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Effects of Btk-inhibitors with varying selectivity and reversibility on platelet aggregation

vorgelegt von
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Affidavit

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I hereby declare that the submitted thesis entitled:

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……

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I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

München, 22,06,2022 — Rundan Duan

place, date — Signature doctoral candidate

This is dedicated to my family, for their unwavering love and support, and to my hometown, which always gives me the direction

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List of abbreviations

AA	arachidonic acid
ADP	adenosine diphosphate
APTT	activated partial thromboplastin time
ATP	adenosine triphosphate
Btk	Bruton’s tyrosine kinase
BTKi	Bruton’s tyrosine kinase inhibitor(s)
COX	cyclooxygenase
CSU	chronic spontaneous urticaria
FcγRIIA	Fc receptor CD32a
GAGs	glycosaminoglycans
GPIb	glycoprotein Ib
GPVI	glycoprotein VI
HIT	Heparin-induced thrombocytopenia
IgG	immunoglobulin G
ITAM	immunoreceptor tyrosine-based activation motif
ITP	immune thrombocytopenic purpura
LAT	Linker of Activation of T cells
MEA	multiple electrode aggregometry
PAR	protease-activated receptor
PF4	platelet factor 4
PFA	platelet function assay
PH	pleckstrin homology
PIP_2	phosphatidylinositol bisphosphate
PIP_3	phosphatidylinositol trisphosphate
PKC	protein kinase C
PLCγ2	phospholipase Cγ2

PRP	platelet rich plasma
PT	prothrombin time
SH	SRC kinase homology
SPA	spontaneous platelet aggregation
TH	Tec homology
TRAP	thrombin receptor-activating peptide
TXA_2	thromboxane A2
VITT	vaccine-induced immune thrombotic thrombocytopenia
vWF	von Willebrand factor
XLA	X-linked agammaglobulinemia

1. List of publications

1.1 Publications included in this work:

Paper I

Duan R, Goldmann L, Brandl R, Spannagl M, Weber C, Siess W, von Hundelshausen P. Effects of the Btk-inhibitors remibrutinib (LOU064) and rilzabrutinib (PRN1008) with varying Btk selectivity over Tec on platelet aggregation and *in vitro* bleeding time. Front Cardiovasc Med. 2021 Sep 24;8:749022. doi: 10.3389/fcvm.2021.749022.

Paper II

Duan R, Goldmann L, Li Y, Weber C, Siess W, von Hundelshausen P. Spontaneous platelet aggregation in blood is mediated by FcγRIIA stimulation of Bruton's tyrosine kinase. *Int. J. Mol. Sci.* 2022, *23*, 76. doi: 10.3390/ijms23010076.

Paper III

Goldmann L, **Duan R**, Kragh T, Wittmann G, Weber C, Lorenz R, von Hundelshausen P, Spannagl M, Siess W. Oral Bruton tyrosine kinase inhibitors block activation of the platelet Fc receptor CD32a (FcγRIIA): a new option in HIT? Blood Adv. 2019 Dec 10;3(23):4021-4033. doi: 10.1182/bloodadvances.2019000617. Erratum in: Blood Adv. 2020 Jan 14;4(1):112.

1.2 Additional publications not included in this work:

- Eckardt V, Miller MC, Blanchet X, **Duan R**, Leberzammer J, Duchene J, Soehnlein O, Megens RT, Ludwig AK, Dregni A, Faussner A, Wichapong K, Ippel H, Dijkgraaf I, Kaltner H, Döring Y, Bidzhekov K, Hackeng TM, Weber C, Gabius HJ, von Hundelshausen P, Mayo KH. Chemokines and galectins form heterodimers to modulate inflammation. EMBO Rep. 2020 Apr 3;21(4):e47852. doi: 10.15252/embr.201947852.

- Leberzammer J, Agten SM, Blanchet X, **Duan R**, Ippel H, Megens RTA, Schulz C, Aslani M, Duchene J, Döring Y, Jooss NJ, Zhang P, Brandl R, Stark K, Siess W, Jurk K, Heemskerk JWM, Hackeng TM, Mayo KH, Weber C, von Hundelshausen P. Targeting platelet-derived CXCL12 impedes arterial thrombosis. Blood. 2022, 139 (17), 2691-2705. doi: 10.1182/blood.2020010140.

- Weber C, von Hundelshausen P, Siess W. VITT after ChAdOx1 nCoV-19 Vaccination. N Engl J Med. 2021 Dec 2;385(23):2203-2204. doi: 10.1056/NEJMc2111026.

2. Contribution to publications

2.1 Contribution to paper I: Effects of the Btk-inhibitors remibrutinib (LOU064) and rilzabrutinib (PRN1008) with varying Btk selectivity over Tec on platelet aggregation and *in vitro* bleeding time

Rundan Duan is the first author of this paper. She identified IC_{50} values of remibrutinib and rilzabrutinib upon the stimulation of atherosclerotic plaque with multiplate electrode aggregometry. She measured the suppression effect of these two Btk inhibitors on platelet aggregation stimulated by the various agonists. Considering the selectivity of different Btk inhibitors, she compared the effect of remibrutinib, rilzabrutinib, and fenebrutinib on platelet aggregation induced by low dose collagen, high dose collagen and ristocetin. She measured *in vitro* closure time using PFA-200 that is provided by Prof. Spannagl at the department of hemostaseology. She analyzed data, created figures, and wrote the manuscript together with Prof. Siess and PD Dr. von Hundelshausen.

2.2 Contribution to paper II: Spontaneous platelet aggregation in blood is mediated by FcγRIIA stimulation of Bruton's tyrosine kinase

Rundan Duan is the first author of this publication. She designed the experiments based on the discussion with Prof. Siess and PD Dr. von Hundelshausen. Luise Goldmann and Rundan Duan measured spontaneous platelet aggregation and the suppression with fenebrutinib. In order to assess the relation between spontaneous platelet aggregation and collagen stimulation, and the effect of PF4 or heparin on spontaneous platelet aggregation, she measured platelet aggregation with multiple electrode aggregometry. She analyzed data, created figures, and wrote the manuscript together with Prof. Siess and PD Dr. von Hundelshausen.

2.3 Contribution to paper III: Oral Bruton tyrosine kinase inhibitors block activation of the platelet Fc receptor CD32a (FcγRIIA): a new option in HIT?

Rundan Duan is the second author of this publication. She established a method to measure platelet surface P-selectin expression and quantify the platelet-neutrophil complexes by flow cytometry.

2.4 Contribution to additional paper: Chemokines and galectins form heterodimers to modulate inflammation

Rundan Duan is a co-author of this publication. She performed immunofluorescence staining on mouse frozen lymph node sections showing the co-localization of Gal-3 and CXCL12. Further, to assess the interaction of Gal-3 and CXCL12, she confirmed the interaction by performing proximity ligation assay on Jurkat cells and mouse tissues.

2.5 Contribution to additional paper: Targeting platelet-derived CXCL12 impedes arterial thrombosis

Rundan Duan is a co-author of this publication. She accomplished *in vivo* bleeding time on WT and $CXCL12^{flox/flox}$ mice by tail bleeding assay and determined *in vitro* bleeding time with various antagonists treated human blood by using PFA-200. To seek the effect of remibrutinib on platelet activation, she stimulated human platelets with CRP and measured PAC-1 and P-selectin expression by flow cytometry on human platelets treated with remibrutinib or DMSO (solvent control). Furthermore, by performing multiple electrode aggregometry, she observed that CXCL12 induced platelet aggregation could be suppressed by inhibitor of intracellular Calcium (BAPTA-AM) and Syk (Syk inhibitor II).

2.6 Contribution to a letter to the editor: VITT after ChAdOx1 nCoV-19 Vaccination

Rundan Duan performed multiple electrode aggregometry to measure the platelet aggregation activity upon vaccine-induced immune thrombotic thrombocytopenia (VITT) patients' sera stimulation with or without Btk inhibitors and found that Btk inhibitors (ibrutinib and fenebrutinib) largely suppressed VITT serum induced platelet aggregation.

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Confirmation

Datum
04.05.2022

We hereby confirm that Ms Rundan Duan, from Yunnan, China conducted the experiments published in the Letter to The Editor entitled "BTK inhibitors against vaccine-induced immune thrombotic thrombocytopenia" in the New England Journal of Medicine. as part of her dissertation and also contributed conceptually to the discussions This letter was published on November 3, 2021, at NEJM.org. As the journal does not allow more than three authors for correspondence, it was not possible to list Ms Duan as a co-author.

Weber C, von Hundelshausen P, Siess W. VITT after ChAdOx1 nCoV-19 Vaccination. *N Engl J Med*. 2021;385:2203-2204. doi: 10.1056/NEJMc2111026

Prof. Dr. med. C. Weber

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3. Introduction

3.1 Platelet aggregation and platelet-related diseases

Platelets – micro anucleate (2-4μm in diameter), discoid blood cells generated by bone marrow megakaryocytes – play a critical role not only in hemostasis, but also in various physiological and pathophysiological processes including angiogenesis, tumor metastasis, atherosclerosis, and host defense[1-4].

Platelet aggregation is an essential step by which platelets adhere to each other when exposed to agonists, such as collagen, adenosine diphosphate (ADP), or thrombin. This property of platelets is critical in maintaining vascular integrity, essentially allowing platelets to serve as “Band-Aids of blood vessels”. But on the flip side, dysregulated aggregation can cause thrombus formation, leading to life-threatening thrombotic diseases, such as ischemic stroke, myocardial infarction, and Heparin-induced thrombocytopenia (HIT)[3]. In addition, the abnormal platelet aggregation associated with the Coronavirus Disease of 2019 (COVID-19) or COVID-19 vaccine-induced immune thrombotic thrombocytopenia (VITT) is of note[5-8].

Multiple pathways are involved in platelet aggregation. The agonists stimulate platelets via corresponding receptors, which then turn on GPIIb/IIIa – the integrin complex and fibrinogen receptor that is the central mediator of platelet aggregation (Figure 1)[9, 10]. Correspondingly, by targeting specific signaling pathways, various antiplatelet drugs have been developed to block platelet activation and aggregation (Figure 1). In thrombotic events, adequate antiplatelet therapy is crucial for improving patient survival and preventing new thrombotic events. However, it remains a daunting dilemma to balance the efficacy of antiplatelet treatment and the adverse events, which most notably is bleeding. The incidence of bleeding events during antiplatelet therapy ranges from 1.9% to 11.6%, depending on treatment strategies and patient condition, and some bleeding events may be fatal [11-14]. Therefore, there is an imperative need for a novel antiplatelet agent that is capable of adequately inhibiting platelet activation and aggregation while causing fewer bleeding problems.

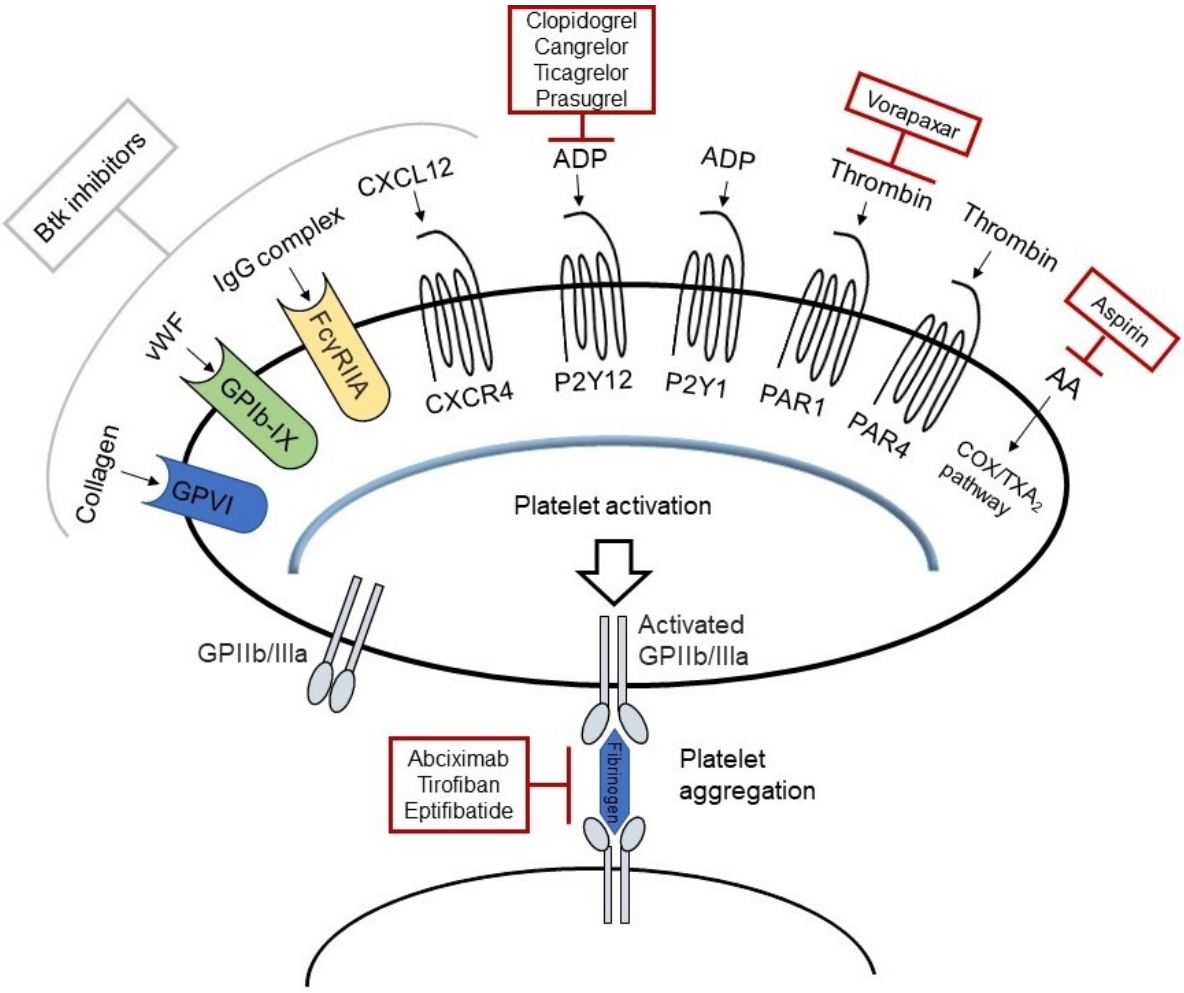

Figure 1. Platelet activation and aggregation pathways. Platelet activation is initiated by the ligation of the receptors (GPVI, GPIb-X-V, FcγRIIA, CXCR4, P2Y12, P2Y1, PAR1, and PAR4) with their corresponding agonists (collagen, vWF, IgG complexes, CXCL12, ADP, and thrombin). The GPIIb/IIIa complex undergoes a conformational change after the activation of platelets. Via activated GPIIb/IIIa complexes, fibrinogen functions to bridge the platelets, further inducing and stabilizing the aggregation. GPVI-, GPIb- and FcγRIIa-, CXCR4- signaling pathways are Btk dependent and are inhibited by Btk inhibitors. The activation of the other G protein-coupled receptors (GPCRs) (P2Y12, P2Y1, PAR1, and PAR4) is Btk independent and can be inhibited by different types of agents. Items in red boxes are approved antiplatelet drugs targeting different pathways. GPVI: glycoprotein VI; vWF: von Willebrand factor; GPIb: glycoprotein Ib; ADP: adenosine diphosphate; PAR: protease-activated receptor; AA: arachidonic acid; COX: cyclooxygenase; TXA_2: thromboxane A_2.

Platelets are able to aggregate spontaneously without agonists *in vitro*, which is known as spontaneous platelet aggregation (SPA). Accumulating evidence

suggests that increased SPA is a hallmark of cardiovascular diseases, and an independent predictor of vascular occlusion in patients with diabetes and myocardial infarction[15-18]. However, at present, there is a lack of a standardized method to evaluate SPA. In previous research, SPA was mainly assessed with light transmission aggregometry or light scattering aggregometry in citrate anticoagulated platelet-rich-plasma (PRP) [15-18]. Instead of PRP, the SPA measurement on hirudin anticoagulated whole blood using multiple electrode aggregometry (MEA) could better preserve the physiological states by not affecting the blood concentrations of Ca^{2+} and Mg^{2+} and accurately reflect platelet reactivity *in vivo*[19]. Furthermore, the mechanism of SPA is completely unknown. Elimination of SPA would help prevent adverse thrombotic events.

MEA is a reliable approach to assess platelet function in anticoagulated whole blood. The device monitors the impedance change of the two electrodes in the cuvette that arise due to platelet aggregation. As described, because the normal concentration of the blood cation is preserved, hirudin-anticoagulated blood is preferable for MEA[20]. MEA is now widely used for the evaluation of the bleeding diathesis, antiplatelet therapies, and thrombotic risks[21, 22].

Platelet function assay (PFA) is another advanced tool evaluating platelet dysfunction and bleeding risk by measuring *in vitro* bleeding time. The blood sample is aspirated constantly through a cartridge containing a perforated filter coated with platelet agonists (collagen/epinephrine or ADP/collagen). The time taken for clot formation blocking the blood flow through the cartridge is recorded as “closure time” or “*in vitro* bleeding time”[23]. The clinical utility of PFA includes the assessment of the platelet-related hemostasis defects, especially in the monitoring of the antiplatelet therapy (e.g., aspirin)[24, 25]. A significantly prolonged *in vitro* bleeding time was observed using PFA and the collagen/epinephrine cartridge in patients taking aspirin[26].

3.2 The Btk pathway in platelets

In 1952, Ogden Bruton first described a case of deficiency of immunoglobin and recurrent infection termed “Agammaglobulinemia”[27]. A condition known as X-linked

agammaglobulinemia (XLA) was found in 1993 to be caused by a defect of the Bruton's tyrosine kinase (Btk) gene via positional cloning and deoxyribonucleic acid cross-hybridization approaches[28, 29]. Subsequently, the determination of Btk deficiency or mutations became the decisive approach for XLA diagnosis. Btk is a nonreceptor cytoplasmic tyrosine kinase that belongs to the Tec family of proteins. Btk possesses five domains: amino terminal pleckstrin homology (PH) domain, proline-rich Tec homology (TH) domain, SRC kinase homology (SH) domains SH2 and SH3, and the catalytic kinase domain (Figure 2)[30, 31]. The catalytic kinase domain is the main target of the Btk inhibitors (BTKi) [32].

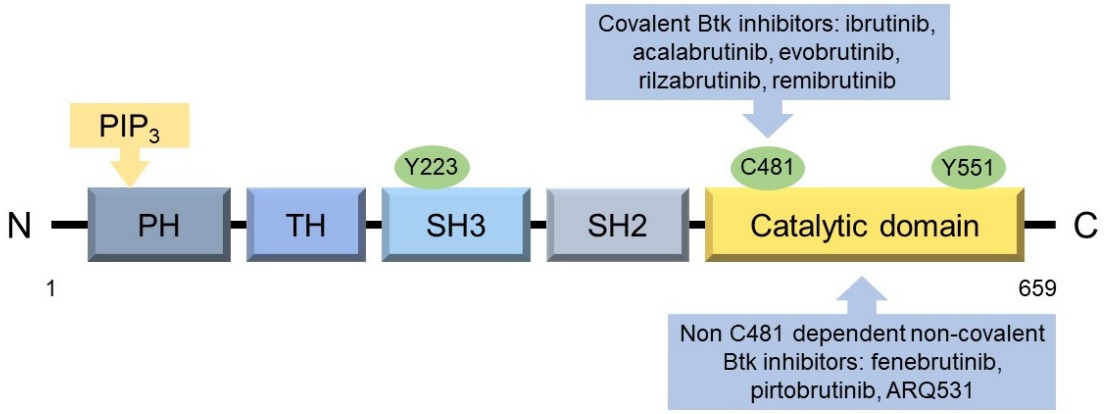

Figure 2. Structural diagram of Btk. The Btk protein consists of 659 amino acids and five domains from the N-terminus to the C-terminus. The catalytic kinase domain is the site recognized by Btk inhibitors. The covalent BTKi selectively bind to the C481 residue, and non-covalent BTKi bind to other residues on the catalytic domain. PH: pleckstrin homology; TH: Tec homology; SH: Src homology; Y223 and Y551: phosphorylation sites.

Btk is expressed in B cells, and plays a critical role in the development and function of the B cells via the B cell receptor pathway[30]. Although Btk is also found in megakaryocytes and platelets, Btk deficiency does not affect megakaryocyte maturation or platelet production[33]. In XLA patients, platelet count was found to be normal, and there are no defects in the production or survival of the Btk-null platelets[33]. Additionally, it is noteworthy that there is no bleeding tendency in XLA patients[33, 34]. In 1998, Quek and colleagues found that GPVI-associated collagen signaling in Btk-null platelets was impaired, while thrombin signaling was not altered[35]. The impairment of collagen stimulation in Btk-null platelets or platelets

incubated with BTKi could be overcome by high concentrations of collagen which induces the activation of Tec[35-38]. Btk-null platelets are able to function *in vivo* with preserved Tec signaling, which may be the reason for the non-bleeding phenotype in XLA patients[31, 39].

Because more research efforts have been channeled towards dissecting the function of Btk in platelets, the role of Btk in platelet activation and aggregation is becoming better understood. Btk phosphorylation takes place downstream of the platelet activation pathway. Platelet activation by the glycoprotein receptors GPVI (via collagen), GPIb (via vWF), FcγRIIA (via IgG complexes) and the GPCR CXCR4 (via CXCL12) is Btk-dependent, whereas platelet activation by other GPCR-stimuli such as thrombin receptor activation peptide (TRAP), arachidonic acid (AA), and ADP is independent of Btk (Figure 1)[31, 37, 38, 40-43]. Figure 3 summarizes the Btk activation and signaling pathways in the platelet. The homologous kinase Tec can be activated through the same pathway as Btk, but only compensates platelet function after GPVI is activated with high dose agonist[36, 44].

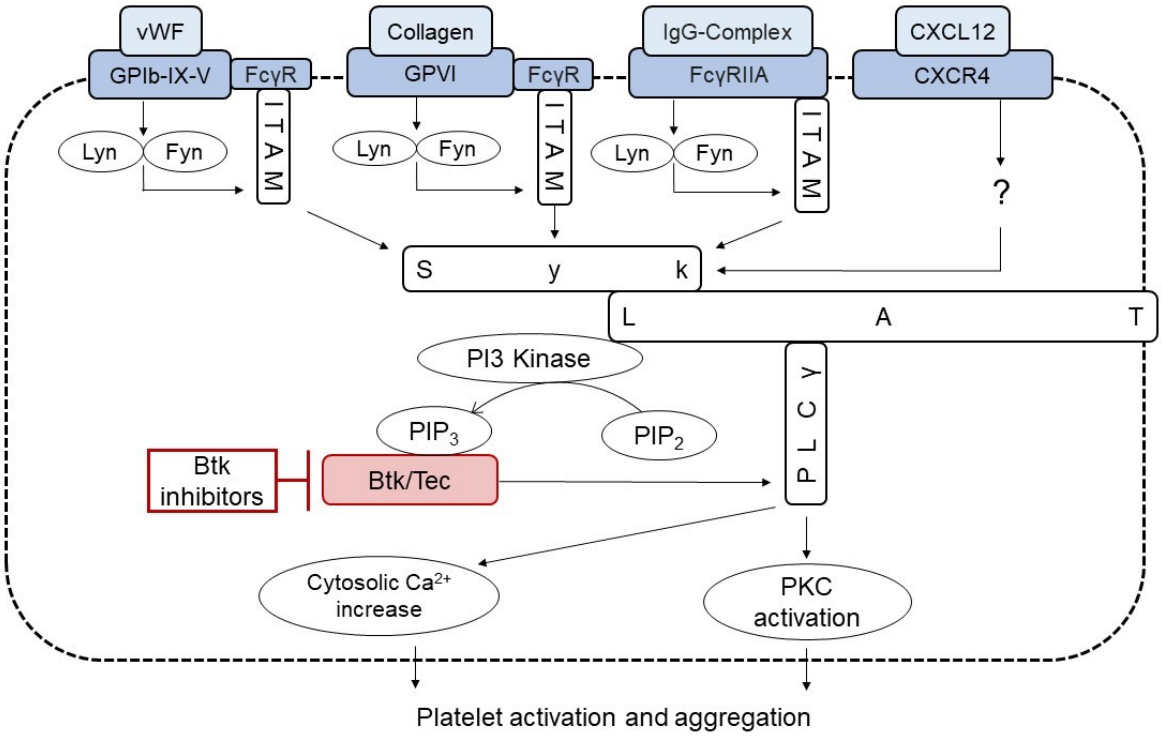

Figure 3. Btk signalling in platelets. After the interaction of the agonists and receptors, the Src-family kinases Lyn and Fyn bind to the cytosolic tail of the GPIb, GPVI, FcγRIIA receptors, then

phosphorylate after GPVI, GPIb or FcγRIIA activation the immunoreceptor tyrosine-based activation motif (ITAM) in the dimeric Fc receptor γ (FcγR)-chain associated with GPVI, GPIb or contained in FcγRIIA[45-47]. Phosphorylated ITAM recruits and activates the spleen tyrosine kinase Syk, which then phosphorylates the transmembrane adapter protein LAT. LAT provides a docking site for PI3 kinase and phospholipase Cγ2 (PLCγ2). Btk activation occurs after the activation of PI3 kinase, which subsequently catalyzes the conversion of phosphatidylinositol bisphosphate (PIP_2) into phosphatidylinositol trisphosphate (PIP_3)[48]. PIP_3 then binds to the PH domain of Btk resulting in the translocation of cytosolic Btk to the membrane. Then, Btk is phosphorylated at the Y551 site in the catalytic domain by Lyn, and autophosphorylated at the Y223 site in the SH3 domain. Activation of phospholipase Cγ2 leads to cytosolic Ca2+ increase and protein kinase C (PKC) activation, which induce platelet activation and aggregation[31, 41]. Recently, the CXCL12-CXCR4 pathway involving Syk, PI3 kinase and Btk in platelets has been described, but more research has to be done to uncover the whole pathway[43].

3.3 Btk inhibitors

In 2003, ibrutinib became the first approved BTKi for the treatment of B cell malignancies. Since then, more BTKi have been developed to not only treat B cell malignancies, but also several autoimmune disorders[42]; BTKi have also recently been tested to treat patients with severe COVID-19 or VITT[49-52]. At present, five irreversible BTKi (ibrutinib, acalabrutinib, zanubrutinib, tirabrutinib and orelabrutinib) have been approved by the FDA, and many more BTKi have been patented and are currently undergoing clinical trials[42, 53].

On the basis of the binding patterns, BTKi are classified into two categories: reversible and irreversible inhibitors. The reversible BTKi bind to a specific site in the catalytic domain through a weak and reversible interaction[42]. In contrast, the irreversible BTKi covalently bind the conserved Cys481 site in the adenosine triphosphate (ATP) binding pocket of Btk (Figure 2)[42]. Since there are other kinases (Bmx, Tec, Txk, Itk, EGFR, Erb2, Erb4, Jak3, and Blk) containing this conserved cysteine site in the ATP binding pocket that is similar to Btk, off-target binding may occur, resulting in a significant reduction in the selectivity of the irreversible BTKi[42]. In platelets, due to the absence of *de novo* Btk synthesis, low-dose irreversible BTKi with covalent binding in the pulsed therapy, similar to low-dose treatment of aspirin, might be able to inhibit platelet activation and aggregation without impairing B cell

function and immune defense[31, 54, 55]. However, the irreversible BTKi with higher selectivity causing less off-target problem should be explored.

