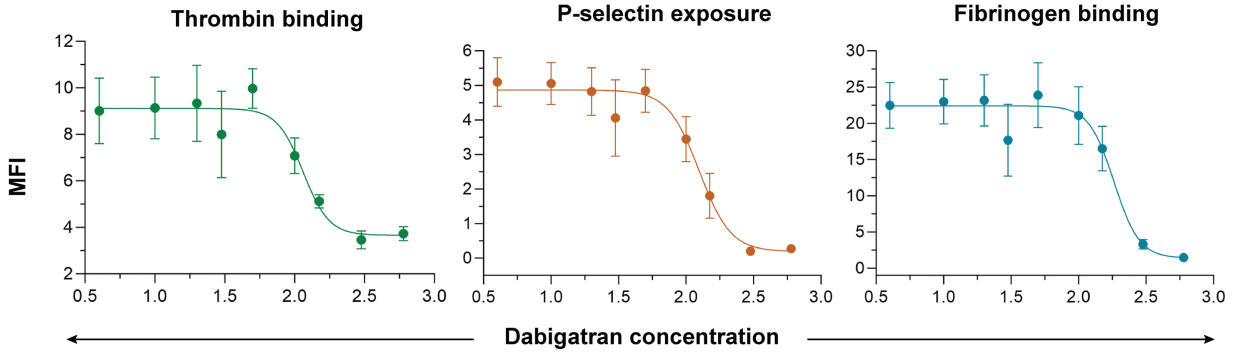


Fig. 6 IC50 of dabigatran for thrombin binding to platelets by flow cytometry. The inhibition potential of dabigatran for thrombin binding to platelets and platelet activation was assessed using flow cytometry. Increasing concentration of the dabigatran (2–600 nM) as log transformation of concentrations to base 10 is plotted against the median fluorescence intensity (MFI) of thrombin binding, P-selectin exposure, or fibrinogen binding and IC50 values were calculated as described in the methods. The results shown are the mean with standard error of mean (SEM) where number of blood donors, $n \geq 5$.

(122 nM) and fibrinogen binding (185 nM) was much higher than the reported 10 nM[15] using aggregometry; however, the same study has reported the IC50 of 560 nM when a thrombin generation assay was used. Similarly, another study[29] has reported the IC50 of 2 µM using thrombin generation method indicating a large variation in the IC50 values obtained, probably dependent on the system and methodology used. However, the IC50 values we obtained were well within the range of reported drug concentrations in patients where the peak concentration in elderly healthy subjects was approximately 540 nM.[13,32]

Our results demonstrate that the small molecules dabigatran, argatroban, and PPACK binding to the active site of thrombin strongly attenuate the binding of thrombin to the surface of platelets, in addition to the well-known inhibition of the enzymatic activity. Our findings are in good agreement with an earlier study where numerous important interactions between PAR1 N-terminus uncleaved fragment and thrombin active site was shown by crystallography[10] and, thus, any disturbance in these interactions can lead to destabilization of binding. Further, thrombin active site occupancy by a ligand has been reported to decrease solvent accessibility of the regions in vicinity of the active site, induce backbone dynamics changes, and inhibit exosite interaction to its ligands.[16,33,34] Among these, the most relevant study supporting our observations has shown that dabigatran attenuates the binding of various substrates to exosite 1 or 2 of thrombin; however, surprisingly in their study argatroban enhanced the exosite binding of its substrates.[16] Contrary to these, it has been shown that dabigatran does not affect thrombin interaction with fibrinogen, which indicates that dabigatran-bound catalytically inactive thrombin may still be able to interact with its substrates via exosite 1. This observation has also been reported earlier.[35,36] However, in these studies cells other than platelets were used and the effect that they have measured, i.e., PAR1 expression on cell surface after prolonged incubation or destabilization of the endothelial barrier, is not relevant in case of the platelets. We also investigated the effect of dabigatran on binding of DNA aptamers to thrombin exosites by MST and SPR, which showed that in the absence of platelets, dabigatran does not inhibit ligand binding to thrombin exosites. Although the shape of the interaction curve for thrombin and HD1 is not a typical curve obtained in SPR measurements it is in good agreement with results published in this field. Yeh et al studied the binding of thrombin and fibrinogen.[16] Wang et al[37] studied the interaction between thrombin and berberin. The resulting curves in both publications were of similar shapes as in this study. The reason for the rapid binding and releasing observed in these curves would be interesting to investigate but is outside of the scope of this study. As SPR is only measured at one thrombin concentration it is not possible to infer detailed conclusions on the possible effect on kinetics that dabigatran might exert. The shapes and intensities of the curves in both presence and absence are very similar and therefor the binding of exosites I and II is not altered to a degree where it may explain the results where dabigatran greatly decreases the binding of thrombin to platelets.

