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Btk Inhibitors as First Oral Atherothrombosis-Selective Antiplatelet Drugs?

Kristina Busygina[1] Viola Denzinger[1] Isabell Bernlochner[2] Christian Weber[1,3,4,*] Reinhard Lorenz[1] Wolfgang Siess[1,3]

[1] Institute for Prevention of Cardiovascular Diseases, LMU (Ludwig-Maximilians University Munich), Munich, Germany
[2] Medizinische Klinik und Poliklinik I, Klinikum rechts der Isar, Medizinische Fakultät, Technische Universität München, Munich, Germany
[3] DZHK (German Centre for Cardiovascular Research), partner site Munich Heart Alliance, Munich, Germany
[4] Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands

Address for correspondence Wolfgang Siess, Prof. Dr. med., Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten, Klinikum Innenstadt, Ludwig-Maximilians Universität München, Pettenkoferstr. 9, D-80336, München, Germany (e-mail: wsiess@med.uni-muenchen.de).

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Abstract

Bruton’s tyrosine kinase (Btk) is essential for B cell differentiation and proliferation, but also platelets express Btk. Patients with X-linked agammaglobulinemia due to hereditary Btk deficiency do not show bleeding, but a mild bleeding tendency is observed in high dose therapy of B-cell malignancies with ibrutinib and novel second-generation irreversible Btk inhibitors (acalabrutinib and ONO/GS-4059). This review discusses recent studies that may explain this apparent paradox and gives mechanistic insights that suggest a unique potential of low dose irreversible Btk inhibitors as atherothrombosis-focused antiplatelet drugs.

Keywords
- Btk inhibitor
- platelet
- bleeding
- GPVI
- GPIb
- X-linked agammaglobulinemia

Introduction

Platelet activation is central for arterial thrombosis and atherothrombosis. Specific mechanisms of platelet activation and shear-resistant platelet–vessel wall interaction assure that the plug adhering to an injured artery (arterial thrombosis) or to an eroded or ruptured atherosclerotic plaque (atherothrombosis) can grow into and obstruct the arterial lumen despite the high-flow velocities in arteries.

The mechanisms of arterial thrombosis and atherothrombosis differ, however, in important aspects[1–7]: The main prothrombotic components of atherosclerotic plaques are collagens type I and III and tissue factor.[2–6,8] Human atherosclerotic plaque material stimulates thrombus formation in vitro in two steps: a rapid 1st phase of glycoprotein (GP) VI-mediated platelet adhesion and aggregation onto plaque collagen is followed by the 2nd phase of plaque tissue factor-induced formation of thrombin and fibrin.[5] A drug which specifically targets plaque-triggered platelet GPVI activation with high local efficacy but leaves physiologic hemostasis intact would substantially improve established antiplatelet therapy with aspirin and/or a $P2Y_{12}$ antagonist, drugs which both interfere with systemic hemostasis and increase bleeding risk.[9,10]

Collagens in atherosclerotic plaques structurally differ from collagens of healthy vascular connective tissue.[11] Plaque collagens show a high turnover due to degradation by matrix metalloproteinases and synthesis by expanded vascular smooth muscle cells.[12–14] Collagen fiber degradation in the arterial intima increases their association with and retention of other proteins including lipoproteins and oxidized lipids, and advanced glycation end products form irreversible covalent cross-links within collagen fibers.[15–17] This alters the collagen fibrillary structure, detectable by increased autofluorescence and decreased second-harmonic generation signal, and

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ultimately leads to changes in platelet activation by plaque collagens as compared with collagens from healthy vascular tissue.[3,4,17–20]

Platelet GPVI as Selective Anti-atherothrombotic Target

The abnormal structure of plaque collagens might explain, why plaques activate mainly GPVI and barely the integrin α2β1 on platelets: recombinant GPVI-Fc (Revacept) which binds to collagen, and anti-GPVI but not anti-integrin α2β1 antibodies inhibit plaque-induced platelet thrombus formation.[4–6,21,22] In flow chamber experiments using hirudin- or heparin-anticoagulated blood, anti-integrin α2β1 antibodies did not impair platelet thrombus formation on human atherosclerotic plaque homogenate from arterially flowing blood.[4,6] Similar results were observed using blood from integrin α2-deficient mice.[4] Thus, targeting GPVI might preferentially inhibit platelet activation after plaque rupture and erosion,[23] whereas in normal hemostasis inhibition of GPVI function may at least in part be compensated by the other major platelet collagen receptor, integrin α2β1.[24–27]

Indeed, GPVI-deficient mouse and human platelets (constitutive or caused by anti-GPVI antibodies) show only a mild bleeding tendency (for reference See Refs. 23 and 28), whereas anti-GPVI antibodies blocked in vitro human plaque-induced platelet thrombus formation more efficiently than aspirin and $P2Y_{12}$ antagonists,[21,29] and inhibited atherothrombosis triggered by plaque injury in vivo in murine models.[30,31] Targeting GPVI might thus allow to selectively inhibit atherosclerotic plaque-induced platelet activation and its sequelae (myocardial infarction, ischemic stroke).