Bleeding is a frequently reported adverse event after the treatment with irreversible BTKi in B cell malignancies[42]. While disease-related mechanisms such as coagulation disorders, thrombocytopenia, and other comorbid conditions that affect vascular permeability could also lead to an increased bleeding risk, BTKi-related bleeding events remain concerning. They are likely caused by inhibition of GPVI- and GPIb- dependent platelet activation pathways[42]. BTKi do not affect coagulation factors related to the intrinsic or extrinsic cascading pathways as assessed by prothrombin time (PT) and activated partial thromboplastin time (APTT)[56]. The exclusive Btk inhibition in platelets, however, cannot explain the bleeding events, since XLA patients do not show a bleeding tendency[34]. The current consensus is that the off-target inhibition of Tec, on top of Btk inhibition that strongly restrains GPVI signaling in platelets, is believed to be the underlying mechanism for BTKi-related bleeding[37, 42, 44, 57, 58]. This makes sense since irreversible BTKi show off-target inhibition on Tec, and bleeding events were observed in B cell malignancies only with irreversible BTKi[42]. However, significantly prolonged *in vitro* bleeding times were observed *in vitro* with irreversible BTKi (ibrutinib, acalabrutinib, and tirabrutinib) only at concentrations 2-3 times higher than their maximal therapeutic plasma levels[36]. Notably, no bleeding events were observed in B cell malignancies patients treated with reversible and highly selective BTKi fenebrutinib according to the data acquired from clinical trials[42, 59]. These findings suggest that reducing the off-target inhibition of Tec could reduce bleeding. However, the question of whether Tec inhibition is the only reason causing bleeding, or that there are other contributing factors that have not been identified, remains unanswered.

In paper I, two novel BTKi - remibrutinib and rilzabrutinib - were investigated. Remibrutinib is a very potent irreversible covalent Btk inhibitor with high selectivity on Btk over Tec[60]. Remibrutinib was developed from fenebrutinib-like scaffolds that bind to the inactive conformation of Btk[60, 61]. Thus, like fenebrutinib, remibrutinib is not expected to increase *in vitro* bleeding time. However, the effect of remibrutinib on platelets *in vitro* or *ex vivo* had not been studied. In the multiple-ascending dose cohort of a phase I placebo controlled clinical trial, remibrutinib intake for 12 days (C_{max} from 0.46μM to 1.11μM) did not increase the risk of bleeding[62]. Furthermore,

in remibrutinib phase 2 clinical trials for chronic spontaneous urticaria (CSU) (NCT03926611) and asthma (NCT03944707), no bleeding events were observed[63, 64]. Remibrutinib is currently under clinical trials not only for CSU (NCT05048342, NCT05030311, NCT05032157, NCT03926611), but also Sjögren syndrome (NCT04035668) and relapsing multiple sclerosis (NCT05147220, NCT05156281).

Rilzabrutinib is a reversible covalent Btk inhibitor with similar IC_{50} values for inhibition of Btk and Tec [65]. It associates rapidly with Btk Cys481 site and dissociates slowly[66]. In an *in vitro* preclinical study, no suppression of platelet aggregation induced by ristocetin and high dose collagen in PRP from health donors and immune thrombocytopenic purpura (ITP) patients with 1µM rilzabrutinib preincubation for 15min was observed[67]. Rilzabrutinib is currently being tested in clinical trials for ITP (NCT03395210, NCT04562766), IgG4-related disease (NCT04520451), CSU (NCT05107115), asthma (NCT05104892), atopic dermatitis (NCT05018806), and warm autoimmune hemolytic anemia (NCT05002777). Notably, despite a median platelet count of only 15,000/µl, only one in 60 patients (2%) treatment-related grade 1 bleeding event was observed in the rilzabrutinib ITP clinical trial[68, 69], while treatment-related bleeding events were more frequent (7% in 27 patients) in the rilzabrutinib pemphigus clinical trial[70].

3.4 Heparin-induced thrombocytopenia (HIT)

Heparin-induced thrombocytopenia is a life-threatening complication that occurs in hospitalized patients undergoing heparin therapy. The incidence of HIT varies from 0.1% to 5% among the population at risk depending on the heparin formulation and clinical context[71]. HIT is characterized by thrombocytopenia and thrombosis due to the abnormal formation of antibody complexes after being exposed to heparin[72, 73].

Platelet factor 4 (PF4) is a positively charged protein released from platelet α-granules upon platelet activation and binds to negatively charged glycosaminoglycans (GAGs) like heparin. The infusion of heparin can lead to the formation of PF4/heparin complexes since PF4 has a higher affinity to heparin than other GAGs[74-76]. Then PF4/heparin complexes preferentially bind to B cells[77],

resulting in the production of immunoglobulin G (IgG) recognizing PF4/heparin complexes[78]. This preferential binding is mediated by complement and complement receptor 2. Subsequently, through the Fc domain, these IgG/PF4/heparin complexes bind and cross-link FcγRIIA on the platelet surface, which triggers platelet activation and aggregation (Figure 4)[79, 80]. The polymorphism of FcγRIIA and the heterogeneous ability of the antibodies to activate FcγRIIA, as well as affinity and specificity to PF4 lead to different consequences of the patients with heparin treatment[81, 82]. In addition, Johnson and colleagues found that PF4 could bind to the surface of extended strings of von Willebrand factor (vWF) released from damaged endothelium, thus forming PF4/vWF complexes[83]. These immune complexes could be recognized by HIT antibodies as well, and IgG/PF4/vWF complexes could promote platelet adhesion and aggregation via FcγRIIA and GPIb signaling pathways[51, 83]. Both FcγRIIA and GPIb signaling are involved in Btk activation as illustrated in Figure 1[39, 41]. Enhanced secretion of prothrombotic factors from activated platelets, increased production of IgG recognizing PF4/heparin or PF4/vWF complexes from activated B cells, enhanced neutrophil extracellular trap formation and increased tissue factor expression on activated monocytes promote thrombin and thrombus formation, resulting in platelet consumption, while at the same time, promoting platelet clearance, ultimately leading to the pathogenesis of thrombocytopenia[71, 73].

Reducing platelet activation and thrombus generation is the key to managing HIT. All sources of heparin should be discontinued, and alternative anticoagulant therapies should be applied instead. Currently, the treatment of HIT mainly relies on non-heparin anticoagulants, such as direct thrombin inhibitors (argatroban, lepirudin, dabigatran) or anti-factor Xa agents (danaparoid, rivaroxaban)[71, 73, 84, 85]. As mentioned, FcγRIIA and GPIb signaling are pathologically activated in HIT, and both receptors act via Btk, suggesting that HIT patients could benefit from BTKi treatment. The efficacy of BTKi in treating HIT warrants further investigation[37].

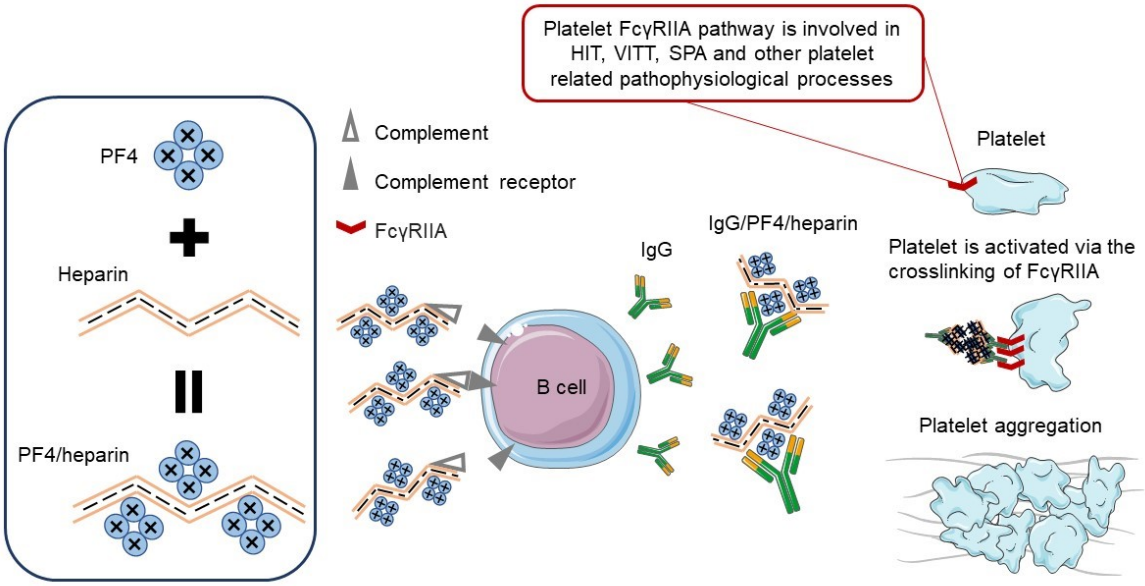

Figure 4. The formation of IgG/PF4/heparin complexes. When exposed to negatively charged heparin, positively charged PF4 binds to heparin forming PF4/heparin complexes. Mediated by the complement and complement receptors on the surface of B cells, PF4/heparin complexes interact with B cells, leading to the production and release of specific IgG molecules. PF4/heparin complexes are then recognized by circulating IgG forming IgG/PF4/heparin complexes. When in contact with platelets in circulation, IgG/PF4/heparin complexes can bind and crosslink FcγRIIA and initiate platelet activation and aggregation.

3.5 Btk inhibitors might be a therapeutic option for VITT

Due to the COVID-19 global pandemic, vaccines against the SARS-CoV-2 virus were developed and administered to the population at an astonishing rate. Vaccine-induced immune thrombotic thrombocytopenia (VITT) was observed in a few of cases following AstraZeneca-Oxford (ChAdOx1 nCov-19) or Johnson&Johnson (Ad26.COV2.S) vaccination[7, 8, 86], which posed a significant concern among healthcare providers and the public leading to the temporary vaccination suspension or restriction on the age of the vaccinated population in many countries.

The clinical features of VITT resembles those of HIT, which includes thrombosis and thrombocytopenia, and the presence of anti-PF4 antibodies[7, 8, 87]. When exposed to

the polyanionic constituents of the adenoviral vector vaccine (ChAdOx1 nCov-19, Ad26.COV2.S), the PF4/polyanion and IgG/PF4/polyanion complexes will likely form in a similar fashion as illustrated in Figure 4. Besides, vWF released from injured endothelium could bind to PF4 forming PF4/vWF that could be recognized by antibodies initiating IgG/PF4/vWF formation[51, 83]. These immune complexes will recognize FcγRIIA and GPIb complexes on the platelet surface and subsequently induce platelet activation and aggregation[51, 88]. Interestingly, Btk inhibitors could suppress *in vitro* VITT serum induced platelet aggregation, suggesting that the application of Btk inhibitors might be a viable alternative treatment option for VITT[51, 52].

Overall, this dissertation focuses on the effect of BTKi with varied selectivity (Btk over Tec) and different Btk binding modes (covalent irreversible, covalent reversible, and reversible) on platelet function. The therapeutic potential of inhibiting Btk-dependent platelet aggregation pathways (GPVI, GPIb, FcγRIIA) in the treatment of atherothrombosis, HIT, and VITT, as well as the BTKi effects on *in vitro* bleeding time, were studied. In addition, we hypothesized that reduced Tec inhibition could avoid BTKi-related bleeding events.

4. Summary

Btk is expressed in all hematopoietic cells, particularly in B cells and notably in platelets. Five Btk inhibitors (ibrutinib, acalabrutinib, zanubrutinib, tirabrutinib and orelabrutinib) have been approved by the FDA for the treatment of various B cell malignancies worldwide. Many more BTKi are now under clinical investigations for other conditions such as chronic lymphocytic leukemia, idiopathic thrombocytopenic purpura, rheumatoid arthritis, and other autoimmune disorders.

Btk participates in the signaling (GPVI/collagen-, GPIb/vWF-, FcγRIIA/IgG complexes- and CXCR4/CXCL12-signalling) leading to platelet activation and aggregation. Selective inhibition of Btk in platelets is expected to not cause severe hemorrhage while suppressing platelet aggregation, since Btk deficiency in XLA patients does not result in impaired of hemostasis. Therefore, Btk is a potential novel therapeutic target for platelet-related prothrombotic diseases. However, it is recognized that bleeding is a frequently reported adverse side effect during irreversible BTKi treatment of B cell malignancies.

4.1 Paper I: Effects of the Btk-inhibitors remibrutinib (LOU064) and rilzabrutinib (PRN1008) with varying Btk selectivity over Tec on platelet aggregation and *in vitro* bleeding time

In Paper I, we compared the effect of two novel BTKi on platelet - remibrutinib and rilzabrutinib. Remibrutinib (IC_{50}=0.03μM) is 5 times more potent than rilzabrutinib (IC_{50}=0.16μM) in the inhibition of platelet aggregation induced by pooled plaque which activates GPVI, and these IC_{50} values are 15-times and 2-fold lower, respectively, than their maximal plasma levels (remibrutinib C_{max}=0.46μM; rilzabrutinib C_{max}=0.33μM), which was determined after optimal dosage in clinical studies. With 0.1μM remibrutinib or 0.5μM rilzabrutinib suppressing over 90% low dose collagen- (GPVI), ristocetin- (GPIb/vWF) and CD32 (FcγRIIA) cross-linking-induced aggregation, a significantly increased *in vitro* closure time was observed, but remibrutinib (0.1μM) showed less hemostatic impairment. Remibrutinib intake for 12 days did not result in an increase of bleeding risk in phase I and II clinical trials,

whereas treatment-related bleeding events (7%) were reported in a clinical trial (of pemphigus) with rilzabrutinib. Although no major (≥ grade 2) treatment-related bleeding was observed in the rilzabrutinib ITP clinical trial, the maximal therapeutic concentrations of rilzabrutinib (0.33μM after 300mg BID) could probably significantly prolong the *in vitro* bleeding time.

In our study, the concentrations of rilzabrutinib that effectively blocked the Btk pathway in platelets and caused significantly increased *in vitro* bleeding time were the same (0.5μM). These results suggest that off-target effects on Tec shutting down completely the GPVI-pathway might be responsible. However, 0.5μM rilzabrutinib treatment showed only a faint inhibition of platelet aggregation (similar to fenebrutinib) upon stimulation with high dose collagen, which argues against off-target effects on Tec.

In contrast, the concentrations of remibrutinib that blocked the Btk pathways of platelet aggregation and caused significantly increased *in vitro* bleeding time were different (0.1μM versus 0.2μM). Thus, remibrutinib shows a higher potency in inhibition of platelet aggregation and less impairment of hemostasis. Further studies should be done to further explore remibrutinib as a novel antiplatelet agent against atherothrombosis and certain platelet-related immune disorders.

4.2 Paper II: Spontaneous platelet aggregation in blood is mediated by FcγRIIA stimulation of Bruton's tyrosine kinase

In Paper II, we described a novel protocol of using hirudin-anticoagulated blood and MEA to measure SPA. High SPA was observed in 8% of healthy donors in our volunteer cohort (n=118) and could be inhibited by anti-FcγRIIA antibody or anti-Fc IgG antibody. This indicates that IgG-complexes and FcγRIIA signaling pathway are involved in SPA. Based on this finding, ibrutinib was orally taken by volunteers (n=3) and fenebrutinib was added to the collected blood. Meanwhile SPA was measured before and after BTKi application. Consequently, SPA was ameliorated after BTKi application both *in vivo* and *in vitro*. In addition, a positive and significant correlation

between high SPA and increased GPVI reactivity to low dose collagen was observed. An increased GPVI reactivity has been also observed in patients with diabetes, COVID-19 and stroke. Considering the inhibitory effect of BTKi on GPVI activation, these patients may benefit from the application of BTKi. This, however, requires further studies. Since we found that the platelet surface FcγRIIA activation by unknown IgG-complexes through the Btk pathway was the underlying mechanism of high SPA, we investigated the hypothesis that anti-PF4/polyanion IgG complexes might be involved. However, we observed that this was not the case, pointing to the involvement of other IgG-complexes in eliciting SPA.

4.3 Paper III: Oral Bruton tyrosine kinase inhibitors block activation of the platelet Fc receptor CD32a (FcγRIIA): a new option in HIT?

In Paper III, a novel Btk dependent therapeutic target – FcγRIIA – for heparin-induced thrombocytopenia type II (HIT) was explored. Five irreversible BTKi (ibrutinib, zanubrutinib, acalabrutinib, tirabrutinib, evobrutinib) and one reversible BTKi (fenebrutinib) were tested in this study. Among the five BTKi, fenebrutinib was found to be the most potent with IC_{50}=0.011μM on the inhibition of FcγRIIA cross-linking–induced platelet aggregation. In addition, a single intake of ibrutinib was sufficient to suppress platelet activation and ATP secretion by FcγRIIA. Since irreversible BTKi were found to prolong the bleeding time *in vitro*, we tested the reversible BTKi fenebrutinib and found that it did not increase bleeding time even after being incubated with high concentrations *in vitro*. The irreversible BTKi inhibited dense granule secretion, P-selectin expression, and platelet-neutrophil aggregates formation that contribute to the thrombosis in HIT. We applied HIT sera from heparin/PF4/IgG antibodies positive HIT patients to induce platelet aggregation in the presence of low dose heparin, and found that ibrutinib, acalabrutinib, and fenebrutinib showed over 90% inhibition on HIT sera-induced platelet aggregation. Given that fenebrutinib, applied at a high dose twice daily, was able to effectively block Btk in blood over time, while preventing increased bleeding events, HIT patients would obtain additional benefit from fenebrutinib treatment. Furthermore, since VITT shares similar pathophysiological mechanism with HIT involving IgG-complex-FcγRIIA pathway, fenebrutinib would as well be a potential therapy for VITT.

In conclusion, we explored the effect of Btk-inhibitors with varying Btk selectivity and reversibility on platelet activation and aggregation. Btk is involved in GPVI, GPIb, FcγRIIA, and CXCR4 pathways in platelets, hence BTKi could be a potential treatment for pathophysiological conditions or diseases associated with these pathways, such as HIT, VITT, atherothrombosis and SPA, while bleeding is a potential adverse event that should always be taken into the consideration. Fenebrutinib was found to be the most potent BTKi (fenebrutinib > remibrutinib > rilzabrutinib) for the suppression of pooled plaque induced platelet aggregation in our study, and did not increase *in vitro* bleeding time. Through the comparison of the *in vitro* bleeding times between the irreversible covalent BTKi remibrutinib (no Tec inhibition), reversible covalent BTKi rilzabrutinib (Tec inhibition) and reversible non-covalent BTKi fenebrutinib (no Tec inhibition), we reported that prolonged *in vitro* bleeding time was observed for remibrutinib and rilzabrutinib treatment, but not fenebrutinib. This argues against the hypothesis that off-target inhibition of Tec is the reason for bleeding. At the same time, this raises the discussion of the irreversibility or the covalent binding patterns of the inhibitors that may be responsible for the adverse bleeding events.

5. Zusammenfassung

Btk wird in allen hämatopoetischen Zellen exprimiert, insbesondere in B-Zellen und vor allem in Thrombozyten. Fünf Btk-Inhibitoren (Ibrutinib, Acalabrutinib, Zanubrutinib, Tirabrutinib und Orelabrutinib) sind weltweit für die Behandlung verschiedener maligner B-Zell-Erkrankungen zugelassen. Weitere BTKi werden derzeit für die Behandlung der chronischen lymphatischen Leukämie, der idiopathischen thrombozytopenischen Purpura, der rheumatoiden Arthritis und vieler anderer Erkrankungen klinisch untersucht.

Btk ist an den Signalwegen der Thrombozytenaktivierung und -aggregation beteiligt (GPVI/Kollagen-, GPIb/vWF-, FcγRIIA/IgG-Komplexe und CXCR4/CXCL12-Signalisierung). Es wird erwartet, dass eine selektive Hemmung von Btk in Thrombozyten keine schweren Blutungen verursacht, während die Thrombozytenaggregation unterdrückt wird, da ein Btk-Mangel bei XLA-Patienten nicht zu einer Beeinträchtigung der Hämostase führt. Daher ist Btk ein potenzielles neues therapeutisches Ziel für thrombozytenbezogene prothrombotische Erkrankungen. Es ist jedoch bekannt, dass Blutungen eine häufig berichtete unerwünschte Nebenwirkung nach der Behandlung von B-Zell-Malignomen mit irreversiblen BTKi sind.

5.1 Paper I: Effects of the Btk-inhibitors remibrutinib (LOU064) and rilzabrutinib (PRN1008) with varying Btk selectivity over Tec on platelet aggregation and *in vitro* bleeding time

In Paper I haben wir die Wirkung von zwei neuen BTKi auf Thrombozyten - Remibrutinib und Rilzabrutinib - verglichen. Remibrutinib (IC_{50}=0,03µM) ist fünfmal potenter als Rilzabrutinib (IC_{50}=0,16µM) bei der Hemmung der GPVI-induzierten Thrombozytenaggregation niedrigen Grades, und diese IC_{50}-Werte sind 15-mal bzw. 2-mal niedriger als ihre maximalen Plasmaspiegel (Remibrutinib C_{max}=0,46µM; Rilzabrutinib C_{max}=0,33µM), die nach optimaler Dosierung in klinischen Studien ermittelt wurden. Bei 0,1µM Remibrutinib bzw. 0,5µM Rilzabrutinib, die zu über 90% die durch niedrige Dosen von Kollagen (GPVI), Ristocetin (GPIb/vWF) und CD32

(FcγRIIA) vernetzungsinduzierte Aggregation unterdrücken, wurde eine signifikant verlängerte In-vitro-Verschlusszeit beobachtet, wobei Remibrutinib (0,1µM) jedoch eine geringere hämostatische Beeinträchtigung zeigte. Die 12-tägige Einnahme von Remibrutinib führte in klinischen Studien der Phasen I und II nicht zu einer Erhöhung des Blutungsrisikos, während in einer klinischen Studie (bei Pemphigus) mit Rilzabrutinib behandlungsbedingte Blutungsereignisse (7%) gemeldet wurden. Obwohl in der klinischen ITP-Studie mit Rilzabrutinib keine schwerwiegenden (≥Grad 2) behandlungsbedingten Blutungen beobachtet wurden, könnten die maximalen therapeutischen Konzentrationen von Rilzabrutinib (0,33µM nach 300mg BID) die In-vitro-Blutungszeit wahrscheinlich erheblich verlängern.

In unserer Studie waren die Konzentrationen von Rilzabrutinib, die den Btk-Weg in Thrombozyten wirksam blockierten und eine signifikant verlängerte In-vitro-Blutungszeit verursachten, dieselben (0,5 µM). Diese Ergebnisse deuten darauf hin, dass Off-Target-Effekte auf Tec, die den GPVI-Signalweg vollständig ausschalten, dafür verantwortlich sein könnten. Allerdings zeigte die Behandlung mit 0,5µM Rilzabrutinib nur eine schwache Hemmung der Thrombozytenaggregation (ähnlich wie bei Fenebrutinib) bei Stimulation mit hochdosiertem Kollagen, was gegen Off-Target-Effekte auf Tec spricht.

Im Gegensatz dazu waren die Konzentrationen von Remibrutinib, die den Btk-Weg der Thrombozytenaggregation blockierten und eine signifikant verlängerte In-vitro-Blutungszeit verursachten, unterschiedlich (0,1µM gegenüber 0,2µM). Somit zeigt Remibrutinib eine höhere Potenz bei der Hemmung der Thrombozytenaggregation und eine geringere Beeinträchtigung der Hämostase. Weitere Studien sollten durchgeführt werden, um Remibrutinib als neuartigen Thrombozytenaggregationshemmer gegen Atherothrombose und bestimmte plättchenbezogene Immunstörungen weiter zu erforschen.

5.2 Paper II: Spontaneous platelet aggregation in blood is mediated by FcγRIIA stimulation of Bruton’s tyrosine kinase

In Paper II haben wir ein neuartiges Protokoll beschrieben, bei dem gerührtes, mit Hirudin antikoaguliertes Blut und MEA zur Messung der SPA verwendet wird. Eine

hohe SPA wurde bei 8 % der gesunden Spender in unserer Probandenkohorte (n=118) beobachtet und konnte durch Anti-FcγRIIA-Antikörper oder Anti-Fc-IgG-Antikörper gehemmt werden. Dies deutet darauf hin, dass IgG-Komplexe und der FcγRIIA-Signalweg an der SPA beteiligt sind. Auf der Grundlage dieser Erkenntnisse wurde Ibrutinib von Freiwilligen (n=3) oral eingenommen und Fenebrutinib dem abgenommenen Blut zugesetzt. Gleichzeitig wurde die SPA vor und nach der BTKi-Gabe gemessen. Das Ergebnis war, dass sich die SPA nach der BTKi-Anwendung sowohl in vivo als auch in vitro verbesserte. Darüber hinaus wurde ein positiver und signifikanter Zusammenhang zwischen hoher SPA und erhöhter GPVI-Reaktivität auf niedrig dosiertes Kollagen festgestellt. Eine erhöhte GPVI-Reaktivität wurde auch bei Patienten mit Diabetes, COVID-19 und Schlaganfall beobachtet. In Anbetracht der hemmenden Wirkung von BTKi auf die GPVI-Aktivierung könnten diese Patienten von der Anwendung von BTKi profitieren. Dazu sind jedoch weitere Studien erforderlich. Da wir feststellten, dass die Stimulation der Thrombozyten-FcγRIIA durch unbekannte IgG-Komplexe über den Btk-Signalweg der zugrunde liegende Mechanismus der hohen SPA war, untersuchten wir die Hypothese, dass Anti-PF4/Polyanion-IgG-Komplexe beteiligt sein könnten. Wir stellten jedoch fest, dass dies nicht der Fall war, was auf die Beteiligung anderer IgG-Komplexe an der Auslösung von SPA hindeutet.

5.3 Paper III: Oral Bruton tyrosine kinase inhibitors block activation of the platelet Fc receptor CD32a (FcγRIIA): a new option in HIT?

In Paper III wurde ein neues Btk-abhängiges therapeutisches Ziel - FcγRIIA - für Heparin-induzierte Thrombozytopenie Typ II (HIT) untersucht. In dieser Studie wurden fünf irreversible BTKi (Ibrutinib, Zanubrutinib, Acalabrutinib, Tirabrutinib, Evobrutinib) und ein reversibles BTKi (Fenebrutinib) getestet. Unter den fünf BTKi erwies sich Fenebrutinib mit einer IC_{50} von 0,011µM als der wirksamste Wirkstoff zur Hemmung der durch FcγRIIA-Vernetzung induzierten Thrombozytenaggregation. Darüber hinaus reichte eine einmalige Einnahme von Ibrutinib aus, um die Thrombozytenaktivierung und ATP-Sekretion durch FcγRIIA zu unterdrücken. Da sich herausstellte, dass irreversible BTKi die Blutungszeit in vitro verlängern, testeten wir das reversible BTKi Fenebrutinib und stellten fest, dass es die Blutungszeit auch nach Inkubation mit hohen Konzentrationen in vitro nicht verlängert. Das irreversible BTKi hemmte die Sekretion von dichten Granula, die Expression von P-Selektin und die

Bildung von Thrombozyten-Neutrophilen-Aggregaten, die zur Thrombose bei HIT beitragen. Wir verwendeten HIT-Seren von Heparin/PF4/IgG-Antikörper-positiven HIT-Patienten, um die Thrombozytenaggregation in Gegenwart von niedrig dosiertem Heparin zu induzieren, und stellten fest, dass Ibrutinib, Acalabrutinib und Fenebrutinib die durch HIT-Seren induzierte Thrombozytenaggregation zu über 90% hemmen. Da Fenebrutinib in einer hohen Dosis zweimal täglich Btk im Blut über einen längeren Zeitraum wirksam blockieren und gleichzeitig vermehrte Blutungsereignisse verhindern konnte, würden HIT-Patienten von einer Behandlung mit Fenebrutinib zusätzlich profitieren. Da die VITT einen ähnlichen pathophysiologischen Mechanismus wie die HIT aufweist, an dem der IgG-Komplex-FcγRIIA-Signalweg beteiligt ist, wäre Fenebrutinib auch eine potenzielle Therapie für die VITT.