Studies using isolated systems of thrombin with its natural or synthetic substrates do not have the similar outcome when cells or platelets have been involved. Thus, our results indicate that dabigatran binding does not affect exosite structure per se to a major extent, allowing small free molecules like peptides and DNA aptamers or even larger molecules like fibrinogen to bind. Dabigatran still induces enough dynamic change to affect its binding to the complex receptor system like platelets. Structural, biochemical, and mutagenesis analysis suggests that in most cases, both exosites are involved in binding and/or catalytic functions, either directly via the substrate interaction or indirectly by facilitating the binding by interacting with the cofactor.[38] PARs are known to have a crosstalk between same or other types of receptors and show diversified interactions and functions. Dimerization is, therefore, a common phenomenon in PAR activation.[39,40] GPIb acts as a cofactor for thrombin to bind to PARs on the platelets[41] and, similarly, PAR1 acts as a facilitator for PAR4 cleavage by thrombin.[42] The observation of various homo- or hetero-dimerization of receptors on platelet surface like PAR1/PAR1, PAR4/PAR4,[43,44] PAR1/PAR4,[43] and PAR4/P2Y12[45] supports the hypothesis that thrombin binding to platelets is complex, possibly requiring all three structural components together, which may explain the dabigatran effect observed in this study. One of the

limitations of this study is the lack of knowledge on how dabigatran inhibited thrombin would react with ligands other than platelets under physiological conditions. The other limitation is that the exact mechanism behind inhibition elicited by dabigatran on the binding of thrombin to platelets is unclear at present. Two insertion loops near the active site cleft, known as the 60-loop and the γ loop (►**Fig. 1**), have been reported to play key roles in determining the substrate and inhibitor specificity as well as catalytic activity of thrombin[46–48] and might have a role in this mechanism. However, it will have to be evaluated structurally which is beyond the scope of the current study.

The inhibition by dabigatran of thrombin binding to platelets may contribute to its antithrombotic effect. So far dabigatran is the only direct oral anticoagulant drug reported to be associated with less ischemic stroke compared with warfarin,[12] although there are no randomized clinical trials comparing DOACs head-to-head. However, the dual mechanism inhibiting thrombin activation of platelets may increase bleeding risk; possibly the effect would be most important at lower concentrations of dabigatran. Interestingly, in a large Swedish registry study, patients with nonvalvular atrial fibrillation on reduced dose of apixaban had lower risk for major bleeding hazard ratio (HR) of 0.62 (95% CI 0.44–0.88) in comparison with patients on a reduced dose dabigatran. There was no such difference for standard doses.[49]

Furthermore, thrombin is probably the most versatile and broad-spectrum enzyme in the cardiovascular milieu, and it has functions that spans from hemostasis, coagulation, inflammation, to immunity, and for all these functions its ability to bind to its substrates is a primary requirement. Thus, the inhibitory effect by dabigatran on thrombin binding to PARs may have wider implications.

What is known about this topic?

- Dabigatran is a small molecule chemical inhibitor of thrombin and binds to the active site to block its function.
- Thrombin binds to the protease-activated receptors on platelet membrane via its exosites and then the active site cleaves the N-terminal to activate the receptor.
- PPACK is a small peptide inhibitor of the thrombin and binds to the active site.

What does this paper add?

- Dabigatran blocks the binding of thrombin to the platelets completely.
- Along with the function, thrombin active site also plays major role in the binding.

Authors' Contribution

A.M. and T.L. conceived the study and designed the flow cytometry and MST experiments. A.M., T.H., A.P.K., and A.A.H. performed MST experiments. A.P.K. and E.A. performed flow cytometry experiments. A.R. and K.U. designed the SPR experiments, analyzed the data, and prepared the figures. A.R. performed the SPR experiments. A.M. and T.L. wrote the manuscript. All authors have read, commented, and approved the final version of the manuscript.

Funding

This study was supported by grants with TL as main and AM as co-applicant from the Swedish Research Council no. 2020–01002 and the Swedish Heart-Lung foundation no. 2019037022 and 20220205 and Swedish Research Council Grant no. 2019–02409 to KU as main applicant, TL was one of the co-applicants.

Conflict of Interest

T.H. is an employee of Nanotemper Technologies GmbH. T.L. is the owner of Diapensia Other authors declare no conflicts of interest.

Acknowledgment

We are thankful to all the blood donors for the study and very grateful to Dr. Dean Derbyshire of ProLinc facility, Linköping University for his valuable discussions and inputs about the structural features of thrombin and its interactions.

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