Optimizing Platelet GPVI Inhibition versus Haemostatic Impairment by the Btk Inhibitors Ibrutinib, Acalabrutinib, ONO/GS-4059, BGB-3111 and Evobrutinib

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Thromb Haemost

Abstract

Ibrutinib and acalabrutinib are approved for B cell malignancies and novel Bruton's tyrosine kinase (Btk) inhibitors undergo clinical testing also in B cell-driven autoimmune disorders. Btk in platelets mediates platelet activation via glycoprotein (GP) VI, which is crucial for atherosclerotic plaque-induced platelet thrombus formation. This can be selectively inhibited by Btk inhibitors. Since patients on second-generation Btk inhibitors apparently show less bleeding than patients on ibrutinib, we compared the effects of ibrutinib and four novel irreversible Btk inhibitors on GPVI-dependent platelet aggregation in blood and in vitro bleeding time. Low concentrations of collagen which induced the same low degree of GPVI-mediated platelet aggregation as atherosclerotic plaque material were applied. IC_{50} values for collagen (0.2–0.5 µg/mL)-induced platelet aggregation after 15-minute pre-incubation were: ibrutinib 0.12 µM, BGB-3111 0.51 µM, acalabrutinib 1.21 µM, ONO/GS-4059 1.20 µM and evobrutinib 5.84 µM. Peak venous plasma concentrations of ibrutinib (0.5 µM), acalabrutinib (2 µM) and ONO/GS-4059 (2 µM) measured after anti-proliferative dosage inhibited collagen-induced platelet aggregation, but did not increase PFA-200 closure time on collagen/epinephrine. Closure times were moderately increased by 2- to 2.5-fold higher concentrations of these inhibitors, but not by BGB-3111 (1 µM) and evobrutinib (10 µM). Prolonging platelet drug exposure to 60 minutes lowered IC_{50} values of any Btk inhibitor for GPVI-mediated aggregation by several fold, and 5- to 10-fold below anti-proliferative therapeutic drug plasma levels. In conclusion, low blood concentrations of ibrutinib and the novel Btk inhibitors suffice for GPVI selective platelet inhibition relevant for atherothrombosis but do not impair primary haemostasis.

Keywords
- Btk inhibitor
- platelet aggregation
- bleeding
- PFA
- GPVI

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Introduction

Bruton's tyrosine kinase (Btk), belonging to the cytosolic Tec-family tyrosine kinases, plays a crucial role in B cell development and proliferation. Ibrutinib, the first-in-class oral irreversible Btk inhibitor, is a mainstay of chronic lymphocytic leukaemia and mantle cell lymphoma (MCL) therapy.[1–4] Btk is also expressed in platelets and involved in signal transduction of the collagen receptor glycoprotein (GP) VI.[5,6]

Direct GPVI antagonists (antibodies, recombinant GPVI-Fc) preferentially inhibit platelet activation by collagenous material exposed after plaque rupture and erosion,[7–11] and we recently demonstrated that Btk inhibitors also selectively prevent atherosclerotic plaque-triggered platelet thrombus formation.[12] Collagen-induced platelet adhesion and aggregation under arterial flow requiring integrin α2β1 not being affected by Btk inhibitors was not inhibited.[12] Exploiting the covalent irreversible inactivation of Btk by ibrutinib and the lack of protein re-synthesis in platelets, we further demonstrated that much lower and less frequent doses of ibrutinib than needed in B cell malignancies suffice to block GPVI-mediated platelet activation, but leave platelet response to several stimuli of physiologic haemostasis largely intact and do not reduce blood B-lymphocytes.[12] This new concept of selective Btk inhibition in platelets holds promise to counteract occluding local atherothrombosis with little impairment of systemic haemostasis.

Although up to 50% of ibrutinib-treated patients with B cell malignancies have a low grade bleeding tendency (spontaneous bruising, petechiae), major bleeding events are rare.[4,13] Patients with X-linked agammaglobulinaemia due to genetic Btk deficiency show reduced GPVI-mediated platelet aggregation without any bleeding phenotype.[5,6,14–16] This apparent discrepancy may be due to off-target co-inhibition of Tec and Src family kinases by ibrutinib.[17] Indeed, at high concentrations ibrutinib irreversibly inhibits platelet Tec in addition to Btk,[18,19] thereby completely blocking GPVI signalling,[6,20] and at even higher concentrations also Src-kinases which mediate integrin αIIbβ3 outside-in signalling and platelet adhesion to immobilized fibrinogen.[18,19,21,22]

Newer highly selective Btk inhibitors, such as acalabrutinib (approved for MCL),[23] ONO/GS-4059 (tirabrutinib) and BGB-3111 (zanubrutinib) (both passed clinical trials for refractory B-lymphoid malignancies),[24,25] and evobrutinib (M2951; under study in multiple sclerosis)[26] are meanwhile available. They appear to have less side effects including bleeding which might be explained by the absence of off-target inhibition of Src family kinases.[17,18,24] We therefore studied ibrutinib, and these novel selective Btk inhibitors to optimize the trade-off between the local anti-atherothrombotic potency by blocking GPVI-mediated platelet aggregation in blood and the impairment of physiologic haemostasis assessed by platelet adhesion to immobilized fibrinogen under flow and in vitro bleeding time.

Materials and Methods

Materials

Ibrutinib, acalabrutinib (ACP-196) and ONO/GS-4059 were obtained from Selleckchem (Houston, Texas, United States). BGB-3111 and evobrutinib were from MedChem Express (New Jersey, United States). Dimethyl sulphoxide (DMSO), fibrinogen and albumin from human serum (fatty acid free) came from Sigma-Aldrich (Taufkirchen, Germany). Collagen (Horm, from equine tendon) was purchased from Takeda (Linz, Austria). Ristocetin was from Roche Diagnostics (Rotkreuz, Switzerland). DiOC6 was obtained from Life Technologies (Eugene, Oregon, United States) and phosphate-buffered saline (PBS) (Dulbecco's Phosphate Buffered Saline) from Gibco (Grand Island, New York, United States). Recombinant lepirudin (Refludan) was generously provided by Professor Christian Sommerhoff (Klinikum LMU, Munich, Germany) and Dr Andreas Calatzis (Dynabyte GmbH, Munich, Germany). Trisodium citrate/citric acid buffer solution (0.129 mol/L; S-Monovette 3.8 mL 9NC PFA) was from Sarstedt (Nümbrecht, Germany).