Indeed, GPVI-inhibiting agents are already studied in clinical trials: Recombinant GPVI-Fc (Revacept) currently undergoes clinical phase II testing in patients with stable coronary artery disease[32] and a phase II trial of Revacept in patients with symptomatic carotid artery stenosis (transient ischemic attacks or stroke) has been completed (October 2018), their final analysis is eagerly awaited.[33,34] A clinical trial with Act017, a high affinity humanized antibody fragment (Fab) against GPVI, is planned in acute ischemic stroke.[35]

Bruton's Tyrosine Kinase in Platelet Signal Transduction

Bruton's tyrosine kinase (Btk) is a nonreceptor cytoplasmic tyrosine kinase named after Colonel Ogden Bruton who in 1952 first described and substituted patients with hereditary X-linked agammaglobulinemia (XLA).[36] XLA is caused by deficiency or dysfunctional mutations of Btk as proven by positional cloning and deoxyribonucleic acid cross-hybridization approaches in 1993.[37,38] Btk plays a critical role in B cell development, and is expressed in pre-B cells and B-lymphocytes, but not in T-lymphocytes. Btk is a member of the cytoplasmic Tec family of tyrosine kinases and carries a pleckstrin homology (PH), a Tec homology, a Src homology 3 (SH3), a SH2, and a SH1 (kinase) domain (►**Fig. 1A**).

Btk is also expressed in megakaryocytes and platelets, but XLA patients lack a bleeding phenotype.[39,40] A role of Btk in platelet GPVI signaling was first shown in 1998 as Btk tyrosine phosphorylation in response to collagen-related peptide (CRP) and collagen that was absent in platelets of XLA patients and accompanied by deficient GPVI-mediated platelet aggregation and secretion.[41] This deficiency could be overcome by high collagen concentrations. Thrombin-mediated platelet responses were not altered.[41]

Collagen also activates Tec, another Tec family kinase.[42] In XLA platelets, collagen stimulated tyrosine phosphorylation of Tec and several other signaling kinases, indicating that Tec activation is independent of Btk, may compensate for the lack of Btk, and sustain XLA platelet function in vivo. This can explain the absence of a bleeding phenotype in XLA patients.[42] The redundancy of Tec and Btk in sustaining GPVI-mediated platelet response could be directly demonstrated in Tec and Btk single- and double-deficient mice.[43] In platelets deficient in Btk or Tec, high concentrations of CRP or collagen could restore platelet reactivity. In double-deficient Tec and Btk platelets aggregation was absent or drastically reduced even on high concentrations of CRP or collagen.[43] These studies unequivocally demonstrate that Btk mediates platelet stimulation by a low degree of GPVI activation that can be bypassed and enforced by Tec at high GPVI agonist concentrations. Interestingly, Btk expression in mice platelets is with $12,146 \pm 1,854$ copies per cell much higher as compared with Tec (508 ± 65 copies per cell).[44]

Btk activation occurs rather downstream within the complex GPVI-signaling cascade. Collagen binding to GPVI first leads to GPVI dimerization, and the activation of the Src-family kinases Lyn and Fyn constitutively bound to the proline-rich region in the GPVI cytosolic tail.[45] These phosphorylate the immunoreceptor tyrosine-based activation motif (ITAM) of the dimeric Fc receptor γ-chain (FcRγ) associated with GPVI.[46] Phosphorylated ITAM recruits and activates the SH2-tandem domain of the tyrosine kinase Syk. Syk phosphorylates then the adapter protein LAT thereby initiating formation of a signaling complex comprising further adapter proteins (SLP-76, GADs, Grb2, Vav1/3) and providing docking sites for PI3-kinase and phospholipase Cγ2 (PLCγ2) (►**Fig. 1B**).[45–48] Btk activation occurs downstream of PI3-kinase activation[42] which increases membrane levels of PIP3 (PI 3,4,5-trisphosphate) that binds with high affinity to the PH-domain of Btk thereby leading to its translocation to the plasma membrane.[49,50] Lyn then phosphorylates Btk at Y-551 in the kinase domain, and subsequent autophosphorylation at Y-223 in the SH3 domain completes the activation of Btk[49,51] (►**Fig. 1A, B**). Y-223 phosphorylation is decisive for kinase activity of Btk. In platelets, Btk has been reported to interact with and of being activated by PKCθ.[52] Active Btk participates in the tyrosine phosphorylation and activation of the effector protein PLCγ2.[41,45,46] This increases cytosolic Ca^{2+} and activates protein kinase C, the two main downstream signals for platelet activation.[53] Btk also can regulate Ca^{2+} entry in platelets without increasing phospholipase C activity.[54] Ca^{2+} entry is inhibited by LFM-A13, the first Btk inhibitor