Abschließend haben wir die Wirkung von Btk-Inhibitoren mit unterschiedlicher Btk-Selektivität und Reversibilität auf die Aktivierung und Aggregation von Blutplättchen untersucht. Btk ist an den GPVI-, GPIb-, FcγRIIA- und CXCR4-Signalwegen in Thrombozyten beteiligt. Daher könnten BTKi eine potenzielle Behandlung für pathophysiologische Zustände oder Krankheiten sein, die mit diesen Signalwegen in Zusammenhang stehen, wie z. B. HIT, VITT, Atherothrombose und SPA, während Blutungen ein potenzielles unerwünschtes Ereignis sind, das immer in Betracht gezogen werden sollte. Fenebrutinib erwies sich in unserer Studie als das wirksamste BTKi (Fenebrutinib > Remibrutinib > Rilzabrutinib) für die Hemmung der durch GPVI stimulierten Thrombozytenaggregation in geringem Maße und verlängerte die Blutungszeit in vitro nicht. Durch den Vergleich der In-vitro-Blutungszeiten zwischen dem irreversiblen kovalenten BTKi Remibrutinib (keine Tec-Hemmung), dem reversiblen kovalenten BTKi Rilzabrutinib (Tec-Hemmung) und dem reversiblen nicht-kovalenten BTKi Fenebrutinib (keine Tec-Hemmung) berichteten wir, dass eine verlängerte In-vitro-Blutungszeit bei der Behandlung mit Remibrutinib und Rilzabrutinib, aber nicht mit Fenebrutinib beobachtet wurde. Dies spricht gegen die Hypothese, dass die Off-Target-Hemmung von Tec der Grund für die Blutungen ist. Gleichzeitig wirft dies die Diskussion über die Irreversibilität oder die kovalenten Bindungsmuster der Inhibitoren auf, die für die unerwünschten Blutungen verantwortlich sein könnten.

6. References

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7. Paper I

frontiers in Cardiovascular Medicine

BRIEF RESEARCH REPORT
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Effects of the Btk-Inhibitors Remibrutinib (LOU064) and Rilzabrutinib (PRN1008) With Varying Btk Selectivity Over Tec on Platelet Aggregation and *in vitro* Bleeding Time

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Background: Bruton tyrosine kinase inhibitors (BTKi) are used in B-cell malignancies and in development against various autoimmune diseases. Since Btk is also involved in specific pathways of platelet activation, BTKi might be considered to target platelet GPVI/GPIb-mediated atherothrombosis and platelet FcγRIIA-dependent immune disorders. However, BTKi treatment of patients with B-cell malignancies is frequently associated with mild bleeding events caused possibly by off-target inhibition of Tec. Here, we compared the platelet effects of two novel BTKi that exhibit a high (remibrutinib) or low (rilzabrutinib) selectivity for Btk over Tec.

Methods and Results: Remibrutinib and rilzabrutinib were pre-incubated with anticoagulated blood. Platelet aggregation and *in vitro* bleeding time (closure time) were studied by multiple electrode aggregometry (MEA) and platelet-function analyzer-200 (PFA-200), respectively. Both BTKi inhibited atherosclerotic plaque-stimulated GPVI-mediated platelet aggregation, remibrutinib being more potent (IC_{50} = 0.03 μM) than rilzabrutinib (IC_{50} = 0.16 μM). Concentrations of remibrutinib (0.1 μM) and rilzabrutinib (0.5 μM), >80% inhibitory for plaque-induced aggregation, also significantly suppressed (>90%) the Btk-dependent pathways of platelet aggregation upon GPVI, von Willebrand factor/GPIb and FcγRIIA activation stimulated by low collagen concentrations, ristocetin and antibody cross-linking, respectively. Both BTKi did not inhibit aggregation stimulated by ADP, TRAP-6 or arachidonic acid. Remibrutinib (0.1 μM) only slightly prolonged closure time and significantly less than rilzabrutinib (0.5 μM).

Conclusion: Remibrutinib and rilzabrutinib inhibit Btk-dependent pathways of platelet aggregation upon GPVI, VWF/GPIb, and FcγRIIA activation. Remibrutinib being more potent and showing a better profile of inhibition of Btk-dependent platelet activation vs. hemostatic impairment than rilzabrutinib may be considered for further development as an antiplatelet drug.

Keywords: atherothrombosis, platelet-aggregation, bleeding, PFA, MEA

INTRODUCTION

Since the first description of a patient with recurrent infections and deficiency of immunoglobulins termed "Agammaglobulinemia" by Ogden Bruton in 1952 (1), it took more than 40 years of research, until Bruton tyrosine kinase (Btk) was identified in 1993 as the responsible protein that is deficient in patients with X-linked agammaglobulinemia (2, 3). Btk belongs to the Tec (tyrosine kinase expressed in hepatocellular carcinoma) family of non-receptor cytoplasmic tyrosine kinase, and contains five different protein interaction domains: an amino terminal pleckstrin homology (PH) domain, a proline-rich Tec homology (TH) domain, the SRC kinase homology (SH) domains SH2 and SH3, and a kinase domain (4). Btk is the best studied member of this tyrosine kinase family and most homologous to Tec, the namesake of this kinase family. Btk plays a crucial role in B-cell receptor function and in immunoglobulin Fc- receptor signaling of monocytes/macrophages and neutrophils (4). Since the approval of ibrutinib, the covalent irreversible first in class Btk inhibitor (BTKi) in 2013 for treatment of certain B-cell malignancies, many more reversible and irreversible BTKi have evolved and the spectrum of diseases that are targeted extends from specific forms of B-cell malignancies to various autoimmune disorders (5). Up to now four BTKi (ibrutinib, acalabrutinib, zanubrutinib, and tirabrutinib) have been approved but at least further eight BTKi are in clinical studies (5).

Btk is expressed not only in B-cells but also in various hematopoietic cells including the megakaryocyte-platelet lineage (6). Btk in platelets is involved in signaling of specific glycoprotein receptors including glycoprotein (GP) VI activation by collagen, GPIb activation by von Willebrand factor (VWF), FcγRIIa activation by IgG immune complexes, and CLEC-2 activation by podoplanin (5). Thus, Btk might be a promising therapeutic target of platelet-related diseases, and BTKi have indeed been proposed as novel antiplatelet drugs as they inhibit selectively platelet GPVI/GPIb-stimulated atherothrombosis (7, 8), platelet FcγRIIA-dependent immune disorders (heparin-induced thrombocytopenia, vaccine-induced immune thrombotic thrombocytopenia) (9, 10), and podoplanin/CLEC-2 mediated venous thrombosis (11, 12). Somewhat surprisingly, XLA patients do not show a bleeding tendency (13). In contrast, mild bleeding events are frequent in patients with B-cell malignancies treated with irreversible covalent BTKi (ibrutinib, acalabrutinib, zanubrutinib, and tirabrutinib) (5). The reasons are not clear but are probably multifactorial. They might be related to the type of diseases treated, but also caused by off-target inhibition as reviewed recently (5).

Beside Btk the homologous kinase Tec is also expressed in platelets. Whereas, FcγRIIa activation and VWF activation of GPIb do not require Tec activation (5, 9), Tec plays a role in GPVI activation. After GPVI-mediated platelet stimulation by high dose collagen, both Btk and Tec support platelet aggregation. Btk-deficient human platelets from patients with XLA and Btk-deficient mouse platelets do not respond to low concentrations of collagen or collagen-related peptide (CRP) indicating that Btk is required for platelet activation after low-degree GPVI stimulation (14, 15). Similar observations have been made by using low Btk-specific concentrations of irreversible BTKi and the reversible BTKi fenebrutinib which inhibits Btk but not Tec and applying human atherosclerotic plaque which also induces only a low-degree activation of GPVI (8, 9, 16–18). After stimulation with high concentrations of collagen, Tec compensates for the absence of Btk (as in XLA patients) or inhibition of Btk (as after platelet pretreatment with Btk-selective concentrations of BTKi) and preserves GPVI-stimulated platelet aggregation. Inhibition of both Tec and Btk abrogates GPVI-activation (15). Since the four approved irreversible covalent BTKi mentioned above have limited selectivity for Btk over Tec as measured by biochemical assays *in vitro* (5), and at higher concentrations prolong bleeding time *in vitro* (19), it is assumed that therapeutic concentrations of these BTKi inhibit in platelets irreversibly Tec in addition to Btk thereby abrogating GPVI signaling. This might contribute to the observed bleeding side effects.

Therefore, we hypothesized that off target effects of BTKi with low Btk selectivity over Tec might explain bleeding of BTKi, and investigated in the present study the effects of two novel BTKi on Btk-mediated pathways of platelet aggregation and bleeding time *in vitro*: the novel selective covalent BTKi remibrutinib (LOU064), a very potent irreversible covalent BTKi, which is highly selective for Btk and barely inhibits Tec (20), and rilzabrutinib (PRN1008) an oral, reversible covalent BTKi which inhibits Btk and Tec with similar IC50 values (21). Both BTKi are in clinical studies of dermatological autoimmune diseases. Rilzabrutinib inhibits very potently Btk and Tec *in vitro* (IC50 values, 1.3 and 0.8 nM, respectively) (22). It forms a reversible covalent bond with Cys481 of Btk, and shows a fast association and a very slow dissociation rate (23). Rilzabrutinib is in clinical trials of pemphigus (24) and idiopathic thrombocytopenic purpura (ITP), a disease exhibiting very low platelet counts (<50.000/μl) and bleeding events. Here, it inhibits platelet destruction mainly via the inhibition of autoantibody/FcγR signaling in splenic macrophages (25). Unexpectedly, in a

previous report clinically relevant concentrations of rilzabrutinib showed no inhibition of platelet activation *in vitro* (26).

MATERIALS AND METHODS

Reagents

Remibrutinib (LOU064), rilzabrutinib (PRN1008) and fenebrutinib (GDC-0853) were purchased from MedChem Express (New Jersey, USA). Dimethyl sulfoxide (DMSO) was from Sigma-Aldrich (Taufkirchen, Germany). Collagen was from Takeda (Linz, Austria). ADP, ristocetin, arachidonic acid (AA) and TRAP-6 (Thrombin Receptor Activator Peptide 6) were obtained from Roche Diagnostics (Mannheim, Germany). The anti-CD32 antibody AT10 (monoclonal mouse IgG1), cross-adsorbed F(ab')2-goat anti-mouse IgG (H + L) and the anti-CD9 antibody Ts9 (monoclonal mouse IgG1) were from ThermoFisher Scientific (Waltham, MA, USA).

Declaration of Helsinki

Informed consent was obtained from healthy volunteers, as approved by the Ethics Committee of the Faculty of Medicine of the University of Munich, and in accordance with the ethical principles for medical research involving human subjects, as set out in the Declaration of Helsinki.

Human Atherosclerotic Plaque Homogenates

Atherosclerotic tissue specimens were obtained from patients who underwent endarterectomy for high-grade carotid artery stenosis. Specimen containing lipid-rich soft plaques were collected. The atheromatous plaques were carefully dissected under sterile conditions from other regions of the atherosclerotic tissue. The plaques were weighed, homogenized with a glass pestle and potter, then stored at −80°C (27, 28). Plaque homogenates from 5 patients were pooled.

Blood Collection

Whole blood from healthy donors who had not taken any antiplatelet drug within 2 weeks was collected by cubital venipuncture into blood tubes (double wall) from Verum Diagnostica GmbH (Munich, Germany) containing hirudin as anticoagulant (final hirudin concentration in blood: 200 U/ml corresponding to 15 μg/ml) for platelet aggregation measurements (29) or buffered trisodium citrate/citric acid solution (citrate concentration 0.129 mol/L; S-Monovette 3.8 mL 9NC/PFA from Sarstedt, Nümbrecht, Germany) for closure time measurements with the PFA-200 (30). The blood was kept at room temperature and measurements were performed with 3 h after venipuncture.

Platelet Aggregation in Blood

Multiple electrode aggregometry (MEA) (Roche Diagnostics, Mannheim, Germany) that monitors the change of conductivity between two sets of electrodes (red and blue traces) caused by the attachment of platelets was applied to measure platelet aggregation, as described (29, 31). In brief, 0.9% NaCl (300 μL) was placed in aggregometer cuvettes (06675590, Roche, Mannheim, Germany) with aliquots (300 μL) of hirudin-anticoagulated blood. BTKi or DMSO (solvent control; 0.6 μL) were added, and mixed well with pipet, covered, and incubated for 1 h at 37°C (19). Then, the cuvettes were transferred into the device, platelet stimuli (collagen, ristocetin, AT10 + Fab2, anti-CD9 antibody, TRAP-6, ADP, or AA) were added at concentrations as detailed in the figure legends, stirring was simultaneously started and aggregation was measured for 10 min. Aggregation was recorded in arbitrary units (AU), and cumulative aggregation (AU*min) from 0 to 10 min was measured by quantifying the area under the curve. The traces selected as representative and displayed in the Figures belonged to a specific experiment whose values were closest to the mean.

IC50 values were obtained by non-linear fitting using the following model:

Fifty = (Top + Baseline)/2
Y = Bottom + (Top-Bottom)/(1 + 10^((LogAbsoluteIC50-X)*HillSlope + log((Top-Bottom)/(Fifty-Bottom)-1)))

Closure Time Measurement

The INNOVANCE® PFA-200 System (Siemens Healthcare, Erlangen, Germany), which simulates primary hemostasis *in vitro*, provides rapid and precise assessment of platelet dysfunction and bleeding risk (32, 33). DMSO (0.1%; solvent control) or various concentrations of remibrutinib or rilzabrutinib were pipetted (0.8 μl) into samples of citrate-anticoagulated blood (0.8 ml) (30) and preincubated for 1 h at 37°C. The Dade® PFA Collagen/EPI Test Cartridge was used, and the time of complete plug formation was reported as "closure time." The normal range of closure time is assessed specifically for each test center and was determined to be 84–170 s. The normal range as recommended by the manufacturer (84–160 s) has been slightly modified at our institution to 84–170 s based on the measurement on 54 healthy unselected persons without any medication according to the approved-level consensus guideline from the Clinical and Laboratory Standards Institute (CLSI EP28).

Statistics

The data are shown as mean ± standard deviation (SD) of the indicated number of the experiments. Normal distribution of values was assessed using the Shapiro-Wilk test. Parallel multi-experimental conditions were analyzed by ordinary one-way ANOVA, followed by Bonferroni's test if the normality test was passed, otherwise a Kruskal-Wallis test for unmatched and a Friedman's test for matched observations followed by Dunn's test were used. Side-by side comparisons were analyzed by Wilcoxon matched-pairs signed rank test.

RESULTS

Remibrutinib (LOU064) and Rilzabrutinib (PRN1008) Dose-Dependently Inhibit GPVI-Mediated Platelet Aggregation in Blood Triggered by Atherosclerotic Plaque

Diverse collagen type I and III fibers are the decisive plaque components that induce platelet aggregation via activation of GPVI (27, 28, 34). Blood was incubated with increasing

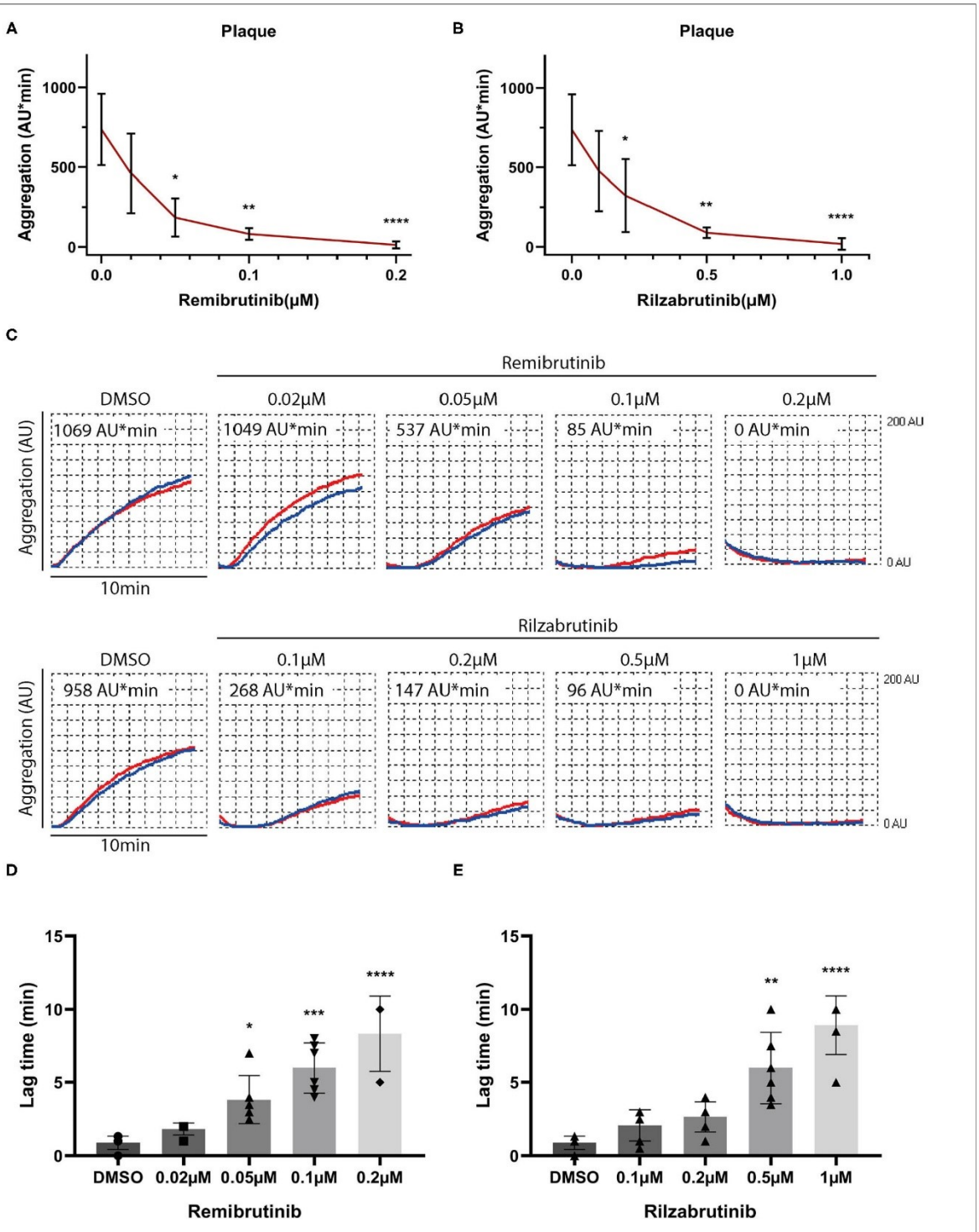

FIGURE 1 | Effects of increasing concentrations of remibrutinib and rilzabrutinib on platelet aggregation in blood stimulated by plaque homogenate. Hirudin anticoagulated blood was preincubated for 1 h at 37°C with solvent control (DMSO, 0.1%), or increasing concentrations of remibrutinib **(A,C,D)** or rilzabrutinib **(B,C,E)**

(Continued)

FIGURE 1 | and aggregation was recorded for 10 min after stimulation by plaque homogenate (833 μg/ml) (19). The dose-response curves of **(A)** remibrutinib and **(B)** rilzabrutinib are shown. **(C)** Representative aggregation traces in red and blue for each electrode, respectively, are shown. **(D,E)** Bar graphs show the dose-dependent delay in aggregation by **(D)** remibrutinib and **(E)** rilzabrutinib. Single data points are shown but are in part not visible due to overlap. Values are mean ± SD ($n = 6$). Statistical analysis was carried out comparing against baseline (without BTKi) using the Friedman test followed by Dunn's test **(A–E)**. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

concentrations of remibrutinib or rilzabrutinib for 1 h prior to plaque stimulation. Remibrutinib and rilzabrutinib inhibited plaque-induced platelet aggregation with IC_{50} values of 0.03 and 0.16 μM, respectively. Remibrutinib (0.2 μM) and rilzabrutinib (1 μM) were able to block plaque-induced platelet aggregation by >90% (**Figures 1A,B**). Accordingly, remibrutinib is more potent than rilzabrutinib.

The aggregation tracings in **Figure 1C** and panels in **Figures 1D,E** show a dose-dependent increase in delay of aggregation (lag time) caused by both inhibitors.

Effects of Remibrutinib and Rilzabrutinib on Platelet Aggregation Stimulated by Collagen, Ristocetin, FcγRIIA- and G-Protein Coupled Receptor-Activation

Next the effects of remibrutinib and rilzabrutinib were investigated on platelet aggregation induced by stimuli known to activate Btk-dependent and Btk-independent platelet signaling pathways. Concentrations of remibrutinib (0.1 μM) and rilzabrutinib (0.5 μM) were chosen that inhibited atherosclerotic plaque-induced platelet aggregation by 89 and 88%, respectively (**Figure 1A**).

Figure 2 shows the results for platelet stimuli that induce aggregation through a Btk-dependent mechanism (5). GPVI-dependent aggregation was inhibited by remibrutinib and rilzabrutinib by 91 and 94%, respectively, on low dose collagen, and by 37 and 41%, respectively, on high dose collagen (**Figures 2A,B**). Glycoprotein Ib/von Willebrand factor (GPIb/VWF)-dependent ristocetin-induced platelet aggregation was blocked by 95% by both BTKi (**Figure 2C**). The inhibitory effects of remibrutinib and rilzabrutinib on GPVI- and GPIb/VWF-dependent platelet aggregation were similar to those of fenebrutinib (0.1 μM) (**Supplementary Figure 2**), which is a reversible and highly selective Btk inhibitor.

Complete suppression of platelet aggregation by both BTKi was also observed on FcγRIIA activation by crosslinking or anti-CD9 antibody stimulation (**Figures 2D,E**). Due to the absence of adenosine 5'-diphosphate (ADP) secretion from platelets (9), anti-CD9 antibody stimulation showed a delayed aggregation response and less maximal aggregation compared with CD32-crosslinking (**Figure 2E**).

Remibrutinib and rilzabrutinib did not compromise Btk-independent pathways of platelet aggregation stimulated by GPCR activation with thrombin receptor-activating peptide (TRAP), arachidonic acid (AA), or ADP under the conditions tested (**Figure 3**).

Effect of Remibrutinib and Rilzabrutinib on *in vitro* Bleeding Time

To investigate whether remibrutinib and rilzabrutinib might impair primary hemostasis, the platelet function analyzer PFA-200 was used. The instrument aspirates citrate-anticoagulated blood under constant vacuum from a reservoir through a capillary and a small hole in a membrane filter which was coated in our experiments with collagen and epinephrine (collagen/epinephrine cartridge). The time required to obtain full occlusion of the aperture is reported as "*in vitro* closure time" (32, 35). The PFA is used for routine screening of patients with potential hemorrhagic risk and is very sensitive to monitor aspirin intake (36, 37).

Closure time was slightly, but significantly prolonged by 0.1 μM remibrutinib (**Figures 4A,B**) which suppressed >85% Btk-dependent platelet aggregation after GPVI activation with low dose collagen and after VWF/GPIb activation with ristocetin (**Figures 1A**, **2A,C**), but it did not exceed the upper limit of the normal range (170 s). Higher concentrations of remibrutinib (0.2 and 0.5 μM) significantly and profoundly prolonged closure time.

For rilzabrutinib it was found that a concentration of 0.2 μM which inhibited GPVI-dependent plaque-stimulated platelet aggregation by 56% (**Figure 1B**) did not affect significantly the closure time. A concentration of 0.5 μM rilzabrutinib equipotent to 0.1 μM remibrutinib suppressed >90% Btk-dependent platelet aggregation after low dose collagen- and ristocetin-stimulated aggregation (**Table 1**; **Figure 2**) and significantly increased closure time by 67% (**Figures 4A,B**). The closure time was significanly more prolonged than by 0.1 μM remibrutinib (**Figure 4B**). A concentration of 1 μM rilzabrutinib prolonged bleeding time maximally. DMSO, the solvent of BTKi, did not affect closure time as shown previously (9), and the DMSO controls showed similar values at the beginning and the end of the experiments (**Figures 4A,B**).

DISCUSSION

We demonstrate here in our study that (i) remibrutinib and rilzabrutinib inhibit and delay dose-dependently atherosclerotic plaque-induced GPVI-mediated platelet aggregation; (ii) remibrutinib (0.1 μM) and rilzabrutinib (0.5 μM) also block Btk-dependent GPVI-, GPIb/VWF- and FcγRIIa-stimulated platelet aggregation; (iii) higher concentrations of remibrutinib (≥0.2 μM) and therapeutic concentrations of rilzabrutinib (≥0.2 μM) prolong the bleeding time *in vitro* as measured by PFA-200.

According to the dose-response curve (**Figures 1A,B**), the potency for platelet inhibition of low degree GPVI-induced

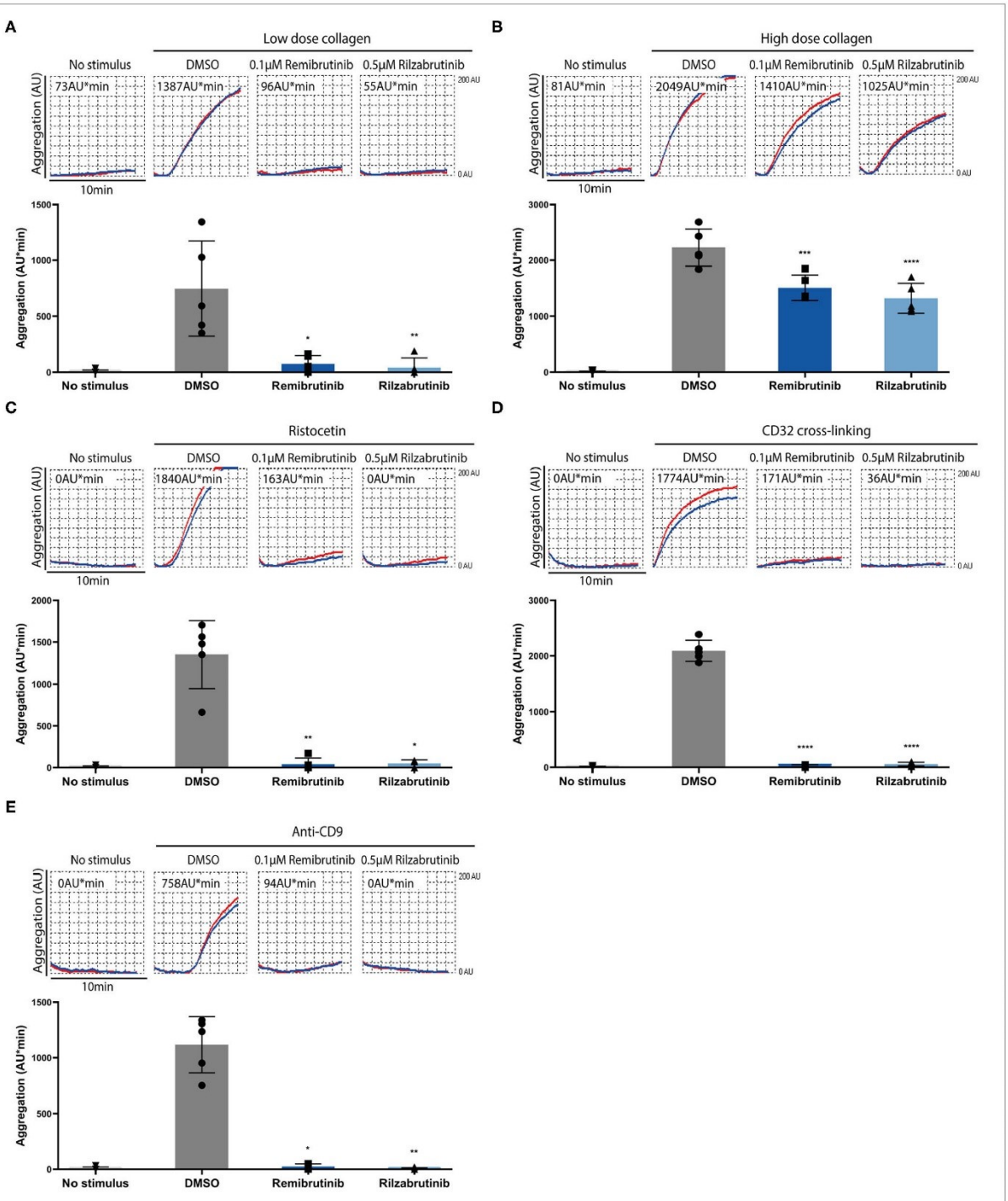

FIGURE 2 | Effects of remibrutinib and rilzabrutinib on platelet aggregation in blood after stimulation by collagen, ristocetin or FcγRIIA activation. Hirudin anticoagulated blood was preincubated for 1 h with DMSO or BTKi (remibrutinib 0.1 μM, rilzabrutinib 0.5 μM) prior to stimulation with **(A)** low dose collagen (0.4–0.6 μg/ml) that was titrated to induce a similar degree of platelet aggregation as plaque homogenate (833 μg/mL) (19), **(B)** high dose collagen (4–6 μg/ml) that was 10× concentrations of the low dose collagen (8, 19), **(C)** ristocetin (0.5 mg/ml), **(D)** CD32 cross-linking (3 min incubation with 2 μg/ml AT10, plus 30 μg/ml Fab2),