Declaration of Helsinki

Informed consent of healthy volunteers to donate blood was obtained 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

Blood was obtained from healthy adults who had not taken any drugs affecting platelet function for at least 2 weeks. Venous blood was drawn into a syringe containing recombinant hirudin (final concentration in blood: ~200 U/mL; 13 µg/mL).

Stock Solutions

Ibrutinib, BGB-3111, acalabrutinib (ACP-196), ONO/GS-4059 and evobrutinib were dissolved in DMSO at concentrations of 10 or 20 mM (evobrutinib). Aliquots (30 µL) were stored at 80°C. Before each in vitro experiment, dilutions were made in DMSO (0.025, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20 mM to obtain final concentrations in blood of 0.025, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 µM, respectively). The final concentration of DMSO in blood was always 0.1% (control). Collagen (1 mg/mL) was dissolved in SKF-Buffer in a 1:10 ratio (stock solution 100 µg/mL) for aggregometry experiments. DiOC6 for platelet labelling was dissolved in DMSO at a concentration of 5 mM. Aliquots (10 µL) were stored at –20°C.

Platelet Aggregation in Blood

Platelet aggregation in hirudin-anticoagulated blood was measured by multiple electrode aggregometry (MEA) using the Multiplate device from Dynabyte (Munich, Germany).[27,28] Ibrutinib, BGB-3111, acalabrutinib, ONO/GS-4059, evobrutinib or DMSO (control) was first added to 0.3 mL of physiological saline in the MEA cuvettes before addition of 0.3 mL of blood. The samples were pre-incubated in the absence of stirring for either 5, 10, 15, 30 or 60 minutes in the test cuvettes. Subsequently, collagen (various concentrations) or ristocetin (0.5 mg/mL) was added, stirring was started and the impedance change was recorded continuously for 10 minutes in duplicate samples. Cumulative aggregation values over the 10-minute time period are expressed as AU*min (AU, aggregation unit).

Platelet Adhesion and Aggregation onto Fibrinogen in Flowing Blood

Glass coverslips (Menzel, 24 × 60mm, #1.5) were coated by spreading 30 µL fibrinogen solution (200 µg/mL in PBS) over a surface of 5 × 5 mm^2. They were subsequently blocked with human serum albumin (HSA) 0.5% in PBS, and stored in a wet chamber at 8°C until use within maximum 24 hours. The coverslips were assembled into parallel plate flow chambers using sticky slides (0.1 Luer sticky slides, ibidi, Martinsried, Germany) which had been blocked before with HSA (4% in PBS). The flow chambers were then mounted on the stage of a fluorescence microscope (TE2000-E, Nikon, Tokyo, Japan) equipped with an incubation chamber (37°C). The flow chambers were perfused with PBS and subsequently blocked with PBS containing 4% HSA for 2 minutes to prevent non-specific binding of platelets to the glass coverslips.

Ibrutinib (0.2 or 1 µM), acalabrutinib (1 or 5 µM) or DMSO (final concentration in blood 0.1%; control) was first added to 100 µL of NaCl (0.9%) placed in Falcon tubes, and blood samples (2.9 mL) were subsequently added. This procedure guaranteed that the Btk inhibitors were completely dissolved in the blood samples.[12] DiOC6 (1 µM) was added to blood for fluorescence labelling of platelets, and the samples were incubated for either 15 or 60 minutes at 37°C. Control runs were performed with DMSO (0.1%).

Blood was perfused through the flow chamber at a shear rate of 600/second with a withdrawal syringe pump (Harvard Apparatus, Holliston, Massachusetts, United States). Fluorescence microscopy (excitation: 485/25 nm, emission: 528/38 nm) was performed for real-time measurement of platelet adhesion and aggregate formation (distinguished by their different sizes) using a 10× air objective (numerical aperture 0.4) and a CoolSNAP HQ2 CCD camera (Photometrics, Tucson, Arizona, United States). Fluorescence images were continuously recorded (1 frame/second) for 5 minutes. They were analysed by quantifying the binary fluorescent area fraction (1.0 = total area) after subtraction of the fibrinogen autofluorescence at time 0 minute using the NIS-Element 3.2 (Nikon) software package 2.[10] The area visualized in this setting was 669 µm × 896 µm.

In Vitro Closure Time

The PFA-200 device (Siemens Healthcare, Erlangen, Germany) is an advanced successor model of the PFA-100 which simulates primary haemostasis and has been validated to measure bleeding time in vitro.[29] The instrument aspirates citrate-anticoagulated blood under constant vacuum from a reservoir through a capillary and a small hole in a membrane filter which is coated with collagen and adenosine diphosphate (ADP) (collagen/ADP test cartridge), or collagen and epinephrine (EPI) (collagen/EPI test cartridge). The time until complete occlusion of the aperture is reported as "in vitro closure time (CT)." Various concentrations of Btk inhibitors, DMSO (solvent control, final concentration in blood 0.1%) or saline were first added to 50 µL NaCl, and blood samples (0.8 mL) anticoagulated with citrate (129 mM/L) were subsequently added. This anticoagulation has been reported to produce stable results, also if measurements are done at later times after blood collection.[30] Blood samples were pre-incubated for 15 minutes at 37°, before measuring the CT in vitro with the collagen/EPI test cartridge. Measurements were performed between 75 and 150 minutes after blood collection.