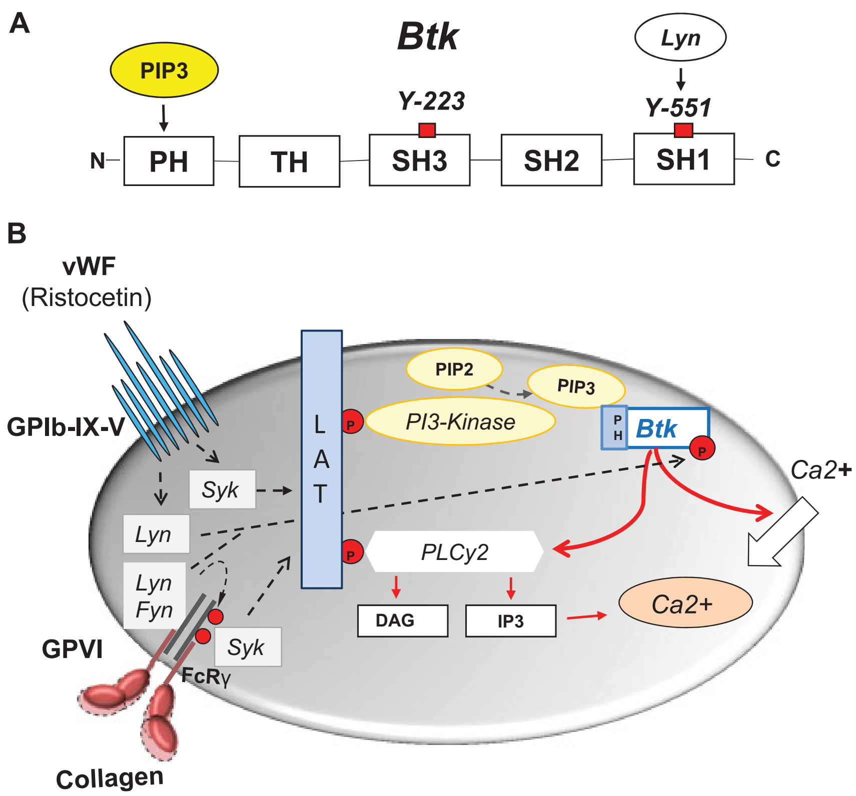

Fig. 1 Bruton's tyrosine kinase (Btk) domain structure, and Btk activation and signaling in platelets. (**A**) PH, pleckstrin homology; TH, Tec homology; SH, Src homology; the SH1 domain is identical to the kinase domain. Y223, autophosphorylation site. (**B**) Glycoprotein (GP) Ib and GPVI stimulation share Btk activation pathways (dashed lines); Btk downstream signaling stimulates independently PLCγ2 and Ca^{2+} entry (red lines). For details see text. Syk phosphorylates the adapter protein LAT which provides docking sites for further adapter proteins (not shown), PI3-kinase, and PLCγ2. The pathway of Tec activation is similar to that of Btk.[48] DAG, 1,2-diacylglycerol; LAT, linker for activation of T cells; IP3, inositol 1,4,5-trisphosphate; PLCγ2, phospholipase Cγ2; PI3-kinase, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol(3,4,5)-trisphosphate.

described.[52,55] Thereby by stimulating PLCγ2 and PLCγ2-independent Ca^{2+} entry, Btk plays a central role in raising cytosolic Ca^{2+} required for platelet aggregation and secretion[53] (►**Fig. 1B**).

Furthermore, Btk has been reported to be involved in botrocetin/von Willebrand factor (VWF) signaling through GPIb: Washed platelets from X-linked immunodeficient mice due to mutated Btk did not aggregate in response to botrocetin/VWF. Interestingly, in botrocetin/VWF-stimulated wild-type platelets Lyn, Syk, SLP-76, and PI3K activation preceded Btk phosphorylation and aggregation similar as in GPVI-stimulated platelets.[56]

Ibrutinib and the Novel Irreversible and Reversible Btk Inhibitors

Ibrutinib, the first-in-class oral irreversible Btk inhibitor, is approved for various B cell malignancies such as chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma, mantle cell lymphoma (MCL), Waldenström's macroglobulinemia, and, recently, chronic graft versus host disease.[57] The dose for MCL is 560 mg, for the other diseases 420 mg orally once daily.[57]