(Continued)

FIGURE 2 | or **(E)** anti-CD9 antibody (1 μg/ml). Representative MEA tracings (top panels) and bar graphs (bottom panels) are shown. Values are shown as mean ± SD ($n = 5$). Statistical analysis was carried out using ordinary one-way ANOVA followed by Bonferroni's test **(B,D)** or Kruskal-Wallis followed by Dunn's test **(A,C,E)**. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

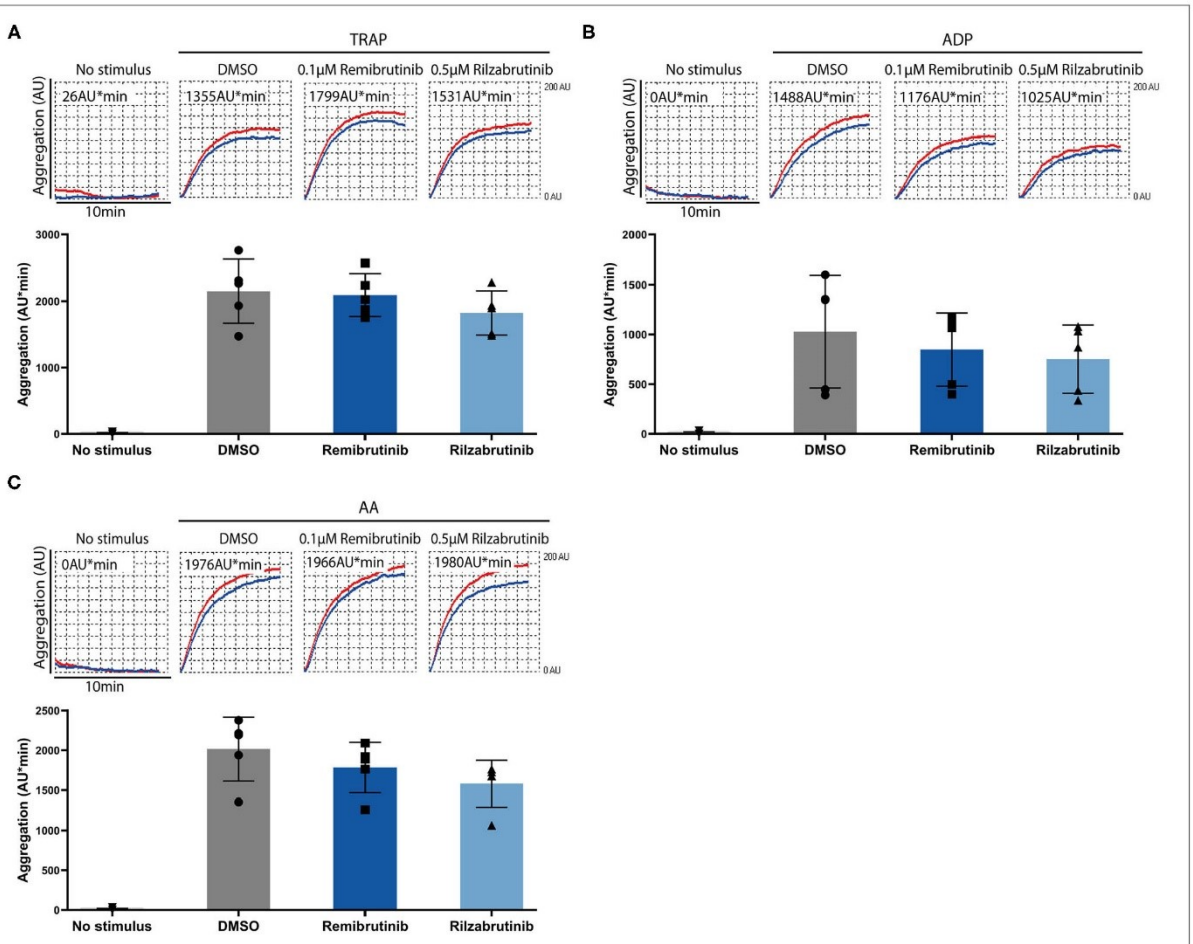

FIGURE 3 | Effects of remibrutinib and rilzabrutinib on platelet aggregation in blood after stimulation by TRAP, ADP, or AA. Hirudin anticoagulated blood samples were pretreated for 1 h at 37°C with solvent control (DMSO, 0.1%), or BTKi (remibrutinib 0.1 μM, rilzabrutinib 0.5 μM) before stimulation with **(A)** TRAP (15 μM), **(B)** ADP (10 μM), or **(C)** AA (0.6 mM). Representative MEA tracings (top panels) and aligned dot blot bar graphs (bottom panels) are shown. Values shown are mean ± SD ($n = 5$). Statistical analysis was carried out using ordinary ANOVA followed by Bonferroni's test **(A,B)** or Kruskal-Wallis test followed by Dunn's test **(C)**, that did not show significant differences.

platelet aggregation of remibrutinib (IC_{50} = 0.03 μM) was 5 times higher than that of rilzabrutinib (IC_{50} = 0.16 μM). Compared with other BTKi (**Table 1**), remibrutinib is only slightly less potent than fenebrutinib (IC_{50} = 0.016 μM) and ibrutinib (IC_{50} = 0.025 μM) and more potent than zanubrutinib, rilzabrutinib, tirabrutinib, acalabrutinib and evobrutinib. The IC_{50} values of remibrutinib (IC_{50} = 0.03 μM) and rilzabrutinib (IC_{50} = 0.16 μM) are 12-times and 2-fold lower than the optimal plasma levels as determined in clinical phase 1 studies, respectively (38, 39). Additionally, both inhibitors induced a dose-dependent increase in delay of atherosclerotic plaque-induced aggregation that was associated with the suppression of aggregation in blood (**Figure 1C**). A delay was also shown in a previous study using ibrutinib- and acalabrutinib-treated washed platelets stimulated by collagen while the maximal aggregation was unaffected (17).

Remibrutinib (0.1 μM) and rilzabrutinib (0.5 μM) significantly suppressed by >90% GPVI-dependent aggregation on low dose collagen, GPIb/VWF-dependent aggregation on ristocetin stimulation, and FcγRIIA-dependent aggregation

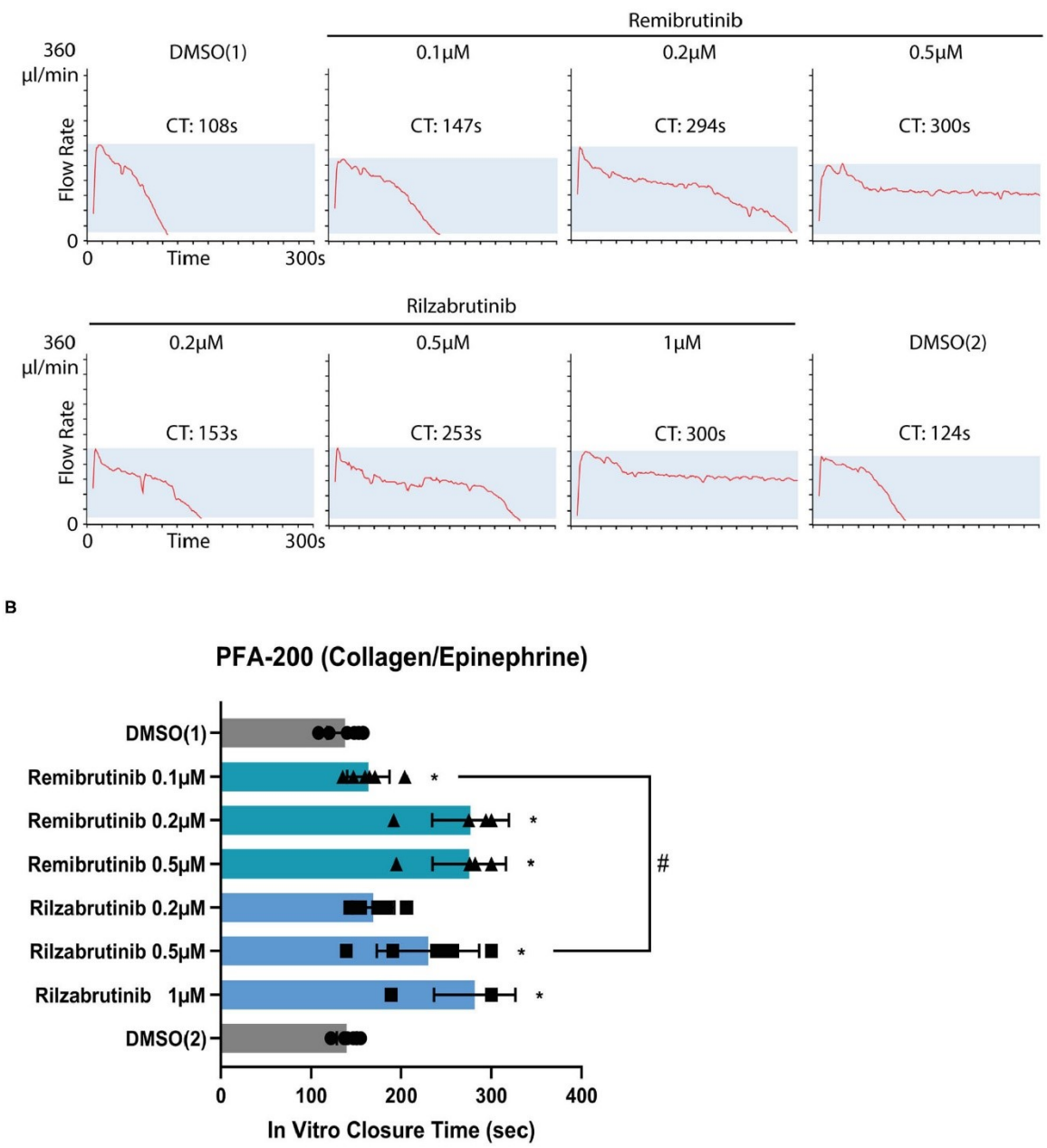

FIGURE 4 | Effect of remibrutinib and rilzabrutinib on bleeding time *in vitro*. Citrate anticoagulated blood samples were pre-incubated for 1 h at 37°C with solvent (DMSO, 0.1%) or increasing concentrations of remibrutinib or rilzabrutinib and then transferred to collagen/epinephrine cartridges. The *in vitro* closure time (CT) was measured with the PFA-200. DMSO (1) and DMSO (2) control samples were measured at the beginning and end of the experiment, respectively. **(A)** Representative PFA-200 tracings. **(B)** The aligned dot plot bar charts show CT values of different concentrations of remibrutinib and rilzabrutinib. Values are mean ± SD ($n = 6$). Statistical analysis was carried out using the Wilcoxon matched-pairs signed rank test comparing against DMSO1 (*$p < 0.05$) or concentrations of remibrutinib (0.1 µM) and rilzabrutinib (0.5 µM) that inhibited Btk-dependent pathways of platelet aggregation by >90% (#$p < 0.05$).

TABLE 1 | IC_{50} values of remibrutinib, rilzabrutinib and other BTK inhibitors for inhibition of low degree GPVI stimulated platelet aggregation, and comparison with therapeutic drug plasma levels.

BTK Inhibitors	IC_{50} (μM)	Therapeutic drug plasma level (μM)
Remibrutinib	0.03*	0.46[a]
Rilzabrutinib	0.16*	0.33[b]
Fenebrutinib	0.016*	0.6[c]
Ibrutinib	0.025#	0.31[d]
Zanubrutinib	0.094#	1.4[e]
Tirabrutinib	0.268#	1.96[f]
Acalabrutinib	0.372#	1.78[g]
Evobrutinib	1.20#	Not known

*Hirudin-anticoagulated blood was pre-incubated with the BTKi for 1 h or 15 min (fenebrutinib, **Supplementary Figure 1**) prior to stimulation with plaque homogenate.
#Hirudin-anticoagulated blood was pre-incubated with the BTKi for 1 h before stimulation with low collagen concentrations (0.2–0.5 μg/ml). Platelet aggregation was measured by multiple electrode aggregometry (MEA).
[a]Remibrutinib, 100 mg q.d, optimal dose in phase I study (38).
[b]Rilzabrutinib, 300 mg b.i.d (39).
[c]Fenebrutinib, 200 mg q.d (40).
[d]Ibrutinib, 420 mg q.d (41).
[e]Zanubrutinib, 320 mg q.d (42).
[f]Tirabrutinib, 320 mg q.d (43).
[g]Acalabrutinib, 100 mg b.i.d (44).

upon CD32 cross-linking, but it had no effect on TRAP, AA, or ADP stimulation as expected according to the results of previous studies with other BTKi (7, 9, 16, 17), although it has to be stated that a non-existing effect is difficult to prove and may depend on the experimental conditions.

High dose collagen GPVI-dependent aggregation was suppressed to a similar degree of about 30% with remibrutinib (0.1 μM), rilzabrutinib (0.5 μM) and the Btk-selective reversible BTKi fenebrutinib (0.1 μM) (**Supplementary Figure 2**). This may indicate that the concentrations and incubation conditions of remibrutinib and rilzabrutinib used are selective for inhibition of Btk and unlikely to also inhibit Tec in platelets. This is unexpected considering the potent inhibition of Tec by rilzabrutinib *in vitro* (22). Rilzabrutinib by inhibiting Tec in addition to Btk would have shut-off GPVI signaling after high collagen stimulation.

As shown in several studies, low-degree GPVI activation only depends on Btk, while high dose collagen-induced GPVI signaling is also dependent on Tec co-activation (9, 15, 19, 45). In a previous study, 50 nM fenebrutinib was applied and only suppression of platelet aggregation on low but not high dose collagen stimulation was observed (9). Our different results may be explained due to the higher concentration of fenebrutinib (0.1 μM) applied in our study providing a more complete inhibition of Btk.

Our results show that the remibrutinib concentration to fully inhibit Btk-dependent pathways of platelet aggregation (0.1 μM) is lower than the reported maximal plasma level (0.46 μM) in a phase I study after intake of 100 mg q.d. for 12 days (38). Thus, this concentration is expected to block completely Btk-dependent signaling in platelets *in vivo*. The equivalent rilzabrutinib concentration (0.5 μM) is higher than the plasma C_{max} reported in clinical studies after therapeutic dosage for autoimmune diseases (0.33 μM) (**Table 1**) (39). Since the IC_{50} of rilzabrutinib for inhibition of plaque-induced platelet aggregation in blood was lower (0.16 μM), it is likely that therapeutic concentrations of rilzabrutinib inhibit Btk-dependent pathways of platelet aggregation, but not entirely. Our results are in contrast to findings showing no inhibition of ristocetin- and high dose collagen- induced aggregation of platelet-rich plasma from healthy donors and ITP patients pre-incubated with 1 μM rilzabrutinib for 15 min *in vitro* (26, 46). The discrepancy might be explained by differences of the experimental system used (blood vs. PRP), different concentrations of collagen (low vs. high) and exposure times of rilzabrutinib (long vs. short). We selected a long exposure time (1 h), since this might better simulate the *in vivo* situation after absorption of the drug, and previous studies have shown that platelet inhibition with irreversible BTKi increases with the exposure time (17, 19).

Bleeding is a frequent side effect of treatment with certain irreversible BTKi such as ibrutinib and the second generation BTKi acalabrutinib, zanubrutinib, and tirabrutinib used to treat B-cell malignancies (5, 47–49). Exclusive inhibition of Btk should not increase bleeding since XLA patients who are deficient of Btk do not show an impairment of haemostasis (13). It has been discussed that bleeding by these BTKi is related to off-target inhibition of Tec, since this kinase is functionally involved in GPVI-induced platelet activation (8, 15). By comparison, for fenebrutinib, a reversible highly selective BTKi, which is the most selective BTKi and which shows no inhibition of Tec (50), no bleeding events were reported in clinical trials (non-Hodgkin lymphoma, chronic lymphocytic leukemia, rheumatoid arthritis, and systemic lupus erythematosus) (5). Also, fenebrutinib *in vitro*, even at very high concentrations up to 1 μM did not prolong bleeding time measured by the PFA-200 (9).

The reversible BTKi fenebrutinib binds to an inactive conformation of Btk (51). Also, remibrutinib which was developed from fenebrutinib-like scaffolds to bind to the inactive conformation of Btk (20, 51) showed a 175-fold higher affinity for Btk over Tec in binding assays *in vitro* (20). Thus, it was expected that remibrutinib would not increase *in vitro* bleeding time measured by PFA-200, similar to fenebrutinib (9). However, we observed that bleeding times *in vitro* were already slightly but significantly increased after blood incubation with 0.1 μM remibrutinib (which inhibited >90% of Btk-dependent pathways of platelet aggregation), and strongly prolonged by remibrutinib concentrations of 0.2 and 0.5 μM. The results for remibrutinib are similar to a previous study, in which low concentrations of the irreversible BTKi ibrutinib, zanubrutinib, acalabrutinib, and tirabrutinib inhibited GPVI- dependent platelet aggregation by >70%, but 2- to 2.5-fold higher concentrations of these BTKi were required to significantly increase the bleeding time *in vitro* (19). The increase of closure time was similar to that observed after treatment with low dose aspirin (52).

In a phase I placebo controlled clinical trial of remibrutinib (total 156 healthy subjects), mild self-limited bleeding events were observed only in 4 persons in the multiple-ascending dose cohorts with remibrutinib intake for 12 days. These included two

subjects in the 600 mg q.d. cohort with epistaxis and two subjects in the 100 mg cohort with trauma-triggered hematomas (38).

Rilzabrutinib in our study slightly but non-significantly increased at 0.2 μM closure time in the PFA device, the increase was at 0.5 μM pronounced (**Figure 4B**). Potent Tec inhibition could contribute to the increased *in vitro* bleeding time (5); however, the results of the aggregation studies upon stimulation with high concentrations of collagen argue against simultaneous Tec inhibition in platelets by 0.5 μM of rilzabrutinib (see above).

Thus, the mechanisms underlying the increase of closure times elicited by remibrutinib as well as rilzabrutinib are unlikely to involve off-target inhibition of Tec. They could be related to effects on the Btk protein itself. Recently it was found that binding of certain irreversible BTKi (except fenebrutinib) to the kinase domain had long-range allosteric effects on the SH2-and SH3- regulatory domains changing their conformation toward an activated state of the protein (53).

In contrast to remibrutinib, there was not a clear difference of rilzabrutinib concentrations that inhibited Btk-dependent pathways of platelet aggregation in the MEA and robustly increased the closure time in the PFA; the concentration of rilzabrutinib of 0.5 μM does both. Maximal therapeutic concentrations of rilzabrutinib (0.33 μM) are expected to significantly increase the closure time in the PFA device, but no treatment-related bleeding had been noted in the ITP clinical trial with rilzabrutinib (25), although the median platelet count at study entry was only 14.173/μl (25). However, 7% (2/27) of patients treated with rilzabrutinib had treatment-related epistaxis as observed in the latest pemphigus clinical trial (24).

LIMITATIONS

Although our *in vitro* study has the advantage of reducing the complexity of the experimental conditions, and the different effects of the two BTKi studied on platelets in blood are obvious, these data cannot directly be translated into the situation *in vivo*. Clinical studies of platelet function *ex vivo* after oral intake of therapeutic dosage are warranted to approach the *in vivo* effects of remibrutinib and rilzabrutinib on platelets.

CONCLUSION

In the present study we found significant differences of the two BTKi remibrutinib and rilzabrutinib on platelets that would favor remibrutinib as a candidate for further development as an antiplatelet drug to inhibit Btk-dependent platelet activation pathways underlying atherothrombosis and certain platelet-related immune disorders. Since *de novo* protein synthesis in platelets is very limited and because low concentrations of irreversible BTKi such as remibrutinib may covalently inactivate platelet BTK already by a single exposure at low concentrations during absorption, it is likely that low doses of such a selective irreversible BTKi are effective in cardiovascular prevention without affecting the immune system (7, 8, 54). Our study further suggests that off-target effects on Tec are unlike to be involved in the increase of closure time measured by PFA, and may not explain the bleeding side effects elicited by BTKi.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Faculty of Medicine of the University of Munich. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

RD designed and performed experiments, analyzed data, and wrote the manuscript. LG performed experiments and analyzed data. RB provided plaque material. MS supervised experiments and contributed to discussions. CW supervised and contributed to discussions. WS conceived the study, designed experiments, and wrote the manuscript. PH supervised and analyzed experiments and wrote the manuscript. All authors have contributed significantly to this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.749022/full#supplementary-material

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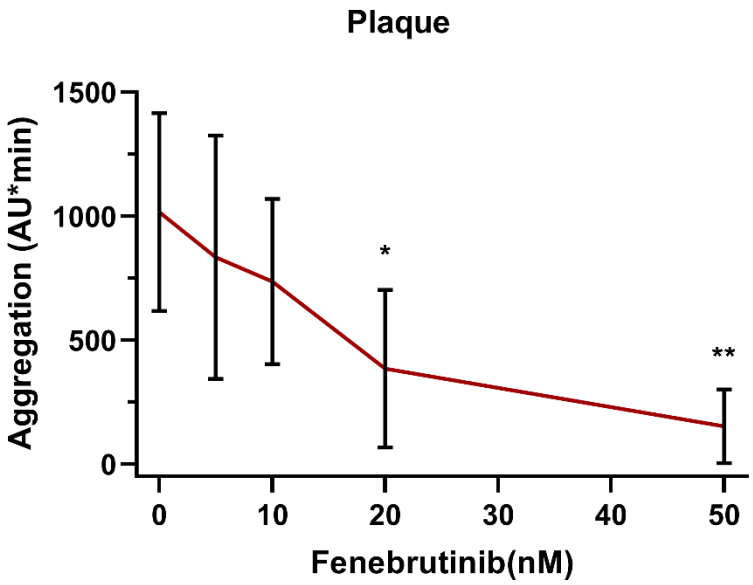
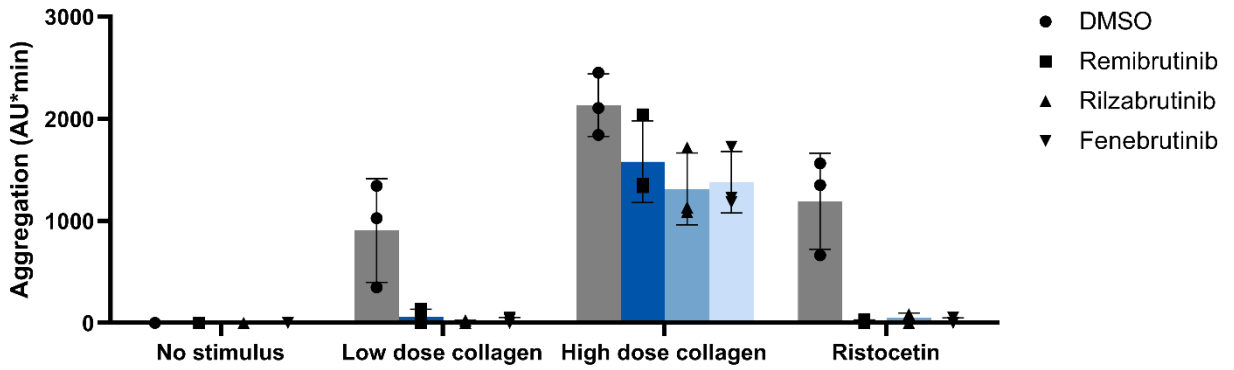
Supplemental Figure 1



Supplemental Figure 1. Dose-response curve of fenebrutinib on plaque homogenate- induced platelet aggregation. Hirudin anticoagulated blood were preincubated with solvent (DMSO, 0.1%) or increasing concentrations of fenebrutinib for 15min at 37°C before stimulation with plaque homogenate (833µg/ml). Statistical analysis was carried out comparing against baseline (without BTKi) using the ordinary one-way ANOVA followed by Bonferroni's test. Values are mean ± SD (n=5). *p<0.05, **p<0.01.

Supplemental Figure 2



Supplemental Figure 2. Comparison of remibrutinib and rilzabrutinib with fenebrutinib on inhibition of GPVI- and GPIb/VWF- dependent platelet aggregation. Hirudin anticoagulated blood samples were treated with solvent control (DMSO, 0.1%), 0.1 µM remibrutinib, 0.5µM rilzabrutinib or 0.1µM fenebrutinib for 1 hour at 37°C prior to stimulation for 10 min with low dose collagen (0.4-0.6µg/ml), high dose collagen (4-6µg/ml), or ristocetin (0.5mg/ml). Bar graphs show the effect of BTKi on platelet aggregation upon stimulation (n=3).

8. Paper II

International Journal of *Molecular Sciences*

MDPI

Article

Spontaneous Platelet Aggregation in Blood Is Mediated by FcγRIIA Stimulation of Bruton's Tyrosine Kinase

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Abstract: High platelet reactivity leading to spontaneous platelet aggregation (SPA) is a hallmark of cardiovascular diseases; however, the mechanism underlying SPA remains obscure. Platelet aggregation in stirred hirudin-anticoagulated blood was measured by multiple electrode aggregometry (MEA) for 10 min. SPA started after a delay of 2–3 min. In our cohort of healthy blood donors (n = 118), nine donors (8%) with high SPA (>250 AU*min) were detected. Pre-incubation of blood with two different antibodies against the platelet Fc-receptor (anti-FcγRIIA, CD32a) significantly reduced high SPA by 86%. High but not normal SPA was dose-dependently and significantly reduced by blocking Fc of human IgG with a specific antibody. SPA was completely abrogated by blood pre-incubation with the reversible Btk-inhibitor (BTKi) fenebrutinib (50 nM), and 3 h after intake of the irreversible BTKi ibrutinib (280 mg) by healthy volunteers. Increased SPA was associated with higher platelet GPVI reactivity. Anti-platelet factor 4 (PF4)/polyanion IgG complexes were excluded as activators of the platelet Fc-receptor. Our results indicate that high SPA in blood is due to platelet FcγRIIA stimulation by unidentified IgG complexes and mediated by Btk activation. The relevance of our findings for SPA as possible risk factor of cardiovascular diseases and pathogenic factor contributing to certain autoimmune diseases is discussed.

Keywords: platelet; FcγRIIA; Btk; PF4; CD32; spontaneous platelet aggregation; multiple electrode aggregometry; ibrutinib; fenebrutinib; GPVI

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1. Introduction

High platelet reactivity leading to spontaneous platelet aggregation (SPA) is a hallmark of cardiovascular diseases; however, the mechanism underlying SPA remains obscure. SPA measured in platelet-rich plasma (PRP) or blood is enhanced in patients with diabetes, acute coronary syndrome, myocardial infarction, and cerebrovascular disease [1–6], and has been reported to be a predictive risk marker of arterial occlusions in patients with diabetes and myocardial infarction [7–9]. Measurement of SPA using blood instead of PRP may reflect better systemic platelet reactivity in vivo, since blood contains other blood cells modifying platelet reactivity, and also the whole heterogeneous platelet population including the larger, more reactive young reticulated platelets [10,11].

Platelet aggregation in stirred anticoagulated blood can be easily and reliably measured by multiple electrode aggregometry (MEA) [12], a method that is now widely used to determine stimulated platelet aggregation in blood in patients with various diseases. However, in stirred or shaken blood, ADP leaking along with ATP from red cells elicits SPA and enhances stimulus-induced platelet aggregation [13–15]. We found that the omission of stirring during the 3 min warming-up phase before start of the MEA measurement virtually eliminated the ATP/ADP release from red cells and the subsequent ADP-mediated

SPA [15]. Using this protocol, we now describe in the present study our observations of a novel type of SPA in blood and delineate the underlying mechanism.