Statistics

Values are given as the mean ± standard deviation (SD) of n experiments. After confirmation of normality, means of two parallel experimental conditions were compared by paired Student's t-test, and more than two concurrent experimental conditions were tested by analysis of variance for repeated measures followed by pair comparisons by Bonferroni's or the least significance difference methods (*: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$) using the SigmaStat package.

Results

Ibrutinib, BGB-3111, Acalabrutinib, ONO/GS-4059 and Evobrutinib Inhibit Platelet Aggregation in Blood Stimulated by Low Concentrations of Collagen

Platelet aggregation induced by fibrous collagen under static conditions is GPVI-dependent. Blood was pre-incubated with increasing concentrations of Btk inhibitors for 15minutes and subsequently stimulated with sub-maximal collagen concentrations titrated in each experiment to induce a similar degree of platelet aggregation as with maximal concentrations of atherosclerotic plaque material.[21] Ibrutinib (1 µM), BGB-3111 (2 µM), acalabrutinib (5 µM), ONO/GS-4059 (5 µM) and evobrutinib (10 µM) reduced collagen-induced platelet aggregation by > 90, 79, 85, 80 and 70%, respectively (►**Fig. 1**). Ibrutinib (IC_{50}, 0.12 µM) was more potent than BGB-3111 (IC_{50}, 0.51 µM),[31] acalabrutinib (IC_{50}, 1.21 µM), ONO/GS-4059 (IC_{50}, 1.20 µM) and evobrutinib (IC_{50}, 5.84 µM) (►**Fig. 1**; ►**Table 1**). Btk inhibitor-mediated suppression of platelet aggregation was maintained over the entire 10-minute observation period (data not shown).

Effect of Ibrutinib, BGB-3111, Acalabrutinib, ONO/GS-4059 and Evobrutinib on In Vitro Bleeding

To test whether the Btk inhibitors increase bleeding time, we performed measurements with the PFA-200 that is established to estimate potential haemorrhagic risk.[29,31] Blood pre-incubated for 15 minutes with ibrutinib (0.5 µM), acalabrutinib (2 µM) and ONO/GS-4059 (2 µM), concentrations that inhibited collagen-induced platelet aggregation by 86, 70 and 67%, respectively (see ►**Fig. 1**), did not significantly increase CT with the collagen/EPI cartridge (►**Fig. 2**). Higher concentrations of ibrutinib (1 µM), acalabrutinib (5 µM) and ONO/GS-4059 (5 µM) moderately, but significantly, increased CT to 243, 215 and 223 seconds, respectively (►**Fig. 2**). The increase was, however, lower than after pre-incubating blood with acetylsalicylic acid (1 mM) which increased CT to 280 ± 9 seconds (mean ± SD, $n = 4$). BGB-3111 (1 µM) and evobrutinib (10 µM) did not significantly increase CT, and DMSO (0.1%), the solvent of the Btk inhibitors, did not affect CT (►**Fig. 2**).

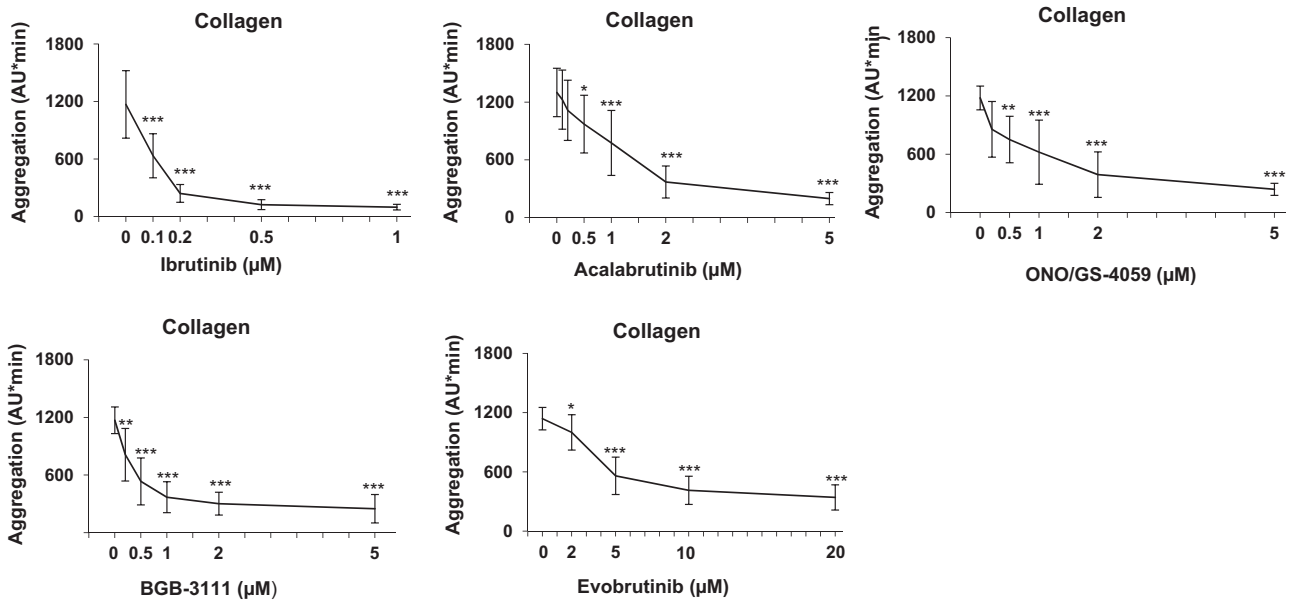

Fig. 1 Effects of ibrutinib, BGB-3111, acalabrutinib, ONO/GS-4059 and evobrutinib on static platelet aggregation in blood stimulated by low concentrations of collagen. Blood samples were pre-incubated for 15 minutes with solvent (dimethyl sulphoxide [DMSO] 0.1%) or Bruton's tyrosine kinase (Btk) inhibitors before stimulation for 10 minutes with sub-maximal collagen concentrations (0.2–0.5 μg/mL) titrated to induce the same degree of platelet aggregation as maximal concentrations of plaque homogenate (833 μg/mL).[21] Dose–response curves of ibrutinib, BGB-3111, acalabrutinib (ACP-196), ONO/GS-4059 and evobrutinib on collagen-induced platelet aggregation are shown. Values are mean ± standard deviation (SD) ($n = 5$).