Ibrutinib binds covalently to cysteine 481 in the active site thus irreversibly inhibiting Btk; it inhibits to a minor extent also other kinases.[58] New, more selective irreversible Btk inhibitors have been developed to reduce side effects. Acalabrutinib has recently been approved for the treatment of relapsed MCL,[59] and ONO/GS-4059 (tirabrutinib) and BGB-3111 (zanubrutinib) have passed phase III trials in relapsed or refractory B-lymphoid malignancies.[60,61] Reversible highly specific and potent Btk inhibitors such as BMS-986142, GDC-0853 (fenebrutinib), and G-744 were developed to target B cells and macrophages in various autoimmune diseases such as rheumatoid arthritis and lupus nephritis.[62–66] The blood–brain barrier passing covalent Btk inhibitors evobrutinib (M2951) and PRN-2246 are studied in patients with multiple sclerosis.[67,68]

In kinase-inhibition assays, acalabrutinib was found to be more specific for Btk than ibrutinib. Ibrutinib inhibited also other kinases including the Src-kinases Lyn and Fyn (with 10- and 20-fold higher IC_{50}'s, respectively) and Tec (with a fourfold higher IC_{50}) as compared with Btk.[59] However, recently four different kinase assay platforms failed to confirm a higher selectivity of acalabrutinib for Btk versus Tec compared with ibrutinib.[69] Three of the kinase panel platforms showed lower IC_{50} values of ibrutinib and acalabrutinib for Btk inhibition than for Tec inhibition. In all platforms, ibrutinib inhibited Btk more potently (10- to 100-fold) than acalabrutinib.[69] Using the KINOMEscan platform that screens the binding to 442 kinases, ONO/GS-4059 bound with similar high affinity only to Tec and Btk.[60] Thus, based on the in vitro kinase panels, ibrutinib and acalabrutinib may inhibit Btk at lower concentrations than required to inhibit Tec in intact cells. Concordant with the results of kinase panels, cellular Btk on-target assays demonstrated a higher potency of ibrutinib as compared with acalabrutinib and ONO/GS-4059.[69]

Adverse events such as rash, diarrhea, arthralgias, myalgias, atrial fibrillation, and bleeding are associated with ibrutinib therapy of patients with CLL and MCL. In an analysis of 15 clinical studies of CLL and MCL of patients ($n = 1{,}768$) on full dose ibrutinib therapy including 4 randomized clinical trials, the most common bleeding events were low-grade bleedings including contusion, petechiae, epistaxis, and hematomas, which occurred in approximately 35% of patients (vs. 15% to the comparator in randomized clinical trials).[70] Of note, approximately 50% of the studied CLL and MCL patients had an additional antiplatelet or anticoagulation therapy. Risk factors for low grade bleeding on full dose ibrutinib in patients with leukemia included low baseline platelet count, and concomitant antiplatelet or anticoagulant therapy.[70] Interestingly, the proportion of major hemorrhage associated with long-term ibrutinib therapy was not higher,[70,71] and use of anticoagulants and/or antiplatelet drugs increased the relative risk for major hemorrhage in both the ibrutinib-treated patients and comparator-treated patients to a similar degree (1.9% vs. 2.4%) indicating that ibrutinib may not alter the effect of additional antiplatelet or anticoagulation therapy on the risk of major hemorrhage in B cell malignancies.[70]

The bleeding side effects of ibrutinib—not observed in Btk-deficient XLA patients—have been attributed to off-target effects on other kinases.[59] However, the Calquence (acalabrutinib) full prescribing information in acalabrutinib-treated patients with hematological malignancies ($n = 612$) reports a similar pattern of side effects including atrial fibrillation (3%), low grade bleeding events (petechiae, bruising; ~50%), and rare grade 3 or higher bleeding events (gastrointestinal, intracranial) (2%).[72] Very recently, the long-term Calquence follow-up data in MCL patients ($n = 121$) showed bleeding events of grade 1 and 2 in 33% of patients, and the ongoing Calquence CLL clinical trial ($n = 99$) showed bleeding events (all grades) in even 64% of patients and in 3% of patients grade 3 bleeding.[73] ONO/GS-4059-treated patients ($n = 28$) had also similar incidence of low grade bleeding events (petechiae, purpura, bruising).[74] Therefore, treatment of CLL and MCL patients with ibrutinib or the new Btk inhibitors apparently does not differ in side effects including bleeding.