2. Results

Figure 1A shows representative aggregation tracings of a donor with high SPA as compared to a donor with normal SPA. SPA in blood from donor B began to increase about 2 min after start of stirring. By defining an aggregation threshold of >250 AU*min we detected in our pool of healthy blood donors (n = 118) 9 donors (8%) with increased SPA on 2 different days of measurements (duplicate or triplicate determinations) with intervals of 2 days to 22 months between the tests. In another seven donors, increased SPA was observed in only one out of two to three different experiments performed 7 days to 28 months apart. Thus, increased SPA could be found in some blood donors over a long time period.

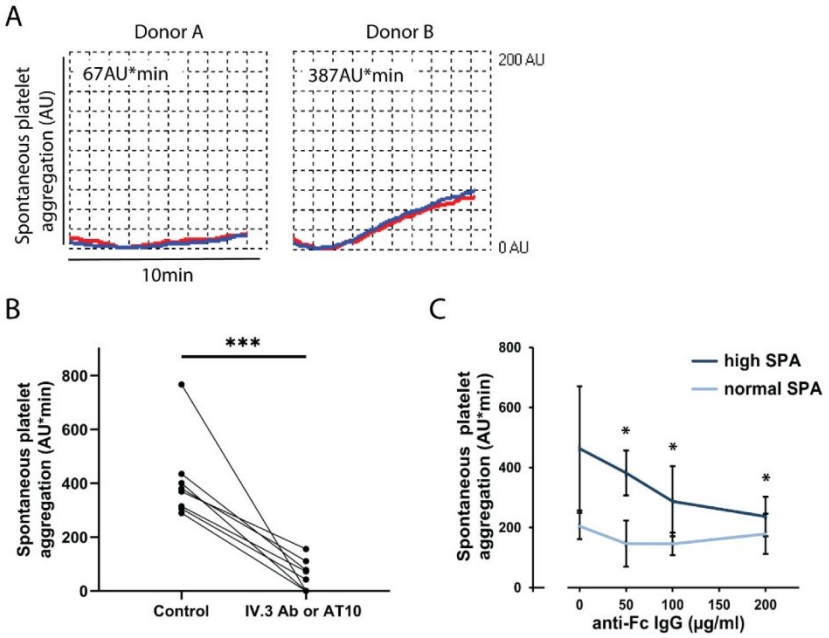

Figure 1. Increased spontaneous platelet aggregation (SPA) in blood is mediated by activation of FcγRIIA. (**A**) Normal and increased SPA in blood from donor A and B, respectively. The anticoagulated blood samples were prewarmed for 3 min without stirring at 37 °C. The stirrer was then added, and the measurement was started. Platelet aggregation was measured for 10 min by MEA. Representative tracings recorded by the two electrode pairs (blue, red) are shown. Increased SPA in donor B begins after 2 min. (**B**) Inhibition of increased SPA by the anti-FcγRIIA antibody IV.3 or AT10. Blood samples from subjects (n = 8) with increased SPA (289-767 AU*min) were incubated for 3 min without (control) or with the CD32 antibody IV.3 or AT10 (2 µg/mL), and then stirred for 10 min. The values are means ± SD (n = 8). *** $p < 0.001$. **C.** Effects of anti-human Fc-IgG antibody on SPA. Blood samples from donors with increased SPA (>250 AU*min, n = 4) and normal SPA (<250 AU*min, n = 4) were pre-incubated for 3 min with different concentrations (50, 100 and 200 µg/mL) of a goat antibody (IgG) directed against the Fc part of human IgG. Then, stirring and measurements (10 min) were started. The values are means ± SD (n = 4). * $p < 0.05$ compared to the samples without Fc-IgG antibodies.

Next, we searched for the underlying mechanisms. Increased SPA was completely inhibited by pre-incubating the blood samples with the integrin αIIbβ3 blocking peptide RGDS (2 mM) or the anti-integrin αIIbβ3 antibody abciximab (20 µg/mL), indicating an

essential role of the integrin αIIbβ3 (data not shown). Then, we used a large range of inhibitors to search for platelet receptors that might signal to activate the integrin αIIbβ3 mediating SPA. The inhibitors were: aspirin, the P2Y12 antagonist ticagrelor, ketanserin (5-hydroxytryptamine 2A receptor antagonist), lysophosphatidic acid receptor-5 (LPA5) receptor antagonists, hirudin in very high concentrations to ensure complete inhibition of thrombin and subsequent fibrin formation, the anti-GPVI antibody 5C4, GPVI-Fc, or the anti-FcγRIIA (CD32a) antibodies IV.3 and AT10.

We found that only pre-incubation of blood samples with the anti-FcγRIIA antibodies significantly inhibited the increased SPA; the mean reduction was 85.9% (Figure 1B). FcγRIIA is typically activated by IgG immune complexes which bind with their Fc-part to FcγRIIA, thereby cross-linking the receptor and inducing intracellular signaling. Blood samples were therefore pre-incubated with increasing concentrations of goat anti-human Fc (IgG) antibody. High SPA was dose-dependently and significantly reduced by the anti-human Fc antibody; values of normal SPA were, however, not affected. This indicates that in certain blood donors the ligation of FcγRIIA by endogenous IgG immune complexes evokes high SPA.

Stimulation of FcγRIIA leads to stimulation of Bruton tyrosine kinase (Btk) [16], and we had recently found that FcγRIIA-induced platelet aggregation, dense and α–granule secretion in blood is abrogated by Btk-inhibitors (BTKi) [17]. Pre-incubation of blood with the reversible BTKi fenebrutinib (50 nM) significantly inhibited SPA in donors with normal and high SPA values (59-432 AU*min) (Figure 2A). In addition, oral intake of a single dose of the irreversible BTKi ibrutinib (280 mg) by healthy volunteers completely inhibited SPA three hours after ingestion (Figure 2B). SPA inhibition was sustained for 2 days, which is explained by the irreversible, covalent Btk inactivation by ibrutinib and the lack of de novo protein synthesis in platelets. The recovery of SPA paralleled the expected physiological platelet renewal rate, and SPA was reversed toward control 7 days after ibrutinib intake. The results of inhibition of SPA by BTKi are in accordance with previous observations of SPA inhibition in blood by pre-incubating blood with various irreversible BTKi in vitro or by oral intake of ibrutinib ex vivo, either by CLL patients or by healthy volunteers [18].

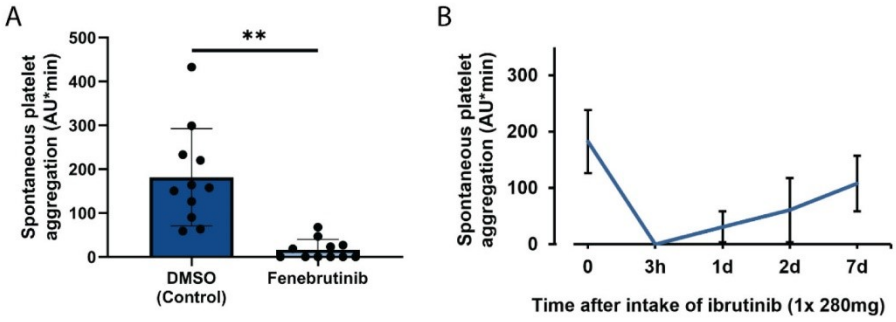

Figure 2. SPA in blood is inhibited by Btk-inhibitors in vitro and ex vivo. (**A**) Effect of the reversible Btk inhibitor fenebrutinib on SPA in vitro. The blood samples were incubated for 15 min with the solvent control DMSO (0.1%) or with fenebrutinib (50 nM) in the MEA cuvettes, then the stir bar was added, and the measurement (10 min) was started. The values are mean ± SD (n = 11). ** $p < 0.01$. The SPA values ranged from 59 to 432 AU*min. (**B**) Effect of oral intake of ibrutinib by a healthy blood donor on SPA ex vivo. SPA was measured before, and 3 hours (h), 1, 2, and 7 days (d) after intake of Imbruvica® (280 mg). Representative results of one of three donors are shown. The values are means ± SD of triplicate determinations.

In order to explore whether SPA might affect platelet aggregation in response to stimuli, platelet aggregation was stimulated by a low concentration of collagen, which induces a low degree of platelet GPVI activation similar to atherosclerotic plaque homogenate [19,20]. By comparison with donors exhibiting normal SPA, donors with increased SPA showed a

significantly increased platelet aggregation in response to collagen (Figure 3A). Furthermore, Figure 3B shows a significant positive correlation (r = 0.63, $p < 0.05$) between SPA and platelet aggregation in response to low collagen concentration. These data provide a link of SPA and enhanced platelet reactivity to GPVI that both have been observed in patients with cardiovascular diseases (see Section 3).

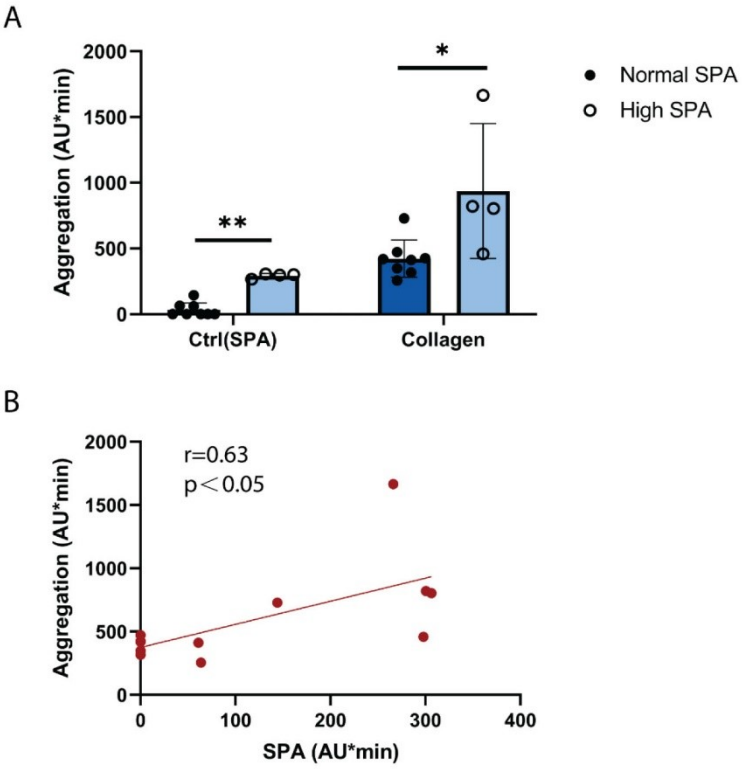

Figure 3. High SPA is associated with increased platelet GPVI reactivity. Hirudin-anticoagulated blood was prewarmed at 37 °C for 3 min. Collagen (0.1 µg/mL) was added, stirring was started, and platelet aggregation was measured for 10 min. (**A**) Blood of donors with high SPA responded more strongly to low-dose collagen than donors with normal SPA. Values are mean ± SD ($n = 4$ with high SPA and $n = 8$ with low SPA). * $p < 0.05$, ** $p < 0.01$. (**B**) SPA and platelet reactivity to collagen (0.1 µg/mL) correlate positively and significantly (Spearman correlation coefficient r = 0.63; $p = 0.03$). Each data point represents an individual subject ($n = 12$).

We also tested the platelet aggregation responses to the weak G-protein receptor coupled stimulus CXCL12 (SDF-1), a platelet chemokine involved in atherothrombosis [21]. However, no differences of platelet aggregation in donors with normal and high SPA and no correlation of SPA and CXCL12 induced aggregation were found (data not shown).

To identify the platelet Fc-receptor activating IgG, antibodies to platelet factor 4 (PF4) in complex with polyvinylsulfonate (a heparin analogue) were measured by ELISA. Platelet factor 4 (PF4) is a positive-charged chemokine, and when binding to polyanions, the conformational change of PF4 is able to induce the generation of IgG antibodies that are able to cross-link and activate FcγRIIA receptors on the platelet surface [22]. Indeed, high titers of anti-PF4/polyanion and anti-PF4/heparin IgG are found in autoimmune heparin-induced thrombocytopenia (HIT) and vaccine-induced immune thrombotic thrombocytopenia (VITT) that stimulate platelet FcγRIIA receptors and subsequent Btk-mediated platelet activation [17,23–27]. Moreover, 5 to 7% of healthy blood donors have detectable PF4/heparin antibodies [28]. However, our data showed only very low anti-PF4/polyanion

IgG titers with no correlation to SPA (correlation coefficient = 0.29; Figure 4A). Moreover, the addition of PF4, heparin or PF4 plus heparin did not increase SPA (Figure 4B). These data show that anti-PF4 IgG is not involved in triggering SPA.

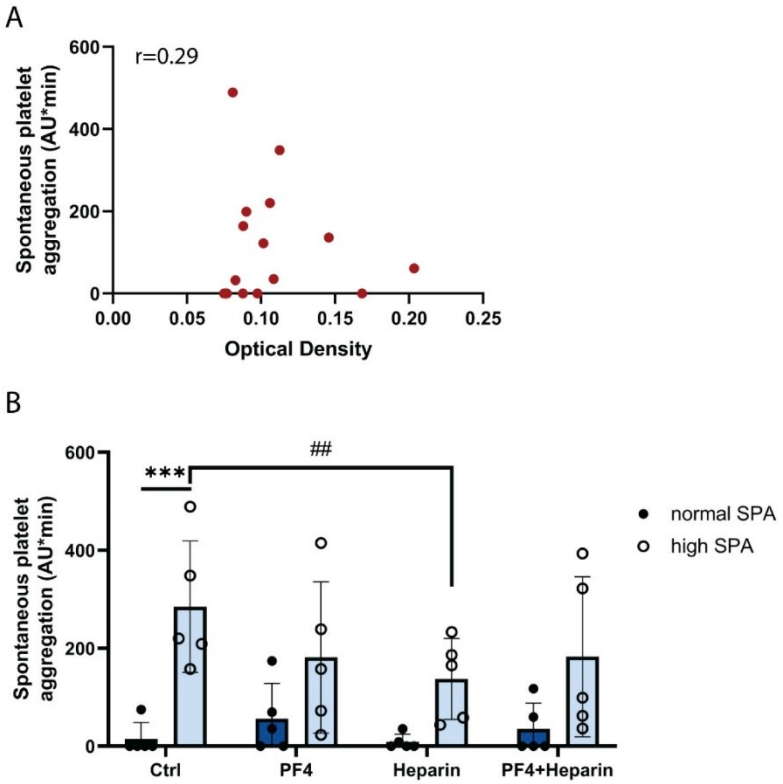

Figure 4. SPA is not mediated by anti-PF4 IgG and not stimulated by PF4 and/or heparin. (**A**) No correlation between SPA and serum titers of anti-PF4/polyanion IgG. The scatterplots display SPA and optical density of the IgG titers. Each dot on the graph represents a subject (n = 17). (**B**) SPA was not increased by incubation with PF4 and/or heparin. The hirudin-anticoagulated blood samples were prewarmed for 3 min at 37 °C. PF4 (5 µg/mL) or heparin (1 U/mL) were added, stirring was started and aggregation was measured for 10 min. The values are means ± SD (n = 5). *** $p < 0.001$, ## < 0.01.

3. Discussion

In the present study, we describe a novel protocol to measure SPA that uses stirred hirudin-anticoagulated blood and MEA, a device that had first been validated in 2006 by comparison with the single platelet counting (SPC) method that measures the decrease in platelet counts upon platelet aggregation [12]. MEA is now widely used to measure platelet function in patients with various hematological and cardiovascular diseases. SPA in previous studies was mostly measured in stirred, citrated anticoagulated PRP using light transmission or laser scattering methods [1,4–9]. However, the use of PRP for SPA measurement as compared to blood has several disadvantages: (a) other blood cells which can influence platelet reactivity are absent in PRP; (b) the preparation of PRP requires blood centrifugation that will per se and through secondary mechanisms modify platelet reactivity [1,12], and (c) will, dependent on the centrifugation speed, eliminate the larger and more reactive platelet populations. Furthermore, the use of citrate as anticoagulant favors platelet secretion and reactivity [29]. In the present study, hirudin was used as an anticoagulant, which does not alter the physiological blood concentrations of free Ca and

Mg crucial for platelet aggregation [12,29]. In other previous studies, SPA was measured in shaken or stirred citrate-anticoagulated blood by SPC [2,3,30,31]. Interestingly, enhanced SPA was found in blood [2,3,30,31] but not PRP of diabetic patients [30] and was found to be related to the red blood cell fragility of diabetic patients [30]. Indeed, it is now known that ADP released from red cells can mediate SPA in shaken or stirred blood [13,14], if no precautions are taken [15]. Our method of SPA measurement described in the present study avoids these possible artefacts, and by using MEA instead of SPC (which requires fixation of blood samples at given time points), SPA can be continuously monitored [12].

Our results indicate that high SPA in blood as observed in a small percentage of human volunteers is due to platelet FcγRIIA stimulation by IgG immune complexes, as documented by the inhibition with antibodies against FcγRIIA and Fc of IgG, respectively. This might be explained by (a) a high reactivity of the platelet FcγRIIA to trace amounts of IgG immune complexes or (b) increased levels of circulating IgG immune complexes.

Supporting the first possibility, platelet FcγRIIA expression was found to be increased in patients with cardiovascular diseases (acute myocardial infarction, unstable angina, or ischemic stroke) and interestingly also in healthy patients with two or more atherosclerosis risk factors [32]. Recently, expression of platelet FcγRIIA was proposed to be included in clinical risk scores to refine the assessment of cardiovascular risk after myocardial infarction [33]. It would be interesting to evaluate SPA according to the protocol described in our study in patients with cardiovascular diseases and evaluate its relation to platelet FcγRIIA expression. Furthermore, our data show a link of SPA and platelet aggregation induced by low-degree GPVI activation. Platelet reactivity to GPVI is enhanced in patients with diabetes and obesity [34,35], and platelet GPVI surface expression was significantly increased in patients with acute coronary syndrome, TIA, and stroke [36,37]. Our findings that Btk inhibitors abrogate SPA suggest that they might thereby reduce also enhanced platelet GPVI sensitivity to GPVI. Such a beneficial action might add to the suppression of atherosclerotic plaque-induced platelet GPVI activation by low concentrations of Btk inhibitors in vitro and ex vivo [18,19].

Our findings could also be related to the sticky platelet syndrome, a thrombophilic qualitative platelet disorder characterized by increased in vitro platelet adhesion and aggregation in response to very low concentrations of the weak platelet agonist ADP and/or epinephrine, and increased risk of arterial ischemic events [38]. This possibility deserves further investigation. However, our data did not show a link between SPA and platelet aggregation induced by another weak platelet agonist, CXCL12.

A high platelet FcγRIIA reactivity might be also explained by a genetic polymorphism of FcγRIIA. Platelets from donors with the homozygous Arg-131-genotype are more sensitive to activating immune complexes [39]. In systemic lupus erythematodes (SLE), the FcγRIIA-Arg-131 genotype is overrepresented, associates with subclinical atherosclerosis [40], and platelets of homozygous Arg-131 SLE patients are more sensitive to stimulation as compared to SLE patients with the His/His-131 genotype [41]. Ligation of platelet FcγRIIA by immune complexes sensitizes platelets to activation by thrombin, which might explain vascular complications associated with SLE [42]. It would be of interest to study SPA in patients with SLE and its relationship to FcγRIIA genotype and vascular complications. Moreover, several Btk-inhibitors (fenebrutinib, branebrutinib) are being studied in clinical trials of patients with SLE. Inhibition of SPA by Btk inhibitors could be related to the clinical outcome.

A further possibility underlying SPA may be the amplification of FcγRIIA-triggered aggregation by subthreshold concentrations of other platelet stimuli. These might particularly be stimuli activating the Gi-protein pathway in platelets [43] such as epinephrine [44] or CXCL12 [45] known to be present at low levels in blood. Additionally, the purinergic P2X1 receptor activated by ATP has been reported to amplify FcγRIIA-induced Ca^{2+} increases and functional responses of platelets [46]. Moreover, ATP as well as ADP might be released from red cells during the 10 min stirring period and could enhance SPA. However, our previous study showed barely any increase in ATP during 3 min of stirring using the

described modified MEA protocol (see Figure 1D of [15]), and in the present study, we could not find significant inhibition of SPA by the PY12-receptor antagonist ticagrelor (data not shown). Furthermore, Btk inhibitors inhibited SPA but do not affect ADP-induced platelet aggregation measured by our modified MEA method [18]. Therefore, it is unlikely that ATP or ADP mediate SPA as measured in our study.

Our results show that ligation of FcγRIIA is required for high SPA. IgG-containing immune complexes must be involved, since increased SPA was inhibited by an anti-human Fc (IgG) antibody. The nature of the immune complexes can only be speculated about. Anti-PF4/polyanion IgG complexes were excluded in our study. They would have been a likely candidate, since in 5–7% of healthy blood donors anti-PF4/heparin IgG titers can be detected [28]. Rheumatoid factors (RF), which are autoantibodies of different Ig subclasses (IgM, IgG) against the Fc-portion of IgG and present in plasma from patients with rheumatoid arthritis and other autoimmune diseases, have been described in up to 5% of healthy 50-year-old persons [47]. However, evidence that RF/IgG complexes activate directly FcγRIIA receptors is lacking.

4. Materials and Methods

4.1. Materials

The CD32 antibody IV.3 (monoclonal mouse IgG2b) was obtained from GeneTex (Irvine, CA, USA). The anti-CD32 antibody AT10 (monoclonal mouse IgG1) was from ThermoFisher Scientific (Waltham, MA, USA). Fenebrutinib (GDC-0853) was purchased from MedChem Express (Monmouth Junction, Middlesex, NJ, USA). Ibrutinib was from Selleckchem (Houston, TX, USA). Collagen was from Takeda (Linz, Austria). Dimethyl sulfoxide (DMSO) was from Sigma-Aldrich (Taufkirchen, Germany). Imbruvica capsules (140 mg) were from Pharmacyclics (Sunnyvale, CA, USA). The goat ImmunoPure® anti-human Fc (IgG) antibody was from ThermoFisher Scientific (Rockford, IL, USA). PF4 was from ChromaTec (Greifswald, Germany). Heparin-Sodium was from Ratiopharm (Ulm, Germany). Abciximab (ReoPro®) was from Janssen Biologicals B.V. (Leiden, The Netherlands).

4.2. Blood and Serum Collection

Venous blood was collected by cubital venipuncture from healthy donors who had not taken any antiplatelet drug for two weeks. A 21-gauge butterfly needle was used, and blood was drawn without venous stasis, into either 3 mL vacuum tubes from Dynabyte (Munich, Germany) containing hirudin (final hirudin concentration in blood: 200 U/mL) or S-monovette hirudin tubes from SARSTEDT (Nümbrecht, Germany) (final hirudin concentration in blood: 525 U/mL). The first 2 mL was discarded. The blood was kept at room temperature for 30 min, and measurements were performed subsequently within 2 h after venipuncture.

S-monovette serum gel CAT/7.5 mL tubes (Nümbrecht, Germany) were used for serum preparation for the IgG-specific PF4/polyvinylsulfonate ELISA. After collection, the blood was kept undisturbed at room temperature for 30 min, followed by 1000× *g* centrifugation for 10 min at 4 °C. The aliquots of serum were stored at −20 °C before measurement.

4.3. Platelet Aggregation in Blood

Platelet aggregation measurements were performed by multiple electrode aggregometry (MEA) (Roche Diagnostics, Mannheim, Germany), which records the change of electrical impedance between two sensor electrode pairs caused by the aggregation of platelets, as described [12]. In brief, saline (300 µL) and aliquots (300 µL) of hirudin-anticoagulated blood were placed in aggregometer cuvettes (06675590, Roche, Mannheim, Germany). In order to avoid ADP leakage from red cells and subsequent ADP-mediated SPA, blood was first pre-warmed in the MEA cuvettes without stirring for 3 min, and then the stir bar was added and measurement was started [15]. Aggregation was recorded for 10 min. In some samples, fenebrutinib or DMSO (solvent control; 0.6 µL) was added and mixed well with a pipet, then covered and incubated for 15 min at 37 °C. Antibodies AT10 or IV.3 were added

3 min prior to the measurement. Platelet aggregation was recorded in arbitrary units (AU), and the area under the curve shows the cumulative aggregation (AU*min) from 0 to 10 min. The traces from a specific experiment whose values were closest to the mean were selected as representative traces.

4.4. Enzyme-Linked Immunosorbent Assay (ELISA) of Anti-PF4 IgG

The IgG-specific PF4/polyvinylsulfonate (PVS) ELISA (Immucor, Waukesha, USA) was performed to detect antibodies recognizing PF4/PVS in human serum, and the optical density (OD) values ≥ 0.400 are regarded as positive results per manufacturer's instruction.

4.5. Statistics

The data are given as mean $\pm$ standard deviation (SD) of the indicated number of the experiments. The Shapiro–Wilk test was applied to assess the normal distribution of values. The Wilcoxon test was performed on the paired data and Mann–Whitney test on the unpaired data that did not pass the Shapiro–Wilk test. For normal distributed paired data, a paired *t*-test was performed. All correlations were calculated using the non-parametric Spearman test.

5. Conclusions

In summary, we describe a novel protocol to measure SPA that uses stirred hirudin-anticoagulated blood and MEA. High SPA was detected in a small percentage of healthy donors. The underlying mechanism was dependent on platelet FcγRIIA stimulation by unknown IgG complexes and mediated by Btk activation. Future studies are warranted to investigate whether SPA as described in the present study might be a risk marker of atherothrombotic cardiovascular diseases and pathogenic factor accelerating SLE or other auto-immune diseases.

Author Contributions: R.D. designed and performed experiments, analyzed data, and wrote the manuscript; L.G. and Y.L. performed experiments and analyzed data; C.W. supervised and contributed to discussions; W.S. conceived the study, designed experiments, and wrote the manuscript; P.v.H. supervised and analyzed experiments, and wrote the manuscript. All authors have contributed significantly to this manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the by the Ethics Committee of the Faculty of Medicine of the University of Munich (AZ 365-14).

Informed Consent Statement: All blood donors gave their informed consent and signed a document approved by the Ethics Committee of the Faculty of Medicine of the University of Munich.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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Conflicts of Interest: The authors declare no conflict of interest.

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9. Paper III

REGULAR ARTICLE

blood advances

Oral Bruton tyrosine kinase inhibitors block activation of the platelet Fc receptor CD32a (FcγRIIA): a new option in HIT?

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Key Points

- Six different BTKi's blocked platelet activation in blood after FcγRIIA stimulation by cross-linking, anti-CD9 antibodies, or HIT serum.
- Established oral irreversible and novel reversible BTKi's may offer a new option to treat HIT.

Activation of the platelet Fc-receptor CD32a (FcγRIIA) is an early and crucial step in the pathogenesis of heparin-induced thrombocytopenia type II (HIT) that has not been therapeutically targeted. Downstream FcγRIIA Bruton tyrosine kinase (BTK) is activated; however, its role in Fc receptor–induced platelet activation is unknown. We explored the potential to prevent FcγRIIA-induced platelet activation by BTK inhibitors (BTKi's) approved (ibrutinib, acalabrutinib) or in clinical trials (zanubrutinib [BGB-3111] and tirabrutinib [ONO/GS-4059]) for B-cell malignancies, or in trials for autoimmune diseases (evobrutinib, fenebrutinib [GDC-0853]). We found that all BTKi's blocked platelet activation in blood after FcγRIIA stimulation by antibody-mediated cross-linking (inducing platelet aggregation and secretion) or anti-CD9 antibody (inducing platelet aggregation only). The concentrations that inhibit 50% (IC_{50}) of FcγRIIA cross-linking–induced platelet aggregation were for the irreversible BTKi's ibrutinib 0.08 μM, zanubrutinib 0.11 μM, acalabrutinib 0.38 μM, tirabrutinib 0.42 μM, evobrutinib 1.13 μM, and for the reversible BTKi fenebrutinib 0.011 μM. IC_{50} values for ibrutinib and acalabrutinib were four- to fivefold lower than the drug plasma concentrations in patients treated for B-cell malignancies. The BTKi's also suppressed adenosine triphosphate secretion, P-selectin expression, and platelet-neutrophil complex formation after FcγRIIA cross-linking. Moreover, platelet aggregation in donor blood stimulated by sera from HIT patients was blocked by BTKi's. A single oral intake of ibrutinib (280 mg) was sufficient for a rapid and sustained suppression of platelet FcγRIIA activation. Platelet aggregation by adenosine 5′-diphosphate, arachidonic acid, or thrombin receptor-activating peptide was not inhibited. Thus, irreversible and reversible BTKi's potently inhibit platelet activation by FcγRIIA in blood. This new rationale deserves testing in patients with HIT.