Table 1 IC_{50} values of Btk inhibitors for inhibition of collagen-induced platelet aggregation after pre-incubation for 15 and 60 minutes, and comparison with therapeutic drug plasma levels

Btk inhibitor	IC_{50} (μM) 15-min pre-incubation	IC_{50} (μM) 60-min pre-incubation	Anti-proliferative therapeutic drug plasma levels
Ibrutinib	0.12 ± 0.04	0.025 ± 0.01	0.31 μM; 0.37 μM[a]
BGB-3111	0.51 ± 0.27	0.094 ± 0.05	Not known
Acalabrutinib	1.21 ± 0.34	0.372 ± 0.09	1.78 μM[b]
ONO/GS-4059	1.2 ± 0.83	0.268 ± 0.14	1.95 μM[c]
Evobrutinib	5.84 ± 2.31	1.20 ± 0.21	Not known

Abbreviations: Btk, Bruton's tyrosine kinase; SD, standard deviation.
Note: Values are mean ± SD ($n = 5$).
[a]Ibrutinib 420 mg q.d. and 560 mg q.d., respectively.[32,38]
[b]Acalabrutinib 100 mg b.i.d.[17]
[c]ONO/GS-4059 320 mg q.[24]

As even supramaximal ibrutinib concentrations (2 μM) did not increase CT with the collagen/ADP cartridge, this type of measurements was not performed with the lower Btk inhibitor concentrations effective with the collagen/EPI cartridge.

Prolonging Platelet Exposure to Btk Inhibitors Potentiates the Inhibition of Platelet Aggregation Induced by Low Collagen Concentrations

After oral application, platelets in portal venous blood will be exposed during absorption to Btk inhibitors for longer time periods thereby inhibiting platelet Btk more effectively. Therefore, the kinetics of platelet inhibition by even lower concentrations of Btk inhibitors (ibrutinib 0.15 μM, BGB-3111 0.15 μM, acalabrutinib 1 μM, ONO/GS-4059 1 μM and evobrutinib 5 μM) were studied by pre-incubating blood 5 to 60 minutes before stimulation with low dose collagen. Prolonging the incubation time with all Btk inhibitors profoundly and significantly enhanced their effect with complete suppression of aggregation on collagen after 60 minutes (►**Fig. 3**). Concentration-response curves of all Btk inhibitors after 60-minute pre-incubation yielded 3 to 5 times lower IC_{50} values as compared with 15-minute pre-incubation (►**Fig. 4**; ►**Table 1**).

Increasing the Collagen Concentration Partially Overcomes Platelet Inhibition by Btk Inhibitors

Blood was pre-incubated for 60 minutes with therapeutically reached plasma concentrations of Btk inhibitors: ibrutinib 0.25 μM,[32] acalabrutinib 2 μM,[17,33] ONO/GS-4059 2 μM[24] or BGB-3111 0.4 μM (its plasma concentration is unknown). Blood was then stimulated with increasing collagen concentrations which induced 71 ± 10% (0.2 μg/mL), 88 ± 8.4% (1 μg/mL) or maximal (100 ± 8.6%; 2.5 μg/mL) platelet aggregation. The inhibition of

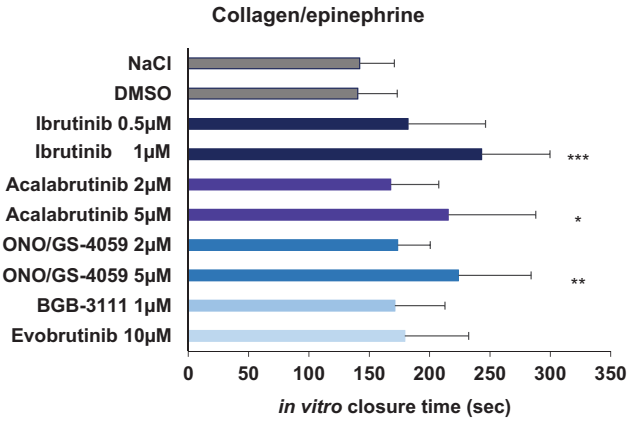

Fig. 2 Effects of ibrutinib, BGB-3111, acalabrutinib, ONO/GS-4059 and evobrutinib on bleeding time in vitro (PFA-200). After pre-incubation time of 15 minutes, blood samples with solvent (dimethyl sulphoxide [DMSO] 0.1%), ibrutinib (0.5 or 1 µM), BGB-3111 (1 µM), acalabrutinib (2 or 5 µM), ONO/GS-4059 (2 or 5 µM) or evobrutinib (10 µM) were transferred to collagen/epinephrine cartridges, and the in vitro closure time was measured with the platelet function analyser PFA 200. Values are mean ± standard deviation (SD) ($n = 8$).

platelet aggregation decreased from 92 to 98% at collagen 0.2 µg/mL to 29 to 33% at an over 10-fold collagen concentration (2.5 µg/mL) with the Btk inhibitors (►**Fig. 5**).