Effect of Btk Inhibitors on Platelets and Possible Mechanisms of Bleeding in CLL and MCL Patients Treated with Btk Inhibitors

In accordance with the role of Btk in GPVI signaling, GPVI-mediated platelet aggregation and secretion induced by collagen and CRP in washed human platelets, platelet-rich plasma (PRP), and blood was inhibited by ibrutinib and acalabrutinib in vitro and ex vivo,[69,75–79] and by ONO/GS-4059, BGB-3111, and evobrutinib in vitro.[80]

In support for the involvement of Btk in botrocetin/VWF-stimulated aggregation of mouse platelets, various Btk inhibitors (ibrutinib, acalabrutinib, ONO/GS-4059, BGB-3111, evobrutinib) have been shown to inhibit ristocetin-induced platelet aggregation in human blood.[79,80] Ristocetin-induced platelet aggregation in blood measured by Multiplate has been used to monitor the bleeding tendency in CLL patients treated with ibrutinib; this method could possibly be used to predict the risk of severe bleeding.[81] It is, however, uncertain whether inhibition of VWF/GPIb signaling by ibrutinib is responsible for the inhibition of platelet adhesion onto immobilized VWF under arterial flow.[76] Also GPVI plays a critical role in this process,[82] probably due to the coassociation of GPIb and GPVI in resting and activated human platelets.[83] Thus, inhibition of GPVI signaling by ibrutinib could explain impairment of platelet adhesion onto VWF surfaces.

A 30% reduction of low dose (5 µM) thrombin receptor activator for peptide (TRAP)-induced aggregation by ibrutinib, acalabrutinib, and ONO/GS-4059 has been found in vitro.[79] However, TRAP-induced aggregation was not inhibited in patients treated with ibrutinib or acalabrutinib.[77] Also, higher dose (15 µM) TRAP-induced aggregation was not impaired in healthy volunteers after ibrutinib intake (own unpublished results). In addition, platelet aggregation by a protease-activated receptor 4-activating peptide was not inhibited by ibrutinib, acalabrutinib, and ONO/GS-4059.[79] Moreover, thrombin does not stimulate Btk phosphorylation in nonaggregating platelets.[42] Btk inhibitors (ibrutinib, acalabrutinib, ONO/GS-4059) also did not impair platelet aggregation in PRP or blood in response to adenosine diphosphate (ADP), epinephrine, arachidonic acid, and U46619. Together these results indicate that G protein coupled receptor-induced platelet signaling and fibrinogen-mediated platelet aggregation is not affected by Btk inhibitors.[75–77,79] Thus, Btk activation downstream of integrin αIIbβ3 in aggregating platelets[84] is apparently not of functional relevance.

When washed platelets were incubated in vitro with high concentrations of ibrutinib only for shorter times (30 seconds–5 minutes) and then washed again, their response on high CRP concentration was almost fully recovered indicating that short-term platelet inhibition under these conditions cannot be due to covalent modification of Btk or Tec.[78] However, in vitro, Btk-specific irreversible platelet inhibition may occur at low drug concentrations but requires longer (> 5 minutes) incubation times. Indeed, inhibition of GPVI-dependent aggregation of platelets in blood by low concentrations of ibrutinib and the novel Btk inhibitors (acalabrutinib, ONO/GS-4059, BGB-3111, evobrutinib) increased with drug exposure time from 5 minutes reaching the full effect within 60 minutes.[80]

The higher potency of ibrutinib (IC_{50} 0.29 nM) compared with acalabrutinib (IC_{50} 2.79 nM) for Btk inhibition in panel kinase assays[69] parallels its higher potency to inhibit in vitro CRP- or collagen-activated aggregation in washed platelets, PRP, or blood (see **►Table 1**). Unexpectedly, despite of protein binding of the lipophilic Btk-inhibitors, the IC_{50} values of ibrutinib and acalabrutinib for inhibition of aggregation were higher in washed platelets as compared with PRP or

Table 1 IC_{50} values of Btk inhibitors for inhibition of GPVI-mediated platelet aggregation in studies of washed platelets, PRP, and blood. For comparison are below the antiproliferative therapeutic maximal plasma concentrations (C_{max}) of Btk inhibitors