Introduction

The platelet Fc receptor CD32a (FcγRIIA) plays a central role in the pathogenesis of heparin-induced thrombocytopenia (HIT).[1-4] HIT is observed in 0.2% to 0.3% of patients receiving heparin[4] and is caused by immunoglobulin G (IgG) antibodies against new epitopes exposed after association of polyanionic heparin with platelet-factor 4 (PF4) secreted from platelets.[1] The immune complexes bind to FcγRIIA on the platelet surface with their Fc domain and cross-link the receptors, which induces platelet aggregation and secretion.[1-4] Formation of procoagulant vesicles by activated platelets and tissue factor

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expression by activated monocytes triggers thrombin formation and thrombosis, that together with enhanced platelet clearance by splenic macrophages results in thrombocytopenia.[1,2,4]

Platelets carry 1000 to 4000 copies of FcγRIIA (CD32a) per cell, the dominant compartment of this receptor in the body.[2] FcγRIIA is a type I transmembrane protein consisting of 2 extracellular Ig-like domains (similar to glycoprotein VI [GPVI]), a single transmembrane domain, and a cytoplasmic tail that contains an immunoreceptor tyrosine-based activation motif (ITAM) domain with dual YXXL amino acid consensus sequences. Signaling through the platelet FcγRIIA is similar to other ITAM receptors such as GPVI in platelets and the B-cell receptor in lymphocytes.[3,5] Cross-linking of the FcγRIIA by immune complexes induces ITAM phosphorylation by Src family kinases, probably Fyn and/or Lyn. Phosphorylated ITAM provides a docking site for the tandem SH2 domains of tyrosine kinase Syk, which recruits and phosphorylates LAT.[6,7] This adapter molecule is important for recruitment and activation of PLCγ2 and PI3K.[5,7] The latter enzyme (by generating phosphatidylinositol(3,4,5)-triphosphate that binds the PH domains of the homologous tyrosine kinases Bruton tyrosine kinase [BTK] and Tec) recruits these kinases to the plasma membrane allowing their tyrosine autophosphorylation in the SH3 domain and tyrosine phosphorylation by Lyn in the catalytic domain.[5,8] After GPVI-mediated platelet activation by collagen, BTK and Tec activation supports PLCγ2 activation.[6] BTK alone mediates platelet activation only after low-degree GPVI activation,[9] whereas Tec compensates for the absence of BTK in signaling downstream of GPVI.[10] PLCγ2 activation then generates the second messengers inositol-1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG), which release Ca^{2+} from intracellular stores and activate protein kinase C (PKC), respectively, causing platelet aggregation and secretion.[11] After FcγRIIA cross-linking, increased BTK and Tec phosphorylation has been demonstrated in human platelets,[12] but their respective causative roles for Fc receptor–stimulated platelet activation are unknown.

The current treatment of HIT patients relies on parenteral application of rapid-acting, non-heparin anticoagulants, such as the direct thrombin inhibitor argatroban or the antithrombin-dependent factor Xa inhibitor danaparoid.[1,4] In the future, direct oral anticoagulants such as the factor Xa inhibitors rivaroxaban and apixaban might be approved.[13] Inhibiting platelet FcγRIIA signaling would block an early crucial step in HIT pathogenesis not targeted so far.

We therefore studied the impact of BTK inhibitors (BTKi's) on FcγRIIA-induced platelet activation and tested the irreversible BTKi's ibrutinib and acalabrutinib (approved for the long-term treatment of various B-cell malignancies and mantle cell lymphoma, respectively),[14,15] zanubrutinib (BGB-3111) and tirabrutinib (ONO/GS-4059) (both with positive results in clinical trials of B-cell malignancies),[16,17] evobrutinib (with positive effects in a recently completed trial in multiple sclerosis),[18] and the reversible highly specific and potent BTKi fenebrutinib (GDC-0853), developed to target B cells and macrophages in autoimmune disorders (rheumatoid arthritis, lupus).[19-21] We stimulated platelet FcγRIIA in blood by antibody cross-linking, with anti-CD9 antibody, and with HIT sera, and measured BTKi effects on platelet activation and the formation of platelet-neutrophil complexes.

Materials and methods

For details regarding materials and methods see supplemental Data. For blood donations, healthy volunteers and patients signed an informed consent as approved by the Ethics Committee of the Faculty of Medicine of the University of Munich, and in accordance with the ethical principles for medical research involving human subjects as set out in the Declaration of Helsinki.

Blood collection for in vitro and ex vivo studies

Venous blood (10-20 mL) obtained from healthy adults who had not taken any platelet inhibitor for more than 2 weeks was anti-coagulated with hirudin for platelet aggregation, secretion, and fluorescence-activated cell sorting measurements,[22-24] or buffered trisodium citrate for measurements of in vitro closure time.[25] To test ibrutinib as a possible therapy for the acute phase of HIT, 3 healthy male physicians took a single dose of ibrutinib (2 capsules of 140 mg each). Blood was collected before and after drug intake.

Platelet aggregation in blood

Blood samples containing BTKi or dimethyl sulfoxide (DMSO; 0.1%) control were preincubated at 37°C for various times in the absence of stirring[23] and were stimulated with AT10 plus Fab2, anti-CD9 antibody (as in a large study of 154 healthy donors[26]) or stored sera from pseudonymized HIT patients who tested positive in particle gel immunoassays and HIT enzyme-linked immunosorbent assays for heparin/PF4 IgG antibodies. Platelet aggregation was measured by multiple electrode aggregometry (MEA) for 10 minutes. Cumulative aggregation values from 0 to 10 minutes were recorded in arbitrary aggregation units per minute (AU/min).[22,24]

ATP secretion

Luciferin/luciferase was added to blood after preincubation with BTKi or DMSO (0.1%) for 30 minutes at 37°C and stimulated by AT10 plus Fab2. Luminescence was recorded using the LUMI-Aggregometer.[23] The luminescence signals were calibrated for each blood donor in every experiment by the addition of adenosine triphosphate (ATP) standard solutions to blood samples.

Platelet P-selectin, platelet-neutrophil conjugates, and in vitro closure time

BTKi or DMSO (0.1%) was preincubated with blood for 30 minutes at 37°C before stimulation with AT10 plus Fab2 for 10 minutes. Platelet P-selectin expression and platelet-neutrophil conjugates were measured by flow cytometry. A PFA-200 (platelet function analyzer-200) device was used to measure in vitro closure time.[27,28]

Statistics

Data are given as the mean ± standard deviation of the number of experiments. Two parallel experimental conditions were analyzed by Student *t* test for paired samples or by Mann-Whitney *U* test if normality was not ensured. Data from more than 2 parallel experimental conditions were analyzed by analysis of variance for repeated measures, and secondary pair comparisons with the control were made by the least significant difference test (indicated in the figures by asterisks). If normality was not ensured, analysis of variance was used on ranks for repeated measures, and secondary pair comparisons with the control were made by Student-Newman-Keuls tests (indicated by in the figures by plus signs).

Results

Platelet effects on FcγRIIA stimulation in blood by CD32 cross-linking or anti-CD9 antibody differ

Platelet FcγRIIA (CD32a) activation induced by binding of the anti-CD32 antibody AT10 and subsequent cross-linking with anti-mouse Fab2 resulted in aggregation and ATP secretion of platelets in hirudin-anticoagulated blood (supplemental Figure 1A). In addition, the anti-CD9 (tetraspanin) antibody (Ts9) was used to stimulate FcγRIIA-dependent platelet aggregation[26,29] (supplemental Figure 1B). In contrast to CD32 cross-linking, the anti-CD9 antibody did not induce ATP secretion (supplemental Figure 1C). The absence of adenosine 5′-diphosphate (ADP) secretion might explain the delayed aggregation after anti-CD9 antibody stimulation compared with CD32 cross-linking, but the maximal aggregation responses were similar on both stimuli (compare aggregation tracings for DMSO in supplemental Figure 2). Because of these differences in platelet responses in blood, we used both stimuli to study platelet activation by FcγRIIA.

The irreversible BTKi's ibrutinib, acalabrutinib, tirabrutinib, zanubrutinib, evobrutinib, and the reversible BTKi fenebrutinib block platelet aggregation in blood on FcγRIIA activation

Blood was preincubated with different concentrations of irreversible BTKi's or the reversible BTKi fenebrutinib before FcγRIIA cross-linking. Ibrutinib (0.2 μM), acalabrutinib (1 μM), tirabrutinib (1 μM), zanubrutinib (0.4 μM), and evobrutinib (2.5 μM) inhibited platelet aggregation by >98%, 90%, 94%, 95%, and 94%, respectively (Figure 1; supplemental Figure 2). The concentrations that inhibit 50% (IC_{50}) of CD32 cross-linking–induced platelet aggregation increased in the following order: ibrutinib<zanubrutinib<acalabrutinib=tirabrutinib<evobrutinib (Table 1).

The BTKi's also dose-dependently inhibited FcγRIIA-dependent aggregation induced by anti-CD9 antibody (Figure 2) with IC_{50} values slightly lower for platelet stimulation by anti-CD9 than by CD32 cross-linking (Table 1). This may be related to the obviously less intense stimulation by anti-CD9. The delayed start of platelet aggregation on anti-CD9 stimulation (supplemental Figure 2) explains the lower cumulative aggregation values measured from 0 to 10 minutes (AU/min; see controls in Figures 1 and 2). These results demonstrate that irreversible BTKi's differ in their potency to inhibit platelet FcγRIIA activation, but their relative order seems independent of the mode of FcγRIIA stimulation.

Platelet aggregation stimulated by CD32 cross-linking was also inhibited by the reversible BTKi fenebrutinib in a dose-dependent manner with 50 nM inducing maximal suppression (Figure 3A). With an IC_{50} of 11 nM, fenebrutinib was the most potent BTKi tested. As expected,[30] fenebrutinib significantly inhibited other BTK-mediated platelet signaling pathways: GPVI-dependent aggregation on low but not high collagen concentration, and glycoprotein Ib/von Willebrand factor (GPIb/VWF)–dependent aggregation on ristocetin stimulation, but it did not compromise aggregation on thrombin receptor-activating peptide (TRAP), arachidonic acid (AA), or ADP, similar to the results of irreversible BTKi's reported previously[24] (Figure 3B). Complete suppression of platelet aggregation on FcγRIIA activation by all BTKi's was maintained over the entire 10-minute observation period (supplemental Figure 2).

Because in vitro high concentrations of certain irreversible BTKi's were recently found to impair primary hemostasis measured with the PFA-200,[31] we investigated the effect of the reversible BTKi fenebrutinib on platelet function in this device. By using the collagen/epinephrine cartridge (which is also sensitive to aspirin[32]), fenebrutinib in much higher concentrations (up to 1000 nM) than required to suppress platelet Fc receptor activation (50 nM) did not alter the closure time in vitro (Figure 3C).

Inhibition of FcγRIIA-mediated platelet aggregation is dependent on the exposure time to irreversible BTKi's

We recently observed that prolonging platelet exposure to irreversible BTKi's potentiated the inhibition of platelet aggregation induced by low collagen concentrations.[31] To study whether this was also observed after FcγRIIA stimulation, we preincubated blood with irreversible BTKi's for 5, 15, 30, and 60 minutes before CD32 cross-linking. The concentrations chosen had shown a complete inhibition after 60 minutes of preincubation (Figure 1). Inhibition of platelet aggregation increased with the duration of exposure with almost complete inhibition after 30 minutes of preincubation with ibrutinib, acalabrutinib, tirabrutinib, and zanubrutinib, and after 60 minutes of preincubation with evobrutinib (Figure 4A). In contrast, even after prolonged incubation with the reversible BTKi fenebrutinib, platelet inhibition was still incomplete (Figure 4B).

BTKi's prevent platelet ATP secretion, platelet P-selectin expression, and the formation of platelet-neutrophil complexes stimulated by FcγRIIA activation

To analyze whether platelet Fc receptor activation in blood might also stimulate platelet granule secretion and the subsequent platelet interaction with other blood cells, we measured ATP secreted from dense granules by luminescence, P-selectin secreted from α granules and expressed on the platelet surface, and the formation of platelet-neutrophil complexes by flow cytometry. FcγRIIA activation induced by CD32 cross-linking stimulated platelet ATP secretion, P-selectin expression, and the formation of platelet-neutrophil complexes in hirudin-anticoagulated blood. These effects were completely blocked by preincubation for 30 minutes with ibrutinib (0.2 μM), acalabrutinib (1 μM), tirabrutinib (1 μM), zanubrutinib (0.4 μM), and evobrutinib (2.5 μM) (Figure 5).

Platelet aggregation induced by HIT serum is prevented by BTKi's

To test whether FcγRIIA activation induced by HIT serum can also be inhibited by BTKi's, we obtained stored sera from 28 patients with HIT who had tested positive for heparin/PF4 IgG antibodies. Seven HIT sera (25%) triggered platelet aggregation in test blood samples of healthy donors as measured by MEA. Platelet aggregation by HIT sera was observed only in the presence of heparin (supplemental Figure 3).[33,34] Platelets from most but not all (8 of 10) test donors responded to HIT sera. The degree of platelet aggregation upon stimulation with HIT serum varied in blood from different donors and ranged from 162 to 1469 AU/min, although all donors showed similar maximal

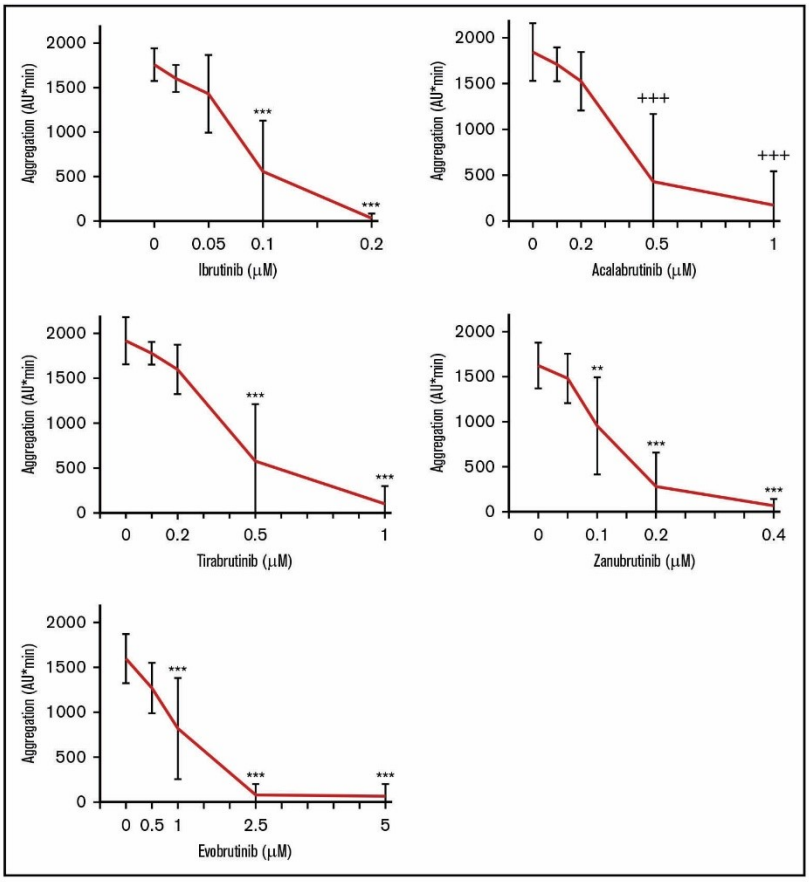

Figure 1. Effects of different concentrations of irreversible BTKi's on platelet aggregation in blood after FcγRIIA stimulation by cross-linking. Blood samples were preincubated for 60 minutes with solvent (DMSO, 0.1%) or BTKi's (ibrutinib, acalabrutinib, tirabrutinib, zanubrutinib, or evobrutinib) before incubation for 3 minutes with the mouse anti-CD32 antibody AT10 (2 μg/mL) and subsequent cross-linking with Fab2 of anti-mouse IgG (30 μg/mL) for 10 minutes. Values are mean ± standard deviation (SD) (n = 6). $^{**}P < .01$; $^{***}P < .001$; $^{+++}P < .001$.

platelet aggregation levels on CD32 cross-linking and anti-CD9 stimulation (data not shown).

The irreversible BTKi's ibrutinib and acalabrutinib as well as the reversible BTKi fenebrutinib blocked the platelet aggregation response to HIT serum. Suppression down to control levels was observed for each HIT serum and donor test blood pair and was complete even in test platelet donors who showed a strong reactivity to HIT serum (supplemental Table; Figure 6A-B).

Table 1. IC_{50} values of BTKi's for inhibition of platelet aggregation induced by CD32 cross-linking and anti-CD9 antibody in blood

	IC_{50}, μM	
	CD32 cross-linking (n = 6)	Anti-CD9 antibody (n = 5)
Ibrutinib	0.08 ± 0.04	0.05 ± 0.01
Zanubrutinib	0.11 ± 0.03	0.09 ± 0.02
Acalabrutinib	0.38 ± 0.16	0.26 ± 0.08
Tirabrutinib	0.42 ± 0.16	0.26 ± 0.06
Evobrutinib	1.13 ± 0.48	0.92 ± 0.15
Fenebrutinib	0.011 ± 0.0039	—

Values are mean ± SD. BTKi's were preincubated for 60 minutes (or 15 minutes for fenebrutinib) before Fc receptor stimulation.

Single intake of ibrutinib prevents platelet activation by FcγRIIA stimulation

By exploiting the covalent irreversible inactivation of BTK by ibrutinib and the lack of protein resynthesis in platelets, we explored whether a single intake of ibrutinib in vivo might suffice to inhibit platelet activation via FcγRIIA as it occurs in the acute phase of HIT. Three healthy male physicians took a single dose of ibrutinib (280 mg). Three hours after ibrutinib intake, platelet aggregation stimulated by CD32 cross-linking was inhibited in donors A, B, and C by 96%, 92%, 98%, respectively, and anti-CD9 activation was inhibited by 97%, 68%, 96%, respectively. The almost complete inhibition of FcγRIIA-induced platelet aggregation by CD32 cross-linking and anti-CD9 activation was sustained for 2 days in donors A and C and was still not fully reversed toward control 7 days after ibrutinib intake (Figure 7A). Platelet aggregation induced by TRAP, AA, or ADP was preserved in all 3 blood donors at all time points. As expected,[30] GPIb/VWF-mediated platelet aggregation on stimulation with ristocetin was inhibited in all donors 3 hours after ibrutinib intake; inhibition lasted for 1 day in donor B and was still not fully reversed in donor A toward control at 7 days after ibrutinib intake. BTK-dependent platelet aggregation after a low degree of GPVI activation upon plaque stimulation was also inhibited (which confirms previous findings[24]) and lasted in all donors up to 2 days after ibrutinib intake. In contrast, a high degree of GPVI-mediated

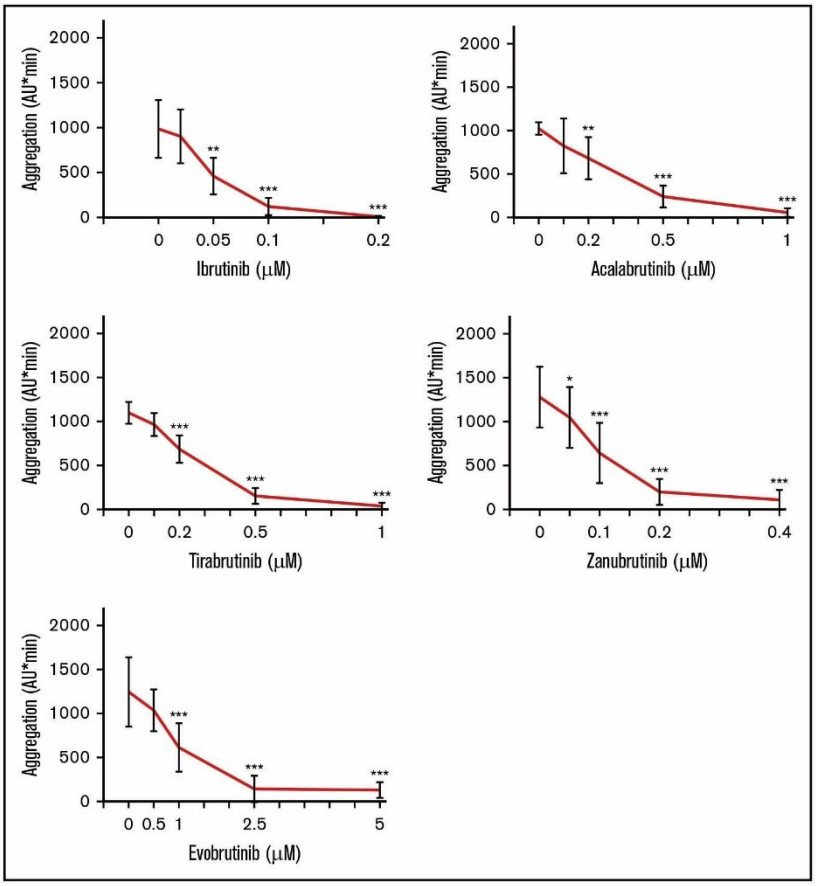

Figure 2. Effects of different concentrations of irreversible BTKi's on FcγRIIA-mediated platelet aggregation in blood stimulated by anti-CD9 antibody. Blood samples were preincubated for 60 minutes with solvent (DMSO, 0.1%) or BTKi's (ibrutinib, acalabrutinib, tirabrutinib, zanubrutinib, and evobrutinib) before stimulation with anti-CD9 antibody (1 μg/mL) for 10 minutes. Values are mean ± SD (n = 5). *$P < .05$; **$P < .01$; ***$P < .001$.

platelet aggregation by high collagen (2.5 μg/mL) was not inhibited in donor B and only 3 hours after ibrutinib intake in donor C (supplemental Figure 4).

Inhibition of FcγRIIA-induced ATP secretion after oral ibrutinib intake was even more pronounced. In all blood donors, ATP secretion after CD32 cross-linking was completely inhibited from 3 hours after ibrutinib intake to at least 2 days, and it was still markedly reduced 7 days after ibrutinib intake (Figure 7B). Primary hemostasis was measured with the PFA-200 in donor B. With the collagen/epinephrine cartridge, a small increase of closure time was detectable only 3 hours after ibrutinib intake. The closure times with the ADP/collagen cartridge were not altered at all (supplemental Figure 5).

Discussion

We demonstrate here, to our knowledge for the first time, that BTKi's block platelet FcγRIIA activation induced in blood by CD32 cross-linking, anti-CD9 antibody, or sera of HIT patients. The 6 BTKi's studied also suppressed platelet aggregation in blood and dense granule secretion and prevented platelet P-selectin expression, which is crucial for platelet interaction with endothelial cells and monocytes.[35] The latter promote tissue factor expression leading to amplification of thrombin formation,[36] a central feature in HIT.[1,4] Moreover, the BTKi's inhibited the formation of platelet-neutrophil aggregates that contribute to thrombosis through generation of neutrophil extracellular traps.[37]

We used several tools to stimulate platelet FcγRIIA in blood: CD32 cross-linking, the anti-CD9 antibody and, of clinical relevance, sera of HIT patients. In contrast to CD32 cross-linking, the anti-CD9 antibody did not stimulate platelet secretion in blood, which might be explained as being a result of inefficient FcγRIIA signaling through Syk, Lyn, and Ca^{2+} release from intracellular stores as observed with washed platelets.[38] In spite of the differences between the 2 stimuli, complete suppression of platelet aggregation in blood by the various BTKi's with similar IC_{50} values after anti-CD9 antibody stimulation and FcγRIIA cross-linking was found. The potencies of the BTKi's differed: the IC_{50} values of BTKi's for inhibition of platelet aggregation induced by FcγRIIA increased in the order of ibrutinib<zanubrutinib<acalabrutinib = tirabrutinib<evobrutinib and were similar to the IC_{50} values for inhibition of platelet aggregation stimulated by low collagen concentrations.[31] The most potent BTKi was fenebrutinib (IC_{50} = 11 nM) which has not been previously studied on platelets.

Platelet FcγRIIA stimulates not only BTK but also the homologous tyrosine kinase Tec, as demonstrated in an early study by Oda et al.[12] So far, the causative role of BTK or Tec for platelet activation

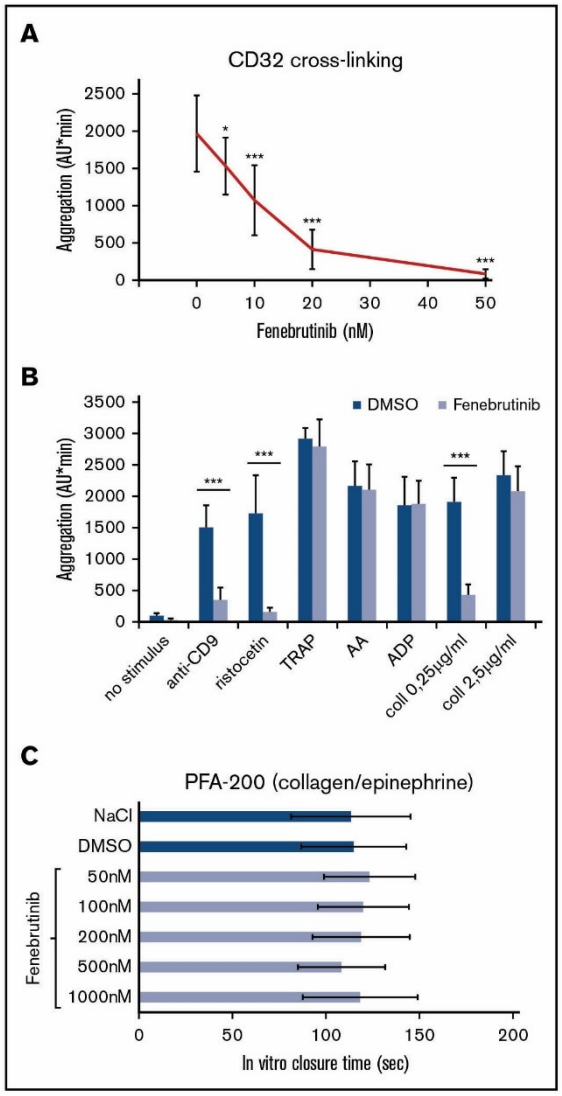

Figure 3. Effects of the reversible BTKi fenebrutinib on platelet aggregation in blood after stimulation by FcγRIIA activation, TRAP, AA, and ADP and on bleeding time in vitro. Blood samples were preincubated for 15 minutes with solvent (DMSO, 0.1%) or fenebrutinib before stimulation with AT10 (2 μg/mL; 3 minutes) plus Fab2 (30 μg/mL), anti-CD9 antibody (1 μg/mL), ristocetin (0.5 mg/mL), TRAP (15 μM), AA (0.6 mM), ADP (5 μM), or collagen (coll) (0.25 μg/mL or 2.5 μg/mL). (A) Dose-response curve of fenebrutinib on platelet aggregation after CD32 cross-linking. (B) Effects of fenebrutinib (50 nM) on anti-CD9 antibody-, ristocetin-, TRAP-, AA-, ADP-, and collagen-induced platelet aggregation and spontaneous platelet aggregation (no stimulus). Values are mean ± SD (n = 5). (C) Effects of fenebrutinib on bleeding time in vitro. Blood samples preincubated for 15 minutes with solvent (DMSO, 0.1%) or increasing concentrations of fenebrutinib were transferred to collagen/epinephrine cartridges, and the in vitro closure time was measured with the PFA-200. Values are mean ± SD (n = 6). *$P < .05$; ***$P < .001$.

after FcγRIIA stimulation has not been studied. We suggest that BTK rather than Tec mediates FcγRIIA-mediated platelet responses in blood for the following reasons. First, all BTKi's studied completely suppressed the maximal platelet responses stimulated by FcγRIIA cross-linking, anti-CD9 antibody, and HIT sera. This is in contrast to GPVI-mediated platelet activation, in which an increase in collagen or collagen-related peptide concentrations overcome platelet inhibition by BTKi's by alternative signaling through Tec.[30,31,39] Second, the suppression was observed by low BTK-specific concentrations of irreversible BTKi's.[30] The IC_{50} values of ibrutinib (0.08 μM) and acalabrutinib (0.38 μM) in blood were much lower than the drug levels in patients treated with approved doses for B-cell disorders (0.31 μM and 1.78 μM, respectively) that are required to fully inhibit BTK in peripheral blood mononuclear cells.[40,41] Third, fenebrutinib, which belongs to the class of reversible BTKi's that do not inhibit Tec in kinase assays,[20] completely suppressed platelet FcγRIIA activation in blood with a very low IC_{50} value (11 nM). This value is identical to the reported IC_{50} value for fenebrutinib for inhibition of BTK autophosphorylation in whole blood.[20] Taken together, and in contrast to GPVI signaling,[10,30] BTK but not Tec seems to be of functional relevance for platelet activation via FcγRIIA in blood.