Ibrutinib, BGB-3111, Acalabrutinib, ONO/GS-4059 and Evobrutinib Inhibit Ristocetin-Induced Platelet Aggregation

Btk is also involved in botrocetin/von Willebrand factor signalling through GPIb leading to aggregation of mice platelets,[34] and ibrutinib has been reported to inhibit ristocetin-induced platelet aggregation in vitro and ex vivo.[12,35] All Btk inhibitors incubated for 15 minutes at concentrations which maximally inhibited collagen-induced platelet aggregation (ibrutinib 1 µM, BGB-3111 2 µM, acalabrutinib 5 µM, ONO/GS-4059 5 µM and evobrutinib 10 µM) also inhibited ristocetin-induced platelet aggregation by > 80% with no major differences between the Btk inhibitors (►**Fig. 6**).

Effect of Ibrutinib and Acalabrutinib on Platelet Adhesion and Aggregation to Immobilized Fibrinogen

Inhibition of integrin αIIbβ3 function can cause bleeding, and ibrutinib has been reported to inhibit in vitro platelet integrin αIIbβ3 outside-in signalling of immobilized fibrinogen.[21] We therefore measured platelet coverage onto immobilized fibrinogen and compared the effects of ibrutinib and acalabrutinib after 15- and 60-minute pre-incubation at concentrations of the Btk inhibitors which inhibited > 95% collagen-induced platelet aggregation (see ►**Figs. 1** and **4**). Confirming previous results,[21] 1 µM ibrutinib incubation for 15 minutes significantly reduced platelet coverage of immobilized fibrinogen at several time points, whereas 5 µM acalabrutinib for 15 minutes did not alter platelet deposition onto immobilized fibrinogen (►**Fig. 7A**). Platelets incubated with five times lower concentrations of the Btk inhibitors for 60 minutes reflecting their drug exposure after therapeutic administration[17,32,33] barely affected platelet coverage onto immobilized fibrinogen (►**Fig. 7B**). Only 5 minutes after the start of flow, platelet coverage after 0.2 µM ibrutinib was significantly reduced by 52%.

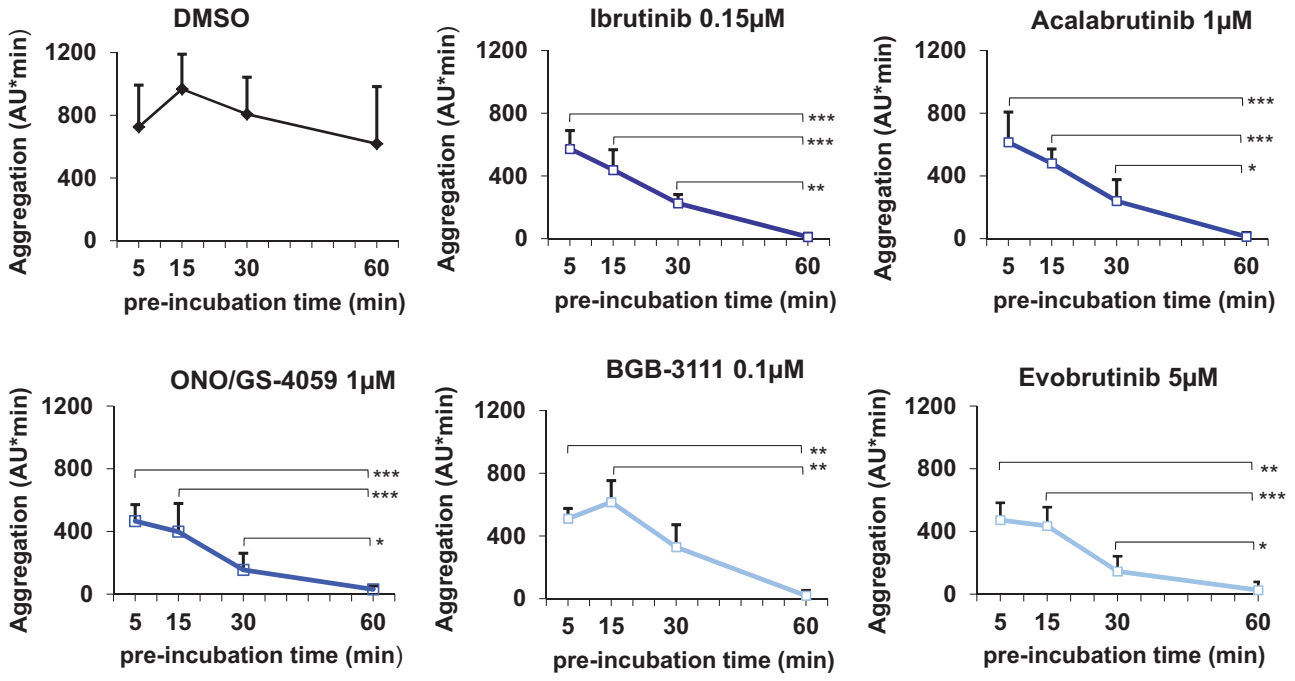

Fig. 3 Prolonging the pre-incubation time increases the inhibitory effects of ibrutinib, BGB-3111, acalabrutinib, ONO/GS-4059 and evobrutinib on collagen-induced platelet aggregation. Blood samples were pre-incubated for 5, 10, 30 and 60 minutes with solvent (dimethyl sulphoxide [DMSO] 0.1%) or ibrutinib (0.15 µM), BGB-3111 (0.1 µM), acalabrutinib (1 µM), ONO/GS-4059 (1 µM) or evobrutinib (5 µM), before stimulation for 10 minutes with collagen (0.2–0.3 µg/mL). Values are mean ± standard deviation (SD) ($n = 4$).