Study	Platelet preparation	Preincubation time (min)	GPVI agonist	Ibrutinib (Mw 440) IC_{50}, µM	Acalabrutinib (Mw 465) IC_{50}, µM	ONO/GS-4059 (Mw 491) IC_{50}, µM
Ref. [77]	Washed pl.	5	CRP-XL (1 µg/mL)	0.501	6.3	n.d.
Ref. [78]	Washed pl.	5	CRP (10 µg/mL)	1.19	21.25	n.d.
Ref. [76]	PRP	10	Submaximal collagen	0.5	n.d.	n.d.
Ref. [69]	PRP	15–45	Half-maximal collagen	0.35 ± 0.07	1.85 ± 0.55	3.15 ± 3.58
Ref. [79]	Blood	15	Plaque	0.18 ± 0.05 (79 ng/mL)	0.34 ± 0.19 (158 ng/mL)	0.79 ± 0.33 (387 ng/mL)
Ref. [80]	Blood	15	Submaximal collagen	0.12 ± 0.04 (53 ng/mL)	1.21 ± 0.34 (562 ng/mL)	1.2 ± 0.83 (589 ng/mL)
Ref. [80]	Blood	60	Submaximal collagen	0.025 ± 0.01 (11 ng/mL)	0.372 ± 0.09 (173 ng/mL)	0.268 ± 0.14 (131 ng/mL)
	Plasma		**Antiproliferative therapeutic C_{max} (µM)**			
				Ibrutinib	**Acalabrutinib**	**ONO/GS-4059**
[a]Ref. [85] Ref. [69] [b]Ref. [59] [c]Ref. [60]				0.31[a]; (136 ng/mL) 0.37[a] (163 ng/mL)	1.78[b] (827 ng/mL)	1.95[c] (957 ng/mL)

Abbreviations: Btk, Bruton's tyrosine kinase; CRP-XL, cross-linked collagen-related peptide; GPVI, glycoprotein VI; PRP, platelet-rich plasma.
[a]Ibrutinib 420 mg daily and 560 mg daily, respectively.
[b]Acalabrutinib 100 mg twice daily.
[c]ONO/GS-4059 320 mg daily.

blood. The higher grade of GPVI activation by CRP as compared with low collagen concentrations and the shorter preincubation times applied in studies of washed platelets may explain, at least in part, these differences. Of note, prolonging the incubation time in blood to 60 minutes yielded 3 to 5 times lower IC_{50} values for inhibition of platelet aggregation by submaximal collagen concentrations as compared with the 15-minute preincubation (►**Table 1**).[80] By comparing these in vitro IC_{50} values with peak plasma concentrations of Btk inhibitors in patients treated for B cell malignancy[59,60,69,85,86] (►**Table 1**), it appears that 5-to15-fold lower than antiproliferative doses of Btk inhibitors will be sufficient to inhibit low grade GPVI-mediated platelet activation by submaximal collagen concentrations or atherosclerotic plaque material. This has indeed been demonstrated in human volunteers taking only 140 mg ibrutinib each day or on alternate days.[79]

The mechanisms of bleeding in patients with B cell malignancies treated with Btk inhibitors (ibrutinib, acalabrutinib) are obviously complex.[87] A longitudinal study of 14 patients treated with ibrutinib reported a correlation between occurrence of bleeding events and decreased platelet response to collagen in PRP and firm adhesion on VWF under arterial flow.[76] To understand, why major bleeding events are apparently more frequent in patients treated with ibrutinib than with acalabrutinib, platelets were incubated with these drugs in vitro and also studied ex vivo in patients on Btk inhibitors.[77,78] In washed platelets short-term incubation (5 and 10 minutes) with low concentrations of ibrutinib and acalabrutinib suppressed CRP-stimulated platelet Btk phosphorylation at Y223,[76–78] that correlated with inhibition of platelet aggregation stimulated by low CRP concentrations.[76,77] Higher CRP concentrations overcame inhibition of aggregation in washed platelet by ibrutinib and acalabrutinib despite still complete suppression of Btk activity,[77,78] and also reversed inhibition of aggregation of PRP in some patients treated with ibrutinib and acalabrutinib.[77]

Lower doses of ibrutinib and acalabrutinib which suffice to inhibit Btk did not inhibit CRP-stimulated Tec phosphorylation in washed platelets but higher concentrations inhibited Tec phosphorylation, too.[77,78] High concentrations of ibrutinib (10- to 30-fold higher than required to inhibit Btk)—but not of acalabrutinib—inhibited also CRP stimulation of Src and Lyn kinases in washed platelets.[77,78] Inhibition of Src kinases might lead to inhibition of integrin αIIbβ3 outside-in signaling and platelet adhesion to immobilized fibrinogen[88] as observed at high ibrutinib concentrations[89] and cause instability of platelet thrombi formed on collagen in flowing blood as observed in two out of six patients on ibrutinib therapy.[77] Thus, major bleeding events (grade 3 and higher: gastrointestinal and intracranial hemorrhage, epistaxis) that are more often observed in patients on ibrutinib than on acalabrutinib therapy might be explained by accumulation of high ibrutinib concentrations in platelets with off-target effects on Src kinases.