A prolonged preincubation of blood in vitro might better reflect the in vivo exposure of platelets after oral drug intake and the typically prolonged absorption phase. We observed that increasing the preincubation time increased the potency of irreversible BTKi's to inhibit FcγRIIA-dependent platelet aggregation, which is in agreement with the findings for inhibition of GPVI-dependent platelet aggregation.[31] The reversible BTKi fenebrutinib did not show this type of kinetics and thus differs pharmacodynamically from irreversible BTKi's. Irreversible BTKi's might require more time than reversible BTKi's to reach and/or inactivate cytosolic BTK in platelets because of their covalent binding to Cys-481.

Stored sera obtained from patients at the time of diagnosing HIT induced stimulated, in the presence of low heparin concentrations, platelet aggregation in hirudin-anticoagulated blood from healthy test donors as measured by MEA. Only a subset of the heparin/PF4 IgG antibodies generated in HIT is able to cross-link the platelet Fc receptors and activate platelets. By using the most responsive test platelet donor, we detected aggregation by MEA in 25% of 28 patients with PF4 IgG antibodies. This is lower than in previous studies, which showed variable MEA-positive results in HIT sera containing anti-PF4 IgG antibodies: 42.5% in 181 HIT patients,[33] 60% in 30 HIT patients,[42] 35% in 37 HIT patients,[43] and 52% in 20 HIT patients[44] (Emmanuel J. Favaloro, Westmead Hospital, written communication, 8 October 2019). These differences might be explained in part by variances in the MEA assays and the responsiveness of test platelets from individual donors. The Fc receptor shows functionally relevant genetic polymorphisms.[2,45] It seems that the Fc receptor Arg/Arg-131 genotype confers a higher platelet sensitivity to several stimuli,[46] and donors with the Arg/Arg-131 genotype are often more responsive to HIT sera than donors with the Arg/His-131 and His/His-131 genotypes.[33,47] This and other Fc receptor polymorphisms[2] might underlie the variable response of test platelet donors to HIT serum.[34] Importantly, the irreversible BTKi's ibrutinib and acalabrutinib as well as the reversible BTKi fenebrutinib suppressed the aggregation response by >95% in any specific reactive combination of a HIT

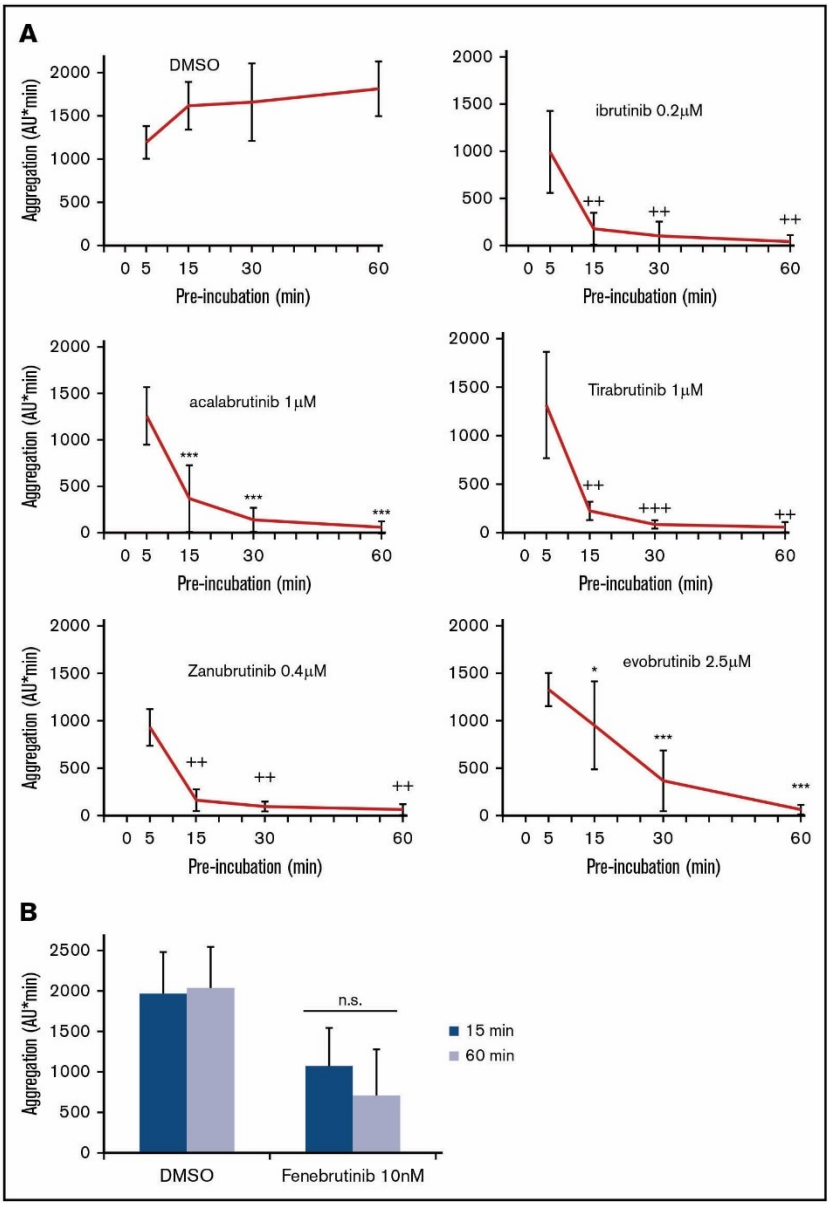

Figure 4. Effects of different times of preincubation with BTKi's on platelet aggregation after FcγRIIA stimulation by cross-linking. (A) Blood samples were preincubated for 5, 15, 30, or 60 minutes with solvent (DMSO, 0.1%) or BTKi's (ibrutinib, acalabrutinib, tirabrutinib, zanubrutinib, or evobrutinib) before incubation with AT10 (2 μg/mL) for 3 minutes and stimulation with Fab2 (30 μg/mL) for 10 minutes. Values are mean ± SD (n = 6). (B) Preincubation for 15 or 60 minutes with 2 concentrations of fenebrutinib before stimulation with AT10 and Fab2. Values are mean ± SD (n = 5). $^{*}P < .05$; $^{***}P < .001$; $^{++}P < .01$; $^{+++}P < .001$. ns, not significant.

serum with test platelets, regardless of the magnitude of the aggregation response.

Our results suggest a potential benefit from BTKi's in HIT treatment. However, grade 1 and 2 bleeding is not infrequent in high-dose therapy for B-cell malignancies with the irreversible BTKi's ibrutinib and acalabrutinib.[14,15,30] The underlying mechanisms in B-cell dyscrasias are complex and are only in part a result of direct platelet inhibition.[30,48] We suggest that a low dosage of the irreversible BTKi that will specifically inhibit BTK in platelets is unlikely to cause bleeding, because patients with X-linked agammaglobulinemia (XLA) as a result of genetic BTK deficiency do not show a bleeding phenotype.[30,49] Similar to BTK-deficient human XLA and mouse X-chromosome-linked immune-deficient (XID) platelets,[9,10,50] irreversible BTKi's at low concentrations inhibit the low-degree GPVI-mediated platelet stimulation and GPIb/VWF-mediated platelet aggregation upon ristocetin stimulation,[24,30,31] but the in vitro bleeding time as measured with the PFA-200 was not increased.[31] We show here that a single low dose of ibrutinib (280 mg) inhibited GPIb/VWF-mediated and low- but not high-degree GPVI-dependent platelet aggregation and did not affect G protein-coupled receptor–mediated platelet aggregation consistent with previous findings of low-dose (140 mg/d) ibrutinib intake for 1 week.[24] However, the single dose of ibrutinib was sufficient to rapidly suppress maximal

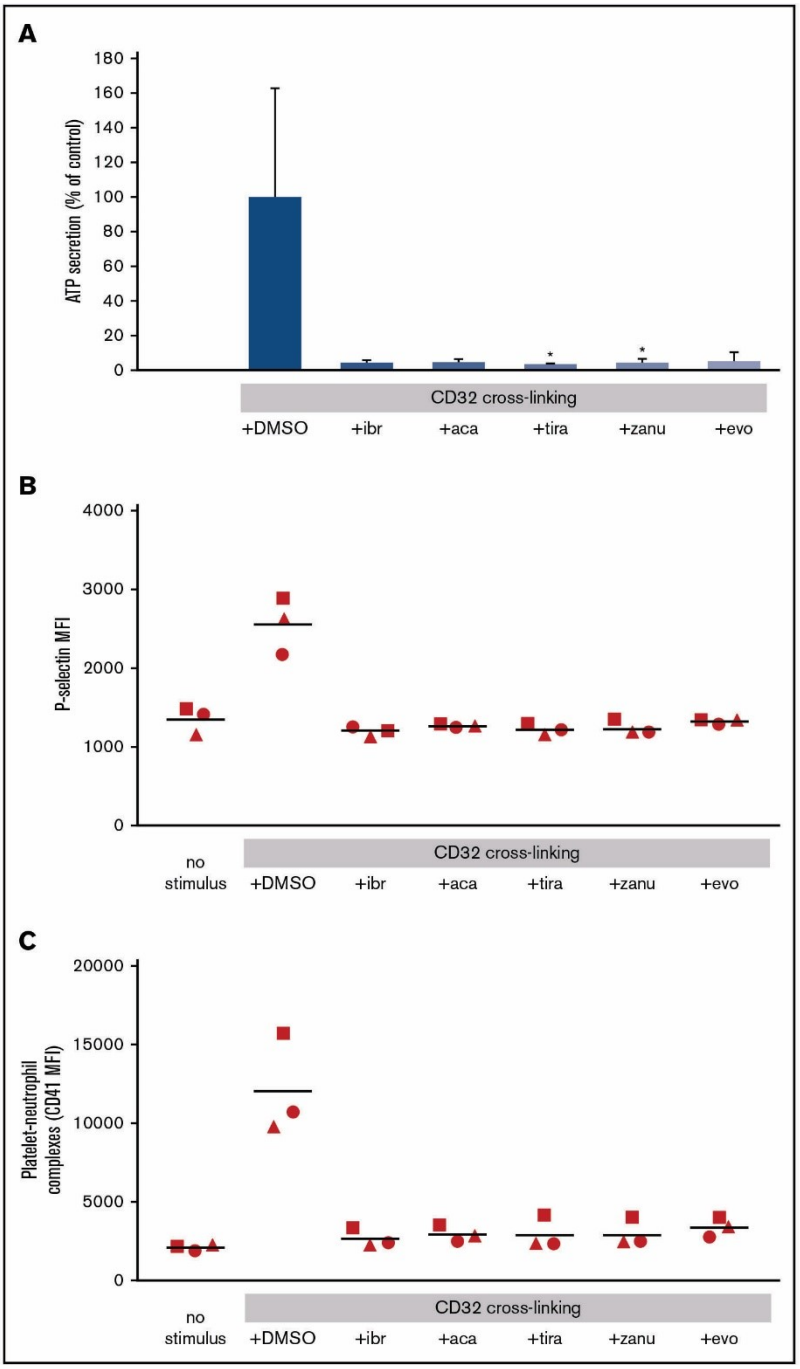

Figure 5. Effects of BTKi's on platelet ATP secretion, P-selectin expression, and platelet-neutrophil complex formation after FcγRIIA stimulation by cross-linking. Blood was incubated for 30 minutes with solvent (DMSO, 0.1%) or BTKi's (ibrutinib [ibr], 0.2 μM; acalabrutinib [aca], 1 μM; tirabrutinib [tira], 1 μM; zanubrutinib [zanu], 0.4 μM; and evobrutinib [evo], 2.5 μM) and for 3 minutes with AT10 (2 μg/mL) before stimulation with Fab2 (30 μg/mL). (A) ATP secretion was measured by using the LUMI-aggregometer. Values are mean ± SD (n = 5). (B) Platelet P-selectin expression (measured by flow cytometry) analyzing the mean fluorescence intensity (MFI) of P-selectin. (C) Platelet-neutrophil complexes determined by flow cytometry as the mean fluorescence intensity of CD41 (platelets) on CD66b cells (neutrophils). (B-C) Scatter plots from 3 experiments with different blood donors. *$P < .05$.

platelet FcγRIIA activation in blood. Inhibition was sustained for at least 2 days, which is explained by the irreversible, covalent BTK inactivation by ibrutinib and the lack of de novo protein synthesis in platelets. The recovery of FcγRIIA-induced platelet aggregation paralleled the expected physiological platelet renewal rate, and nearly complete aggregation was restored 7 days after the single ibrutinib dose. Platelet dense granule secretion stimulated by CD32 cross-linking was surprisingly even more suppressed than aggregation and was still decreased 7 days after ibrutinib intake. Platelet inhibition was more pronounced in donors A and C than in donor B, which is probably the result of the better bioavailability of ibrutinib when it is taken with food (as in donors A and C).[51]

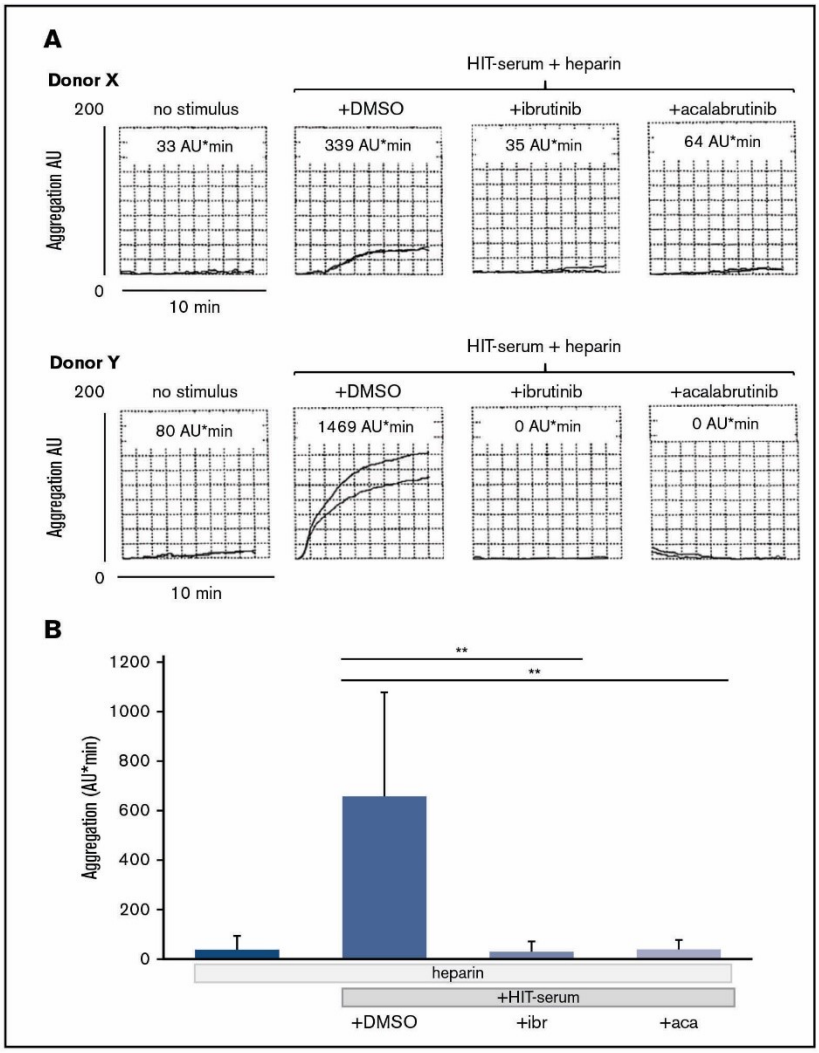

Figure 6. Effects of the BTKi's ibrutinib and acalabrutinib on platelet aggregation in blood stimulated by HIT serum and heparin. Blood samples were incubated for 30 minutes with solvent (DMSO, 0.1%) or BTKi's (ibrutinib, 0.2 μM and acalabrutinib, 1 μM) and subsequently for 30 minutes with HIT-positive serum (100 μL) before addition of heparin (0.5 U/mL) for 3 minutes and start of stirring. Aggregation was measured for 10 minutes. (A) Representative MEA tracings of 2 healthy blood donors showing the effect of BTKi's on low (donor X) and high (donor Y) platelet aggregation upon stimulation by HIT serum. The numbers above the tracings indicate cumulative aggregation values (AU/min) measured for 10 minutes. (B) Bar diagram showing the effects of BTKi's on platelet aggregation stimulated by HIT-serum and heparin. Values are mean ± SD (n = 5 different blood donors). $^{**}P < .01$.

Concentrations of irreversible BTKi's higher than that required for BTK inhibition also inhibit Tec in various kinase panel platforms and in platelets (for references, see Busygina, et al[30]) leading to inhibition of GPVI-mediated platelet activation after high collagen concentrations.[10] This may underlie the observed increase in closure time (as measured by the PFA-200) with the collagen/epinephrine cartridge after blood was incubated with high concentrations of irreversible BTKi's,[31] and may contribute to bleeding in patients with B-cell malignancies treated with continuous high dosages of ibrutinib and acalabrutinib.[14,15,30]

Treatment of HIT with reversible BTKi's might be safer than treatment with irreversible BTKi's. Even at very high concentrations, reversible BTKi's do not inhibit Tec in in vitro kinase assays,[20,52,53] and therefore patients might not be burdened with bleeding. Indeed, we found that the reversible BTKi fenebrutinib, even at 20-fold higher concentrations than required to maximally suppress platelet FcγRIIA activation, did not increase the PFA-200 closure time in vitro. We demonstrate here that fenebrutinib also inhibited platelet aggregation upon low but not high collagen concentrations and inhibited GPIb/VWF-mediated platelet aggregation upon stimulation with ristocetin. Thus, fenebrutinib treatment resembles the platelet phenotype of BTK-deficient XLA patients and XID mice.[9,10,30,50] Indeed, oral intake of multiple and increasing doses of fenebrutinib in a phase I study did not show any bleeding events.[21] In addition, fenebrutinib has shown favorable pharmacokinetic and pharmacodynamic characteristics in healthy volunteers.[21] A low single dose of 15 mg of fenebrutinib resulted in a maximal plasma concentration of 30 nM after 1 hour, inhibited BTK autophosphorylation in whole blood cells by 80%, and completely blocked B-cell activation; inhibition was only slightly reversed 24 hours after intake.[21] In our study, a blood concentration of 50 nM fenebrutinib completely inhibited FcγRIIA stimulation by CD32 cross-linking and HIT sera, which suggests that low oral doses of fenebrutinib will efficiently shut down the platelet Fc receptor signaling pathway and should effectively prevent platelet consumption in HIT.

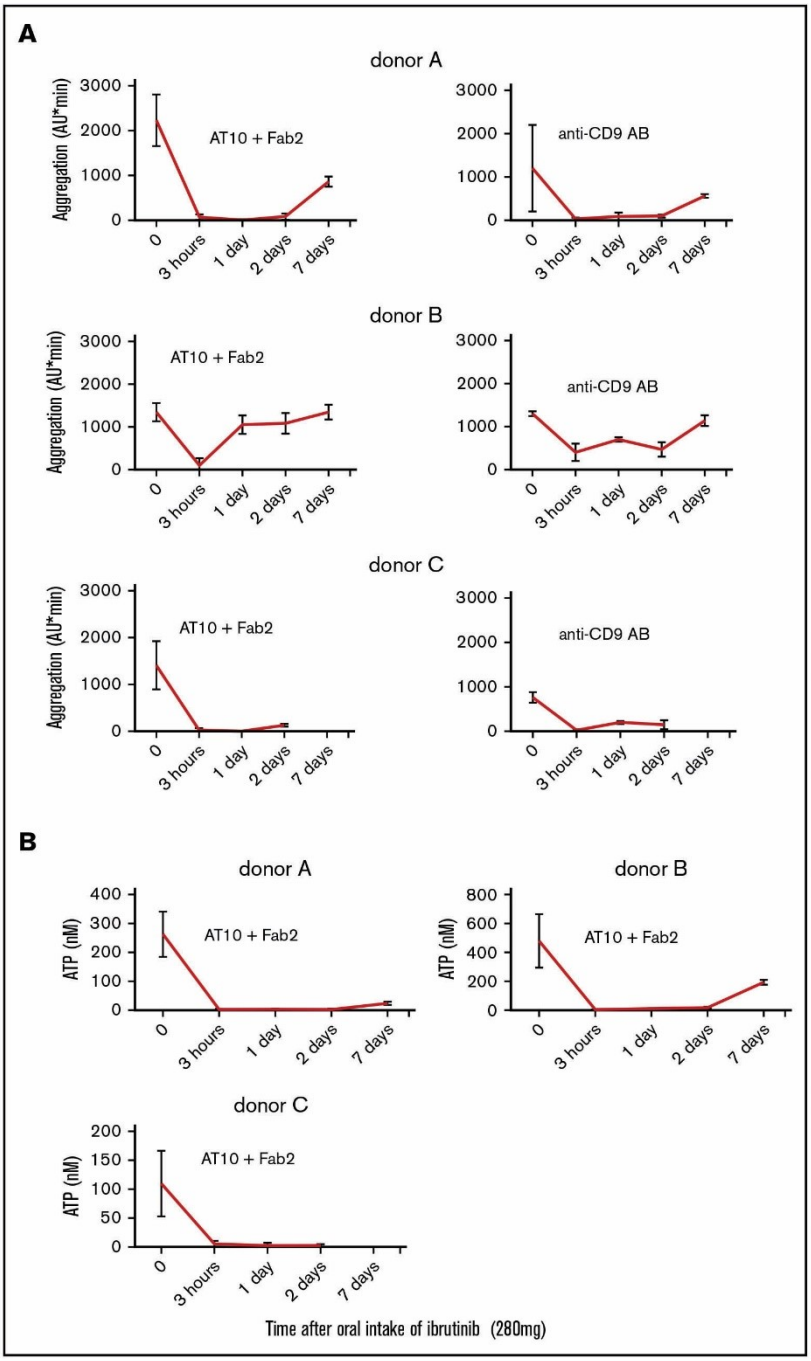

Figure 7. Effects of a single oral intake of ibrutinib on platelet FcγRIIA stimulation. Three healthy donors (A, B, and C) received ibrutinib, 2 doses of 140 mg each. Blood was drawn just before the intake and then 3 hours, 1 day, 2 days, and 1 week after intake. Blood samples were preincubated for 3 minutes before platelet Fc receptor stimulation by CD32 cross-linking with AT10 and Fab2, or anti-CD9 antibody (see legends for Figures 1 and 2). Platelet aggregation (A); ATP secretion (B). The luminescence signals were calibrated for each blood donor in every experiment by the addition of ATP standard solutions. Values are mean ± SD of triplicate determinations. AB, antibody.

In conclusion, BTK inhibition by reversible or low-dose irreversible BTKi's allows blocking of platelet activation by FcγRIIA, an early and crucial step in the pathogenesis of HIT not targeted by current thrombin-directed standard therapy. BTKi's prevent FcγRIIA-induced platelet aggregation and also dense granule secretion, P-selectin expression (critical for platelet interaction with endothelial and monocytes thereby amplifying thrombin formation),[35] and platelet-neutrophil aggregate formation contributing to NETosis and thrombus formation.[37,54] Similar to aspirin,[55] low dosage of irreversible BTKi's will selectively inhibit platelets because platelets lack de novo enzyme synthesis, and because irreversible BTKi's may covalently inactivate platelet BTK already by a single exposure

at low concentrations during absorption. In contrast, reversible BTKi's block platelet FcγRIIA activation, as demonstrated in our study, and will also inhibit activation of B cells, monocytes,[20] and neutrophils,[56,57] and thereby also reduce HIT antibody formation, tissue factor expression, and neutrophil extracellular trap formation, which contribute to thrombus formation in HIT.[37,58-60] This might provide additional treatment benefit in HIT.

The mouse in vivo HIT model might be helpful for studying whether irreversible or reversible BTKi's might be more suitable for treating HIT.[61] The model uses transgenic mice that express both human platelet FcγRIIA and human PF4 and was previously used to test an Syk inhibitor for treatment of HIT.[62] In that model, the effect of BTKi's on neutrophil activation in HIT could also be investigated.[56,57] Recent reports of neutrophils activated via FcγRIIA, neutrophil-platelet interaction, and subsequent NETosis driving thrombosis put several mechanisms amenable to BTKi's in the center of HIT pathophysiology.[37,60]

Acknowledgments

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Authorship

Contribution: L.G., R.D., and T.K. designed the research, performed the experiments, and acquired and analyzed the data; L.G. and W.S. drafted the article; G.W. provided HIT sera and revised the manuscript; P.v.H. and M.S. provided key reagents and equipment and critically revised the manuscript; C.W., P.v.H., M.S., and W.S. handled funding and supervision; R.L. performed statistical analysis and helped write the article; and W.S. conceived the project and designed the research.

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SUPPLEMENTAL DATA

1. Methods

Reagents and antibodies

Ibrutinib, acalabrutinib and tirabrutinib (ONO/GS-4059) came from Selleckchem (Houston, USA). Zanubrutinib (BGB-3111), evobrutinib and fenebrutinib (GDC-0853) were from MedChem Express (New Jersey, USA). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (Taufkirchen, Germany). Luciferin-luciferase reagent and adenosine 5'-triphosphate disodium salt hydrate (ATP) were from Chronolog (Havertown, PA, USA). The anti-CD32 antibody AT10 (monoclonal mouse IgG1), cross-adsorbed F(ab')2-goat anti-mouse IgG (H+L) and the anti-CD9 antibody Ts9 (monoclonal mouse IgG1) were obtained from ThermoFisher Scientific (Waltham, MA, USA). Ristocetin, arachidonic acid (AA) and TRAP-6 were from Roche Diagnostics (Mannheim, Germany). Collagen was from Nycomed Pharma (Unterschleissheim, Germany) and ADP from Biopool (Wicklow, Ireland). Heparin was obtained from Ratiopharm (Ulm, Germany). Red blood cell (RBC) lysis/fixation solution, PE-conjugated isotype control antibody IgG1κ, pacific blue-conjugated anti-CD66b and APC-conjugated anti-CD62P were from Biolegend (San Diego, CA, USA). PE–conjugated anti-CD41a and CD14-FITC were from BD Biosciences (Heidelberg, Germany). .

Blood collection

Blood was donated by healthy adult volunteers, who had not taken any drugs that could influence platelet function for at least two weeks. Blood was sampled by venipuncture using a 21-Gauge Butterfly needle and vacuum collection tubes (3ml) of Dynabyte (Munich, Germany) containing hirudin as anticoagulant (final hirudin concentration in blood: 200 U/ml corresponding to 15 µg/ml) for platelet aggregation

measurements [1] or buffered trisodium citrate/citric acid solution (citrate concentration 0.129mol/L; S-Monovette 3.8mL 9NCPFA from Sarstedt, Nümbrecht, Germany) for PFA-measurements [2].

Stock solutions

Ibrutinib, zanubrutinib, acalabrutinib, tirabrutinib, evobrutinib and fenebrutinib were dissolved in dimethyl sulphoxide (DMSO) at concentrations of 10mM or 1mM (fenebrutinib). Aliquots (30 µl) were stored at -80°C. Before experiments, dilutions were made in DMSO as needed (5mM-0.01mM) toyield an equal final DMSO concentration of 0.1% in all experimental blood samples. AT10 antibody (1mg/ml) was kept in aliquots of 10µl at -20°C, Fab2 (1.3mg/ml) and anti-CD9 (0.5mg/ml) in aliquots of 80-100µl at 4°C.