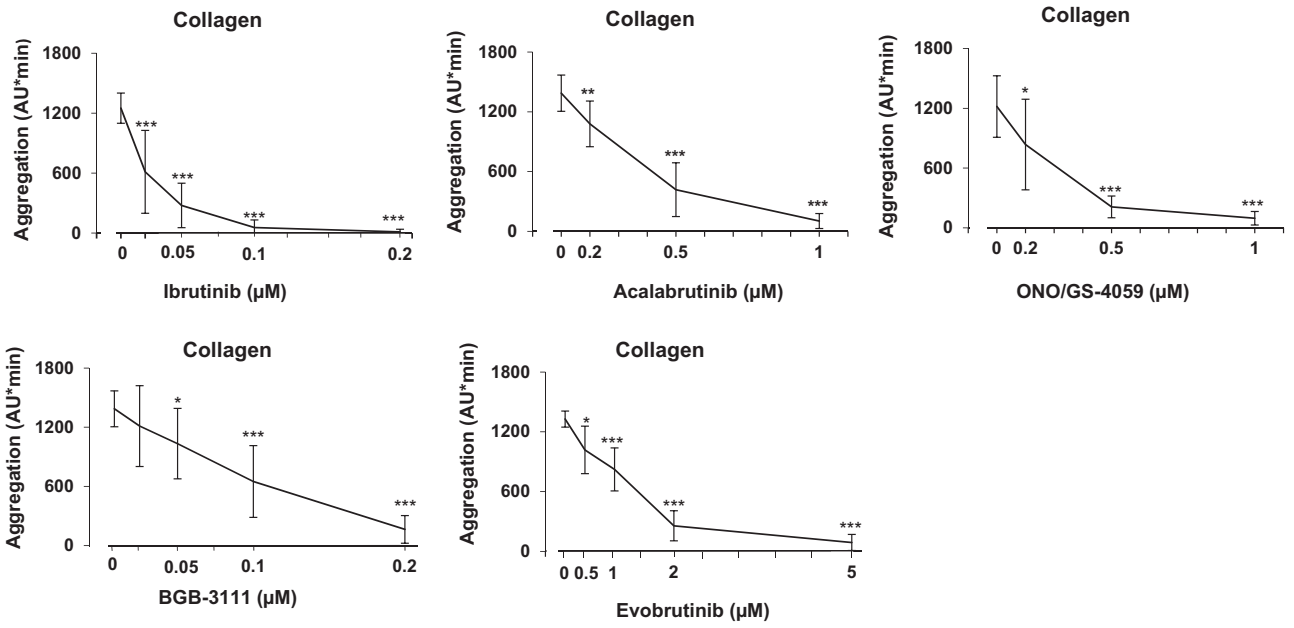

Fig. 4 Effects of ibrutinib, BGB-3111, acalabrutinib, ONO/GS-4059 and evobrutinib after 60-minute pre-incubation on platelet aggregation stimulated by low concentrations of collagen. Blood samples were pre-incubated for 60 minutes with solvent (dimethyl sulphoxide [DMSO] 0.1%) or increasing concentrations of Bruton's tyrosine kinase (Btk) inhibitors before stimulation for 10 minutes with collagen (0.2–0.5 μg/mL). Values are mean ± standard deviation (SD) ($n = 5$).