Bleeding of grade 1 and 2 (petechiae, ecchymosis, bruising) has been reported to be associated with ibrutinib and acalabrutinib therapy in up to 50% of CLL and MCL patients.[72,90] Obviously, the drug concentrations required for treatment of B cell malignancies—and not needed for inhibition of the GPVI-

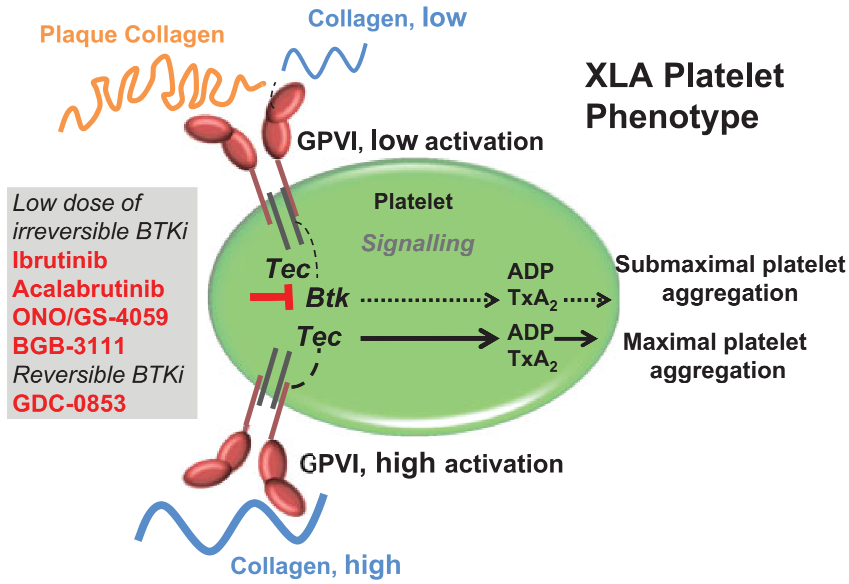

Fig. 2 Model of the selective anti-atherothrombotic effect of low dose irreversible Bruton's tyrosine kinase inhibitors (BTKi). Low concentrations of ibrutinib and second-generation irreversible Btk inhibitors selectively inhibit Btk in human platelets thereby inhibiting the low degree of glycoprotein (GP) VI and platelet activation by atherosclerotic plaque and low collagen concentrations. Reversible Btk inhibitors which do not inhibit Tec are expected to show the same effect. High collagen concentrations can surmount Btk inhibition by activating Tec. This situation resembles the platelet phenotype of X-linked agammaglobulinemia (XLA) patients who are deficient of Btk and do not show bleeding.

mediated response of platelets to low collagen or plaque[79,80]—unnecessarily inactivate Tec in addition to Btk which completely shuts off GPVI signaling. Inhibition of Tec in addition to Btk might be one explanation for the low grade bleeding observed after ibrutinib and acalabrutinib therapy, but absent in XLA patients (►**Fig. 2**).

However, the low grade bleeding observed in CLL and MCL patients on ibrutinib and acalabrutinib might not be explained solely by a direct drug effect on platelets. Patients with B cell malignancies have an intrinsic increased risk for bleeding based on low platelet counts, coagulation disorders, and other comorbidities.[70] Moreover, thrombocytopenia in the context of inflammation leads to loss of vascular integrity and localized hemorrhage.[91] Furthermore, CLL lymphocytes express the ectonucleotidase CD39 degrading extracellular ADP and thus reducing platelet aggregation.[92] The latter might explain why already before start of ibrutinib primary hemostasis measured with the platelet function analyzer (PFA-100, epinephrine-collagen cartridge) was impaired in 22 out of 84 CLL patients, and why CLL patients showed a decreased platelet aggregation in blood on ADP and collagen as compared with normal controls.[93] When lymphocyte counts fell on ibrutinib treatment, the aggregation response to ADP improved, whereas response to collagen was further reduced, although not substantially different from XLA patients which do not show bleeding.[93] This suggests that inhibition of Btk and collagen-mediated aggregation is not exclusively accountable for ibrutinib-related bleeding. This is also supported by a study in mice demonstrating that ibrutinib treatment which inhibited GPVI-mediated platelet activation did not cause bleeding in models of inflammatory hemorrhage.[94] Furthermore, new ibrutinib analogs administered orally to nonhuman primates for 10 days did not increase template skin bleeding time.[95] A recent in vitro study showed that it is possible to achieve platelet GPVI inhibition without hemostatic impairment by prolonged blood incubation with low concentrations of ibrutinib and the novel Btk inhibitors acalabrutinib, ONO/GS-4059, BGB-3111, and evobrutinib.[80] In this regard it should be emphasized that bleeding has not been reported so far in healthy volunteers taking ibrutinib.