Platelet aggregation in blood

Platelet aggregation was measured by multiple electrode aggregometry (MEA) (Multiplate®, VerumDiagnosticaGmbH Roche, Rotkreuz, Schweiz), as described [1,3]. The test cuvettes were filled with 300µl of physiological saline, then 0.6µl Btk-Inhibitor solution or DMSO (solvent control), and finally 300µl of blood were addedAfter gentlemixing the samples were pre-incubated for 5 or 15 minutes at 37°C in the Multiplate device, or for 30 or 60 minutes after covering cuvettes in the incubator. Then AT10 (2µg/ml) and after 3 min Fab2 (30µg/ml), or anti-CD9 antibody (1µg/ml) were added, and stirring and impedance measurement were started. The impedance was recorded for 10 minutes, and aggregation was quantified as area under the curve (AU*min) at 10 min.

For platelet activation by HIT serum, aliquots of sera from five patients tested positive for HIT-specific antibodies and stored at -20°C were used. Saline (200µl) containing

DMSO (control), ibrutinib or acalabrutinib was mixed with blood from healthy donors (300µl), and samples were pre-incubated for 30 minutes at 37°C in the test cuvettes, before addition of 100µl of HIT-serum and incubation for further 30 minutes. Then 0.5U/ml heparin was added, and after incubation for 3 minutes, stirring was started, and aggregation recorded for 10 minutes.

ATP Secretion

ATP secretion from platelets was measured in blood using the LUMI-Aggregometer from Chronolog (Havertown, Pennsylvania) [3,4]. The siliconized glass test cuvettes were filled with 200µl of physiological saline, then 0.4µl Btk-Inhibitor solution or DMSO (solvent control), and 200µl of blood were added and incubated for 30 minutes at 37°C. AT10 (2µg/ml) was added, and after incubation for 3 minutes stirring and recording was started, 30µl luciferin/luciferase reagent was added, followed by Fab2 (30µg/ml), or the samples were stimulated by anti-CD9 antibody (1µg/ml). The increase of luminescence signal was recorded until it started to decline again. Maximal ATP secretion was measured as scale units. Values for BTKi are expressed as % of DMSO controls. The luminescence signals were calibrated for each blood donor in every experiment by addition of ATP standard solutions to non-stimulated blood samples providing final ATP concentrations of 50nM, 100nM and 200nM.

Measurement of P-selectin expression on platelets and analysis of platelet-neutrophil complexes by flow cytometry

Saline (300µl) plus blood samples (300µl) were preincubated in Eppendorf cups for 30 minutes at 37°C with DMSO or the BTKi. The samples were activated with AT10 followed by anti-mouse Fab2 for 10 minutes as described above for the MEA measurements, but not stirred. Aliquots (100µl) of each sample were transferred into

FACS tubes containing anti-CD41a–PE antibody (5µL), isotype-matched IgG1-PE (6µL diluted 1:100), anti-CD14–FITC antibody (2µL), anti-CD66b-Pacific blue antibody and anti-P-selectin-APC antibody (2µl) and incubated for 15 minutes at room temperature in the dark. After lysis of red blood cells with 450µl 1X RBC lysis/fixation buffer, the samples were fixed for 15 minutes in the dark at room temperature. Measurement and analysis were performed on a FACS Canto II flow cytometer (BD Biosciences). Platelet P-selectin expression was determined by P-selectin-APC expression on CD41 positive cells, and platelet-neutrophil complexes determined by CD41 and CD66b positive events were assessed by the mean fluorescence intensity of CD41. The positive regions were determined using the IgG1-PE isotype control and FMO (fluorescence minus one).

In vitro closure time with PFA-200

The PFA-200 device (Siemens Healthcare, Erlangen, Germany) simulates primary haemostasis and is validated for the identification and therapeutic monitoring of primary hemostatic disorders [5,6]. The instrument aspirates citrate-anticoagulated whole blood under constant vacuum from a reservoir through a capillary and a small hole in a membrane filter coated with collagen and epinephrine or collagen and ADP. The time until complete occlusion of the aperture is reported as “in vitro closure time”. Various concentrations of fenebrutinib, DMSO (solvent control, final concentration in blood 0.1%) or saline were first pipetted (0.8µl) into 50 µL NaCl and then blood samples (750 µL) anticoagulated with citrate (129 mM) [2] were added. Samples were preincubated for 15 minutes at 37°C before measuring the closure time with the collagen/epinephrine test cartridge. Measurements were performed between 75 and 150 minutes after blood collection.

Closure time was also measured in donor B before and after oral intake of ibrutinib.

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2. Supplemental Table

Effects of the irreversible BTKi's ibrutinib and acalabrutinib, and of the reversible BTKi fenebrutinib on platelet aggregation in blood stimulated by HIT-serum and heparin.

	Donor 418	**Donor 277**	**Donor 355**
	*Aggregation (AU*min)*		
control	149; 96	0; 30	379;144
heparin 0.5U/ml	0; 38	49;0	276; 174
HIT serum W + heparin	1891;1682	1216	1614; 970
Ibrutinib +HIT serum W+ heparin	35	0	38
Acalabrutinib +HIT serum W+ heparin	40	38	234
Fenebrutinib +HIT serum W+ heparin	28	0	250
HIT serum E + heparin	1327; 1105	229	
Ibrutinib +HIT serum E+ heparin	0	41	
Acalabrutinib +HIT serum E+ heparin	0	0	
Fenebrutinib +HIT serum E+ heparin	0	0	
HIT serum U + heparin	784		
Ibrutinib +HIT serum U+ heparin	0		
Acalabrutinib +HIT serum U+ heparin	30		
Fenebrutinib +HIT serum U+ heparin	95		
HIT serum G+ heparin			750
brutinib +HIT serum G+ heparin			149
Acalabrutinib +HIT serum G+ heparin			134
Fenebrutinib +HIT serum G+ heparin			87

Blood samples of three different responsive donors were incubated for 30 minutes with solvent (DMSO, 0.1%) or Btk-inhibitors (ibrutinib 0.2µM, acalabrutinib 1µM, fenebrutinib 50nM) and subsequently for 30 minutes with HIT-positive serum (100µl) from patients W,E,U or G before addition of heparin (0,5U/ml) for 3 minutes and start of stirring. The numbers are cumulative aggregation values (AU*min) measured for 15 minutes.

3. Supplemental Figures

Figure S1

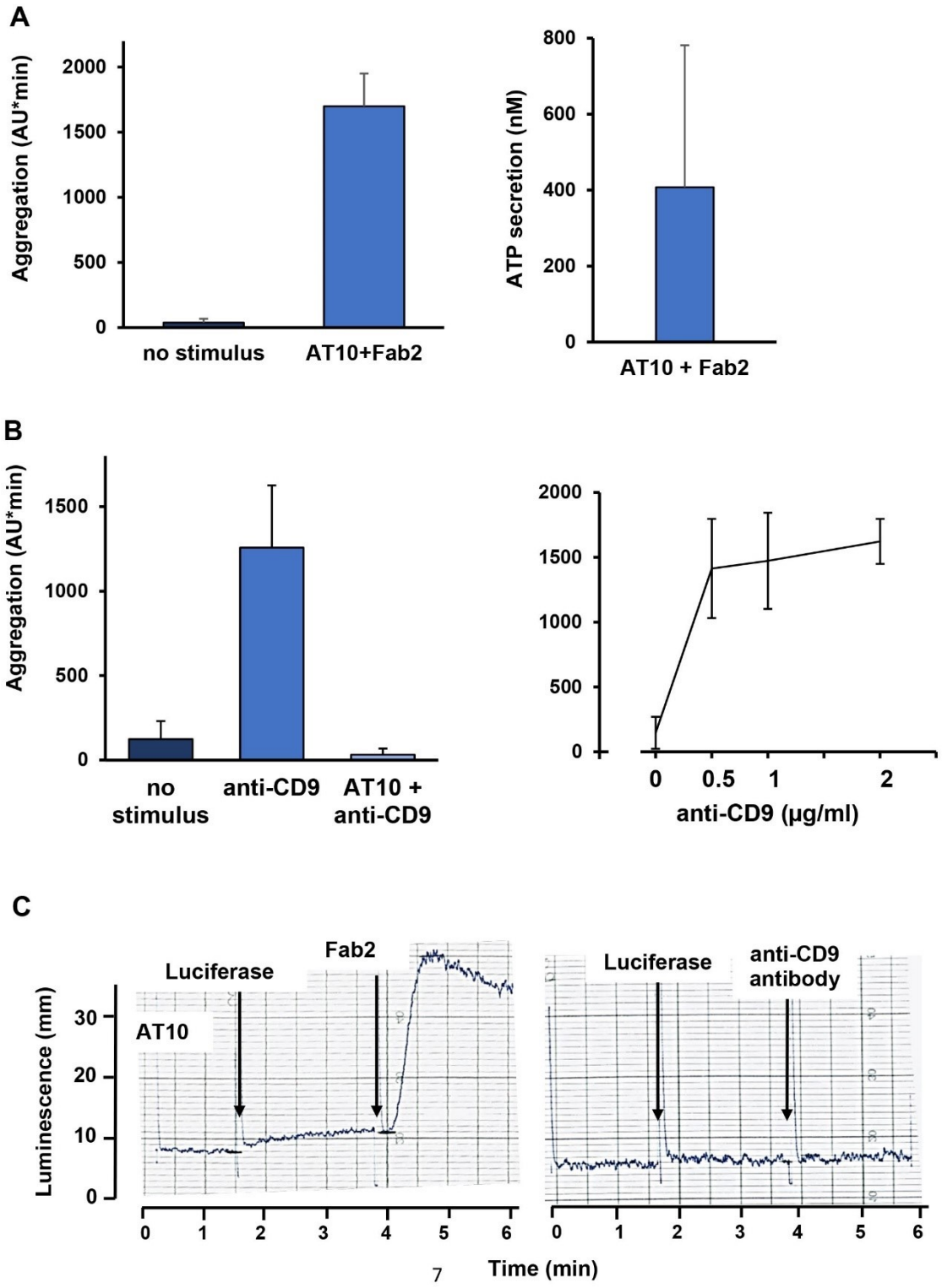


Figure S1

Platelet Fc-receptor activation induced by CD32 crosslinking and anti-CD9 antibody.

Blood samples were pre-incubated for 60 minutes (right) with solvent (DMSO, 0.1%), before incubation with monoclonal AT10 (2µg/ml) for 3 minutes and subsequent stimulation with anti-mouse Fab2 (30µg/ml), or before stimulation with anti-CD9 antibody (1µg/ml). **(A)** CD32 crosslinking stimulates platelet aggregation (left; values are mean ± SD, n=12; aggregation was measured for 10min) and ATP secretion (right, values are mean ± SD, n=5). **(B)** anti-CD9 antibody stimulates platelet aggregation in increasing concentrations (right, values are mean ± SD;n=5) that is inhibited by pre-incubation with the anti-CD32 antibody AT10 (2µg/ml) for 3 min (values are mean ± SD, n=4). **(C)** Platelet stimulation with anti-CD9 antibody (1µg/ml) does not stimulate ATP secretion (right) in contrast to CD32 crosslinking (left). The ATP secretion tracings (measured with the LUMI-aggregometer) shown are representative of experiments with n=5 different blood donors.

Figure S2

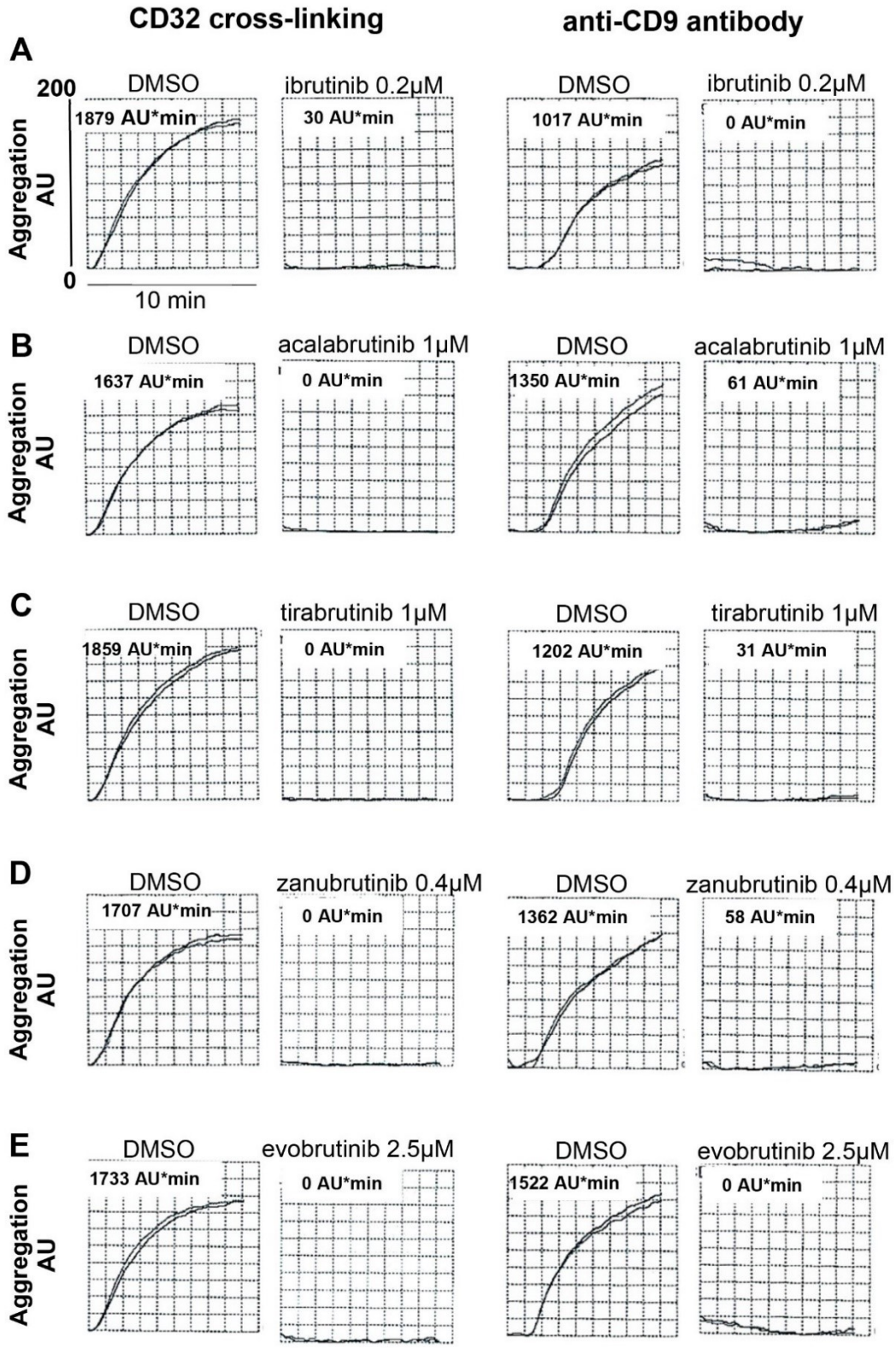

CD32 cross-linking
anti-CD9 antibody
A
B
C
D
E
Aggregation AU
200
0
10 min
DMSO
ibrutinib 0.2μM
1879 AU*min
30 AU*min
1017 AU*min
0 AU*min
acalabrutinib 1μM
1637 AU*min
0 AU*min
1350 AU*min
61 AU*min
tirabrutinib 1μM
1859 AU*min
0 AU*min
1202 AU*min
31 AU*min
zanubrutinib 0.4μM
1707 AU*min
0 AU*min
1362 AU*min
58 AU*min
evobrutinib 2.5μM
1733 AU*min
0 AU*min
1522 AU*min
0 AU*min

Figure S2

Effects of Btk-inhibitors on tracings of platelet aggregation in blood after FcγRIIA stimulation by crosslinking or anti-CD9 antibody. Blood samples were pre-incubated for 60 minutes with solvent (DMSO, 0.1%) or the Btk-inhibitors **(A)** ibrutinib 0.2μM, **(B)** acalabrutinib 1μM, **(C)** tirabrutinib 1μM, **(D)** zanubrutinib 0.4μM and **(E)** evobrutinib 2.5μM. Samples were then incubated for 3 minutes with AT10 (2μg/ml) before stimulation with Fab2-antibody (30μg/ml) (CD32 crosslinking), or with anti-CD9 antibody (1μg/ml). The numbers above the tracings indicate cumulative aggregation values (AU*min) measured for 10 minutes.

Figure S3

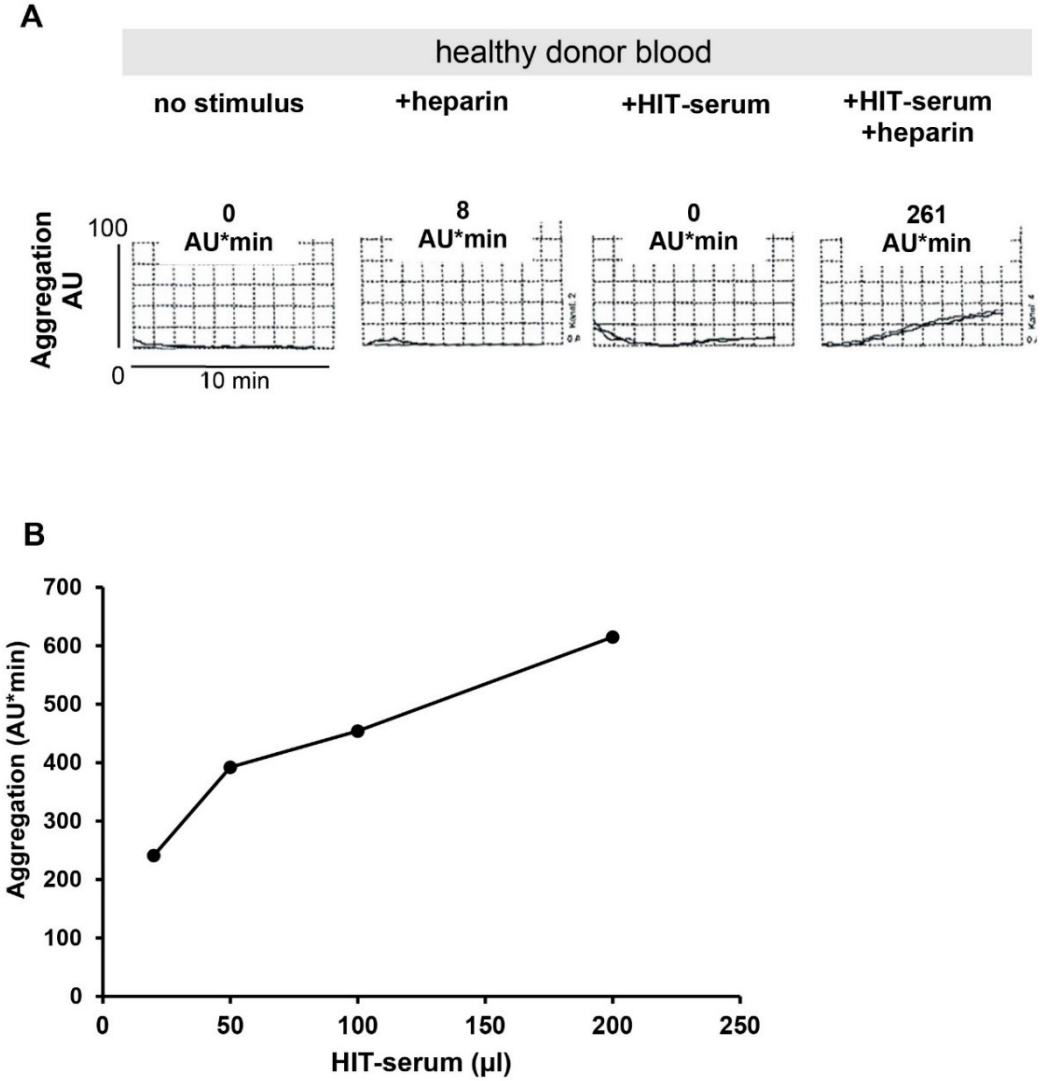


Figure S3

Platelet aggregation in blood stimulated by HIT-serum requires addition of heparin. (A) Representative aggregation tracings. Blood samples were pre-incubated without or with heparin (0,5U/ml), HIT serum (100µl) or heparin plus HIT-positive serum. The numbers above the tracings indicate cumulative aggregation values (AU*min) measured for 10 minutes. **(B)** Blood was pre-incubated with increasing volumes of HIT-serum for 30min. Heparin (1U/ml) was added and aggregation was measured for 10 minutes (mean of duplicate determinations).

Figure S4

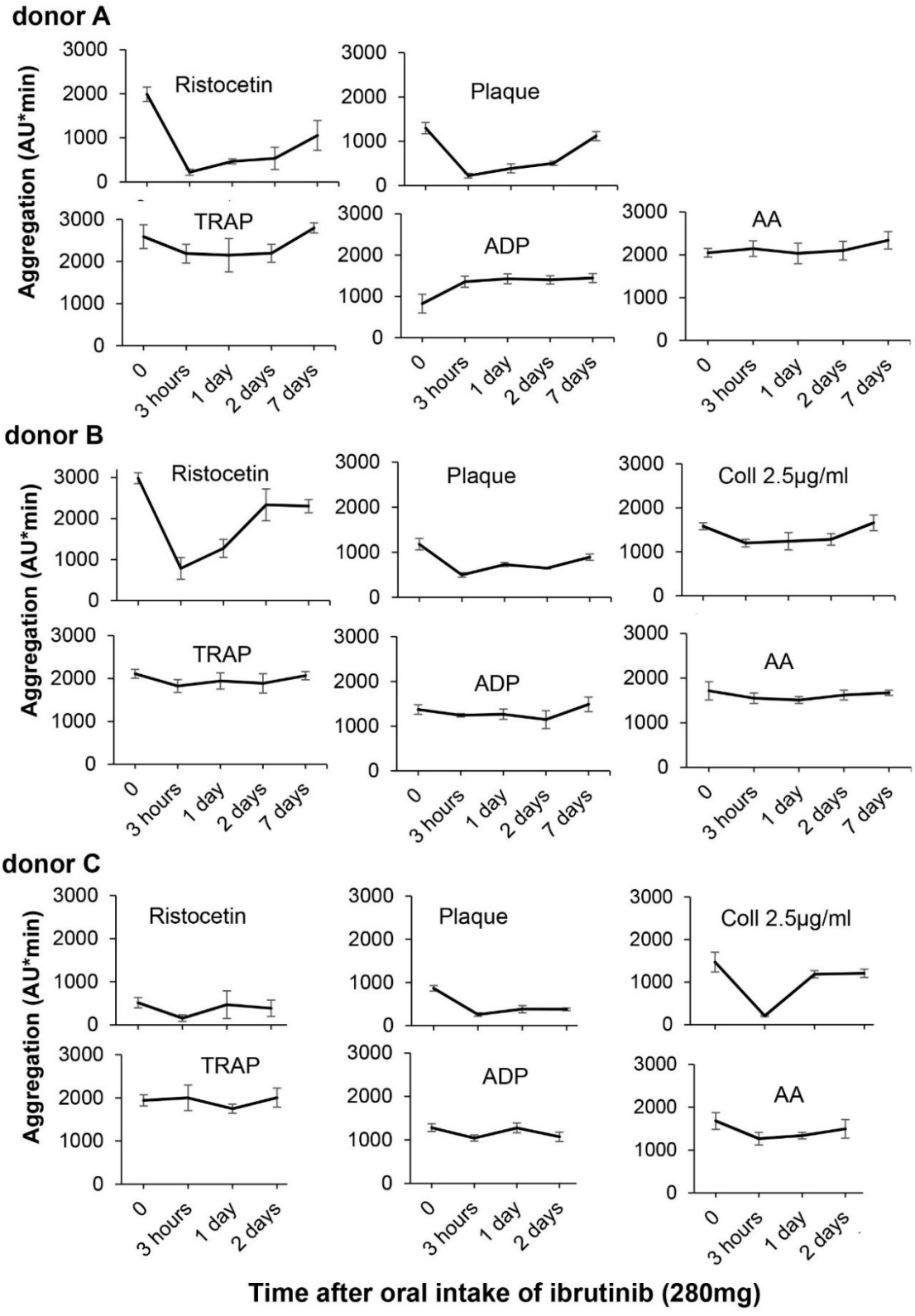
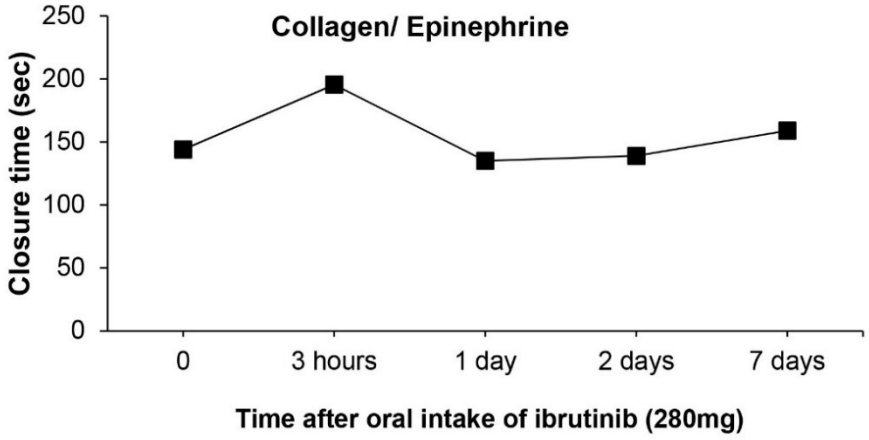

donor A
donor B
donor C
Aggregation (AU*min)
Ristocetin
Plaque
Coll 2.5µg/ml
TRAP
ADP
AA
3000
2000
1000
0
0
3 hours
1 day
2 days
7 days
Time after oral intake of ibrutinib (280mg)

Figure S4

Effects of oral intake of a single intake of ibrutinib on stimulus-induced platelet aggregation.

Three healthy donors A, B, C took 2x140mg Imbruvica® capsules. Blood was drawn just before the intake and 3 hours, 1 day, 2 days and one week after intake. Blood samples were pre-incubated for 3 minutes before stimulation with ristocetin (0.5mg/ml), plaque (833µg/ml), collagen (2.5µg/ml), TRAP (15µM), arachidonic acid (AA) (0.6mM) or ADP (5µM). Platelet aggregation was measured for 10 min. Values are mean ± SD of triplicate determinations (n=3).

Figure S5



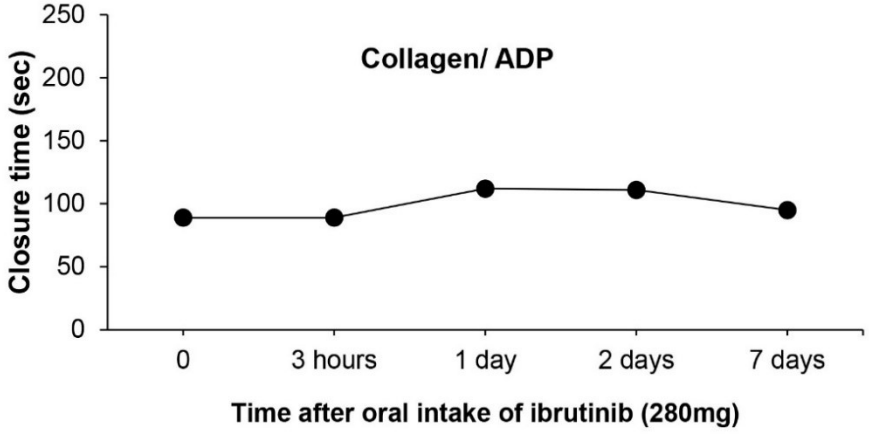


Figure S5

Effects of oral intake of a single intake of ibrutinib (280mg) by donor B on *in vitro* bleeding time (PFA-200).

Citrated blood was drawn just before the intake and 3 hours, 1 day, 2 days and one week after intake, and tested with the collagen/epinephrine and the collagen/ADP cartridges, and the closure times were measured with the PFA-200.

10. Acknowledgements

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