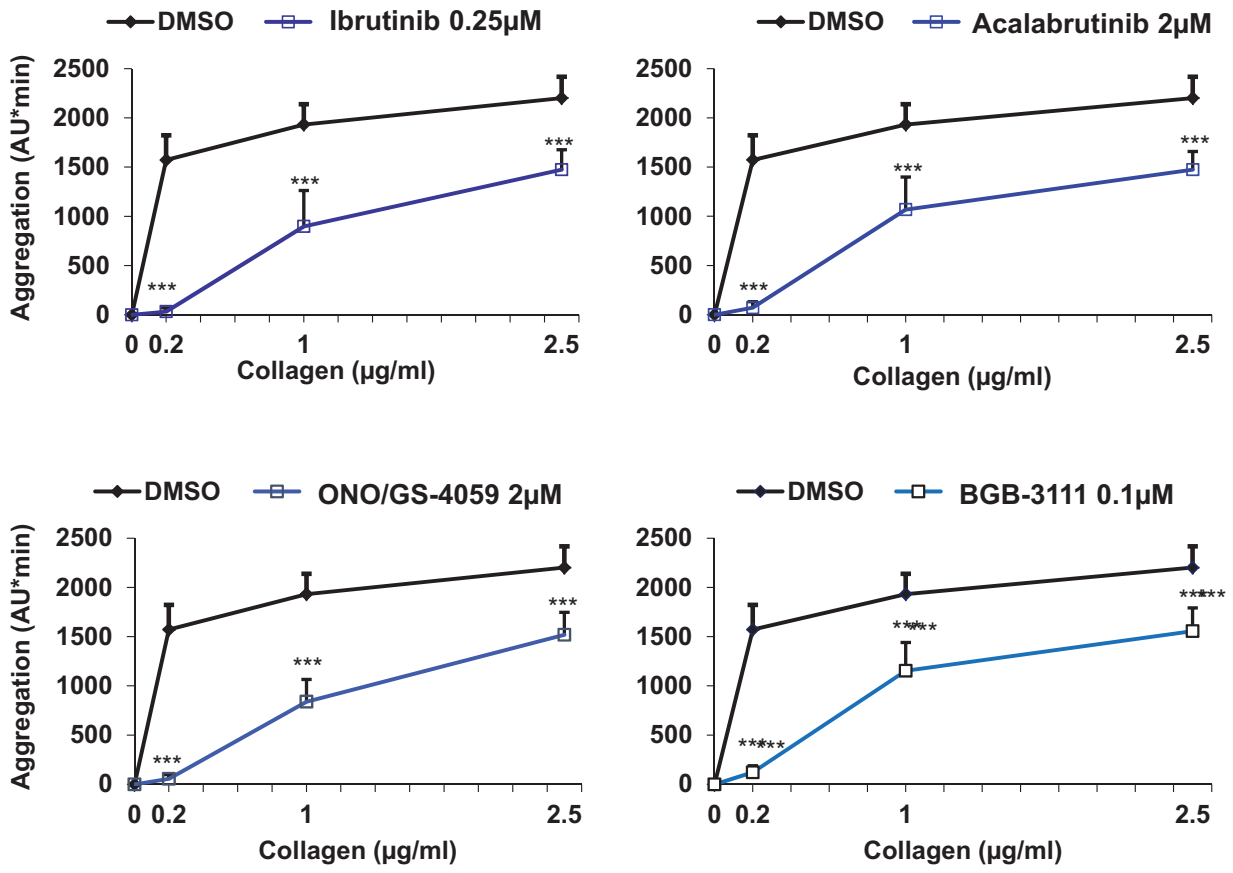

Fig. 5 Increasing concentrations of collagen surmount inhibition of platelet aggregation by Bruton's tyrosine kinase (Btk) inhibitors. Blood samples were pre-incubated for 60 minutes with solvent (dimethyl sulphoxide [DMSO] 0.1%), ibrutinib (0.25 μM), BGB-3111 (0.4 μM), acalabrutinib (2 μM) or ONO/GS-4059 (2 μM) before stimulation with increasing concentrations of collagen (0.2, 1 and 2.5 μg/mL) for 10 minutes. Values are mean ± standard deviation (SD) ($n = 5$).

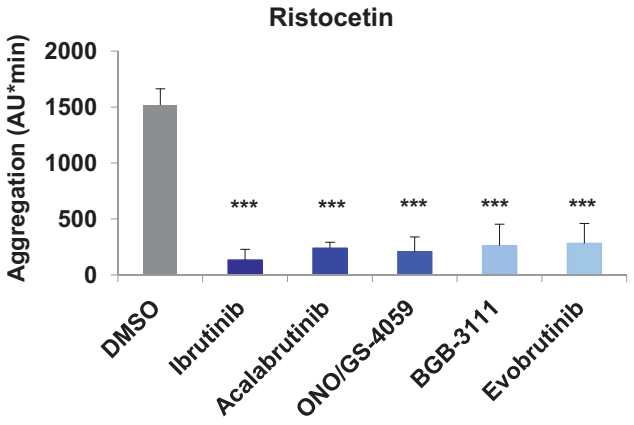

Fig. 6 Effects of Bruton's tyrosine kinase (Btk) inhibitors on ristocetin-induced platelet aggregation. Blood samples were pre-incubated for 15 minutes with solvent (dimethyl sulphoxide [DMSO] 0.1%), ibrutinib (1 μM), BGB-3111 (2 μM), acalabrutinib (5 μM), ONO/GS-4059 (5 μM) or evobrutinib (10 μM) before stimulation with ristocetin (0.5 mg/mL) for 10 minutes. Values are mean ± standard deviation (SD) ($n = 5$).

Discussion

We demonstrate that low concentrations of ibrutinib and several novel Btk inhibitors inhibit GPVI-dependent collagen-induced platelet aggregation in blood but not primary haemostasis. Only high concentrations of ibrutinib, acalabrutinib and ONO/GS-4059 increased in vitro bleeding time measured by PFA-200 using the EPI/collagen cartridge, but still less than aspirin. Ibrutinib therapy for B cell malignancies is associated with low-grade bleeding events in up to 50% of patients.[4,13] The pathophysiology of bleeding associated with ibrutinib therapy is complex, and not only explained by a direct drug effect on platelets.[16,36] According to recent reports, also patients treated with the novel selective Btk inhibitors acalabrutinib and ONO/GS-4059 may show a similar frequency of low-grade bleeding events.[23,37] However, therapeutic concentrations of all these Btk inhibitors in B cell disorders are higher than required for platelet effects and will not only inhibit Btk but also Tec in platelets thereby shutting down GPVI signalling.[6,18–20]

Ibrutinib in contrast to the novel Btk inhibitors has also off-target effects on platelet Src family kinases that may compromise platelet adhesion to fibrinogen, inhibit thrombus stability and explain rare major bleeding events reported on ibrutinib but not acalabrutinib therapy.[18,21] Such differences might explain the impaired platelet adhesion and aggregation onto immobilized fibrinogen after 15-minute pre-incubation that we observed with high concentrations of ibrutinib but not with acalabrutinib.

A prolonged blood pre-incubation at low concentrations might better reflect the in vivo exposure of platelets after low oral drug dosage and retarded absorption kinetics. Indeed, we observed that 60 minutes of pre-incubation increased the potency of all Btk inhibitors to inhibit GPVI-dependent platelet aggregation. The IC_{50} values required for inhibition of platelet aggregation stimulated by collagen were then 5-fold (acalabrutinib), 7-fold (ONO/GS-4059) and 12-fold (ibrutinib) lower than the peak