Low Dose Irreversible Btk Inhibitors as Focal Antiplatelet Therapy

Atherosclerotic plaques stimulate static platelet aggregation and platelet thrombus formation under flow via GPVI by their collagen type I and III content.[4–6,21,96] Recently, it was shown that ibrutinib at therapeutic concentrations inhibited in vitro, as well as ex vivo in blood from patients on CLL dose and from volunteers on low dose, platelet aggregation induced by atherosclerotic plaque material.[79] Of note, ibrutinib inhibition was demonstrated with complete human plaque material containing all potential platelet-activating compounds under static conditions as well as under flow. Moreover, microscopic studies with superfusion over human atherosclerotic plaque homogenates and tissue sections at shear rates present in intact (600/s) or mildly stenotic (1,500/s) atherosclerotic coronary arteries demonstrated that plaque collagen initiated platelet arrest via GPVI and Btk inhibitors suppressed continued thrombus growth.[21,79] Acalabrutinib and ONO/GS-4059 added in vitro had similar effects on plaque-induced static platelet aggregation and thrombus formation under flow, albeit with IC_{50} values for inhibition of plaque-induced static platelet aggregation twofold (acalabrutinib) and fourfold (ONO/GS-4059) higher than ibrutinib.[79]

Moreover, platelet inhibition by ibrutinib, acalabrutinib, and ONO/GS-4059 under arterial flow was plaque-specific. Platelet thrombus formation onto collagen fibers under flow was not inhibited by ibrutinib neither in vitro nor ex vivo after oral drug intake. This was explained mainly by preserved Btk function on integrin α2β1-dependent platelet adhesion to native collagen.[79] Collagen requires both collagen receptors to ensure optimal platelet thrombus formation under flow.[24–27]

A further difference between collagen types I and III of connective tissue and atherosclerotic plaques is the limited capacity of plaque to bind GPVI.[6] Already low concentrations of ibrutinib and other Btk inhibitors effectively prevent the low degree of GPVI-dependent static platelet aggregation induced by saturating plaque concentrations,[21,79] low collagen, and CRP concentrations[77–80] (►**Fig. 2**). Also, in a study using PRP stimulated with half maximal collagen concentrations, Btk inhibition was found to be the primary brake on platelet aggregation: The potencies at which Btk inhibitors suppressed collagen-induced platelet aggregation correlated with their potencies in on-target Btk-assays, but not with Tec-assays; this is exemplified by platelet inhibition with RN486, a highly selective reversible Btk inhibitor with only little Tec impairment.[69] Furthermore, platelet inhibition by Btk inhibition or lack of Btk in XLA or Btk-deficient mice platelets can be surmounted by high collagen and CRP concentrations providing more GPVI-binding sites and a higher degree of GPVI signaling which then bypasses Btk by activating Tec[41–43,77,78,80] (►**Fig. 2**).

It is unlikely that suppression of Btk-signaling downstream of GPIb plays a role in the plaque-specific platelet suppression by Btk inhibitors. Although Btk signaling after VWF activation of GPIb was effectively suppressed by ibrutinib, as indicated by the low IC_{50} (0.085 µM) for inhibition of ristocetin-induced static platelet aggregation, platelet thrombus formation onto collagen at the high shear rate of 1,500/s which requires binding of VWF to GPIb was not significantly inhibited.[79] This indicates that Btk signaling after VWF binding to GPIb under blood flow at high shear is not essential for platelet thrombus formation.

A small pilot study showed that lower doses of Btk inhibitors than used for B cell malignancies may suffice for antiplatelet therapy. Ibrutinib (140 mg) each day or on alternate days for 1 week caused full suppression of atherosclerotic plaque-induced platelet aggregation under static and flow conditions and was more effective than aspirin (100 mg/day).[79] A preferential Btk inhibition in platelets without impairing B cell function and immune defense might be achievable in vivo by exploiting the lack of de novo enzyme synthesis in platelets and the covalent binding of irreversible Btk inhibitors to Btk, similar to the situation after low dose aspirin intake.[97] Portal venous blood levels reached during absorption of low doses of Btk inhibitors may suffice to inhibit the low grade GPVI-dependent platelet activation relevant for atherothrombosis. All these mechanistic insights suggest a unique potential of Btk inhibitors as new atherothrombosis focused oral antiplatelet drugs. This could be tested in clinical studies of patients receiving a low dose of approved irreversible Btk inhibitors (ibrutinib, acalabrutinib) prior to elective percutaneous coronary interventions.[79,98] An alternative would be the short-term application of reversible Btk inhibitors such as fenebrutinib and G-744, which may avoid bleeding due to their absent activity on Tec.[64,66]

Concerning potential cardiovascular applications of Btk inhibitors the pricing of these drugs is still high, but similar to other tyrosine kinase inhibitors, prices are expected to drop sharply as soon as generic drugs become available.[99] For ibrutinib, this could be the case after 2026, and various companies already manufacture ibrutinib as active pharmacological ingredient.[100]

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Conflict of Interest
W.S. has a patent WO2018002316 pending. The other authors report no conflict of interest.

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