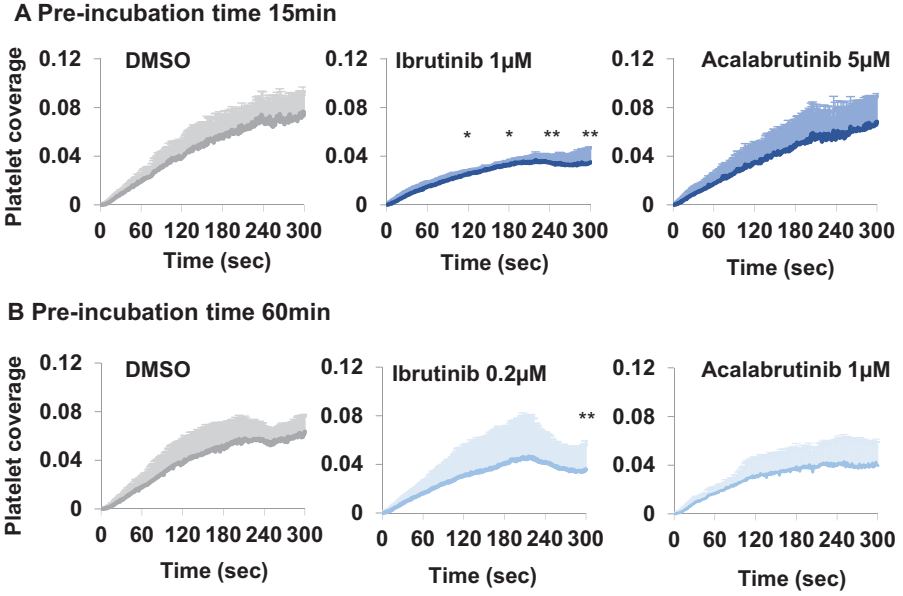

Fig. 7 Effects of ibrutinib and acalabrutinib on platelet aggregation and adhesion to immobilized fibrinogen under arterial flow. Blood samples were pre-incubated with DiOC6 for platelet labelling, and with solvent (dimethyl sulphoxide [DMSO] 0.1%), ibrutinib or acalabrutinib (all in DMSO, final concentration 0.1%) for either 15 or 60 minutes before start of blood flow at a shear rate of 600/second. Values are mean ± standard deviation (SD) ($n = 6$). (**A**) Effects of solvent (DMSO 0.1%), ibrutinib (1 μM) and acalabrutinib (5 μM) after pre-incubation time of 15 minutes. (**B**) Effects of solvent (DMSO 0.1%), ibrutinib (0.2 μM) and acalabrutinib (1 μM) after pre-incubation time of 60 minutes. Significances compared with DMSO control are indicated; $^{*}p < 0.05$; $^{**}p < 0.01$.

plasma levels after established dosage for B cell disorders (►**Table 1**).[17,24,32]

Low assay concentrations of collagen were applied in this study to induce a similar degree of GPVI-mediated platelet aggregation as observed with maximal concentrations of human atherosclerotic plaque homogenates.[10,12] Low-degree GPVI activation signals only through Btk as shown by studies of Btk-deficient human and mice platelets,[5,20] and by a recent study using 12 different Btk inhibitors where Btk and not Tec inhibition correlated with the suppression of platelet aggregation by half-maximal collagen concentrations.[38] Suppression of the low-grade GPVI-mediated platelet activation by irreversible Btk inhibitors could be overcome by increasing the collagen stimulus confirming previous studies with washed platelets using Btk-selective concentrations of ibrutinib and acalabrutinib.[18] This is explained by the compensatory function of Tec co-activation in high collagen-induced GPVI signalling.[6,20]

In conclusion, inhibition of GPVI-mediated platelet aggregation and increase of bleeding time can even be more dissociated by exposing platelets to lower concentrations of Btk inhibitors. Taking into account the different plasma concentrations reached after oral drug application, no major differences of ibrutinib and the novel Btk inhibitors acalabrutinib and ONO/GS-4059 on platelet inhibition and bleeding time were observed. At prolonged pre-incubation times, very low, in current indications sub-therapeutic plasma concentrations of ibrutinib, acalabrutinib and ONO/GS-4059 were sufficient to inhibit GPVI-dependent platelet aggregation. Similar to aspirin,[39] portal venous blood levels reached during absorption of very low doses of Btk inhibitors may suffice to irreversibly inhibit Btk in platelets and prevent the low-grade GPVI-dependent platelet activation relevant for atherothrombosis.[12] Our study provides a rational to explore the new application of low-dose irreversible Btk inhibitors in the prevention and treatment of acute atherothrombotic events.

The concept of atherosclerotic plaque-focused platelet inhibition by targeting the GPVI axis undergoes at present clinical testing in two phase 2 trials using a recombinant protein of the extracellular GPVI domain fused to the Fc region of human immunoglobulin G1 (dimeric GPVI-Fc, Revacept).[40] Its benefit on top of standard anti-platelet therapy is assessed in symptomatic carotid artery stenosis and in elective percutaneous coronary intervention of stable coronary artery disease.[41,42] In vitro, GPVI-Fc inhibits atherosclerotic plaque-induced platelet adhesion and aggregation by binding to collagen, most potently under high shear arterial flow.[10] Although recent studies suggest that platelet GPVI is also a receptor for fibrin and fibrinogen,[43–45] recombinant GPVI-Fc does not bind to fibrin.[46] GPVI-Fc did not increase bleeding in vitro, nor in mice and human volunteers in vivo even given on top of standard anti-platelet drugs (aspirin, P2Y12 receptor antagonists).[11,40,47] However, GPVI-Fc was less effective than Btk inhibitors in inhibiting plaque-induced platelet aggregation under static conditions and at low arterial shear flow in vitro,[10–12,48] and in contrast to Btk inhibitors has to be given intravenously.

What is known about this topic?

- Oral irreversible Btk inhibitors are used, or in clinical studies, to treat patients with B cell malignancies and autoimmune diseases. Recently, they also have been suggested to selectively inhibit atherothrombosis.
- Patients treated with ibrutinib have apparently more frequently major bleeding events than patients treated with the more selective novel second-generation Btk inhibitor, acalabrutinib

What does this paper add?

- Considering differences in plasma levels, ibrutinib and the novel Btk inhibitors acalabrutinib, ONO/GS-4059, BGB-3111 and evobrutinib showed no differences on inhibition of collagen- and ristocetin-induced platelet aggregation in blood and the increase of PFA-200 closure time
- Lower concentrations of the Btk inhibitors inhibited GPVI-dependent platelet aggregation relevant for atherothrombosis without increasing bleeding time.
- Prolonging platelet drug exposure to 60 minutes lowered IC_{50} values of the Btk inhibitors for GPVI-mediated platelet aggregation by 5- to 10_fold below anti-proliferative therapeutic drug plasma levels. Thus, much lower doses of Btk inhibitors than used for treatment of B cell disorders will suffice for inhibition of atherothrombosis and not increase bleeding.
- The results are relevant for the therapeutic application of different Btk inhibitors and the potential future use of Btk inhibitors as anti-platelet therapy.

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Conflict of Interest
None declared.

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