# Ibrutinib Inhibits Platelet Integrin $\alpha_{IIb}\beta_3$ Outside-In Signaling and Thrombus Stability But Not Adhesion to Collagen

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***Objective***—Ibrutinib is an irreversible Bruton tyrosine kinase inhibitor approved for treatment of Waldenstrom macroglobulinemia, chronic lymphocytic leukemia, and mantle cell lymphoma that increases the risk of bleeding among patients. Platelets from ibrutinib-treated patients exhibit deficiencies in collagen-evoked signaling in suspension; however, the significance of this observation and how it relates to bleeding risk is unclear, as platelets encounter immobile collagen in vivo. We sought to clarify the effects of ibrutinib on platelet function to better understand the mechanism underlying bleeding risk.

***Approach and Results***—By comparing signaling in suspension and during adhesion to immobilized ligands, we found that the collagen signaling deficiency caused by ibrutinib is milder during adhesion to immobilized collagen. We also found that platelets in whole blood treated with ibrutinib adhered to collagen under arterial shear but formed unstable thrombi, suggesting that the collagen signaling deficiency caused by ibrutinib may not be the predominant cause of bleeding in vivo. However, clot retraction and signaling evoked by platelet adhesion to immobilized fibrinogen were also inhibited by ibrutinib, indicating that integrin $\alpha_{IIb}\beta_3$ outside-in signaling is also effected in addition to GPVI signaling. When ibrutinib was combined with the $P2Y_{12}$ inhibitor, cangrelor, thrombus formation under arterial shear was inhibited additively.

***Conclusions***—These findings suggest that (1) ibrutinib causes GPVI and integrin $\alpha_{IIb}\beta_3$ platelet signaling deficiencies that result in formation of unstable thrombi and may contribute toward bleeding observed in vivo and (2) combining ibrutinib with $P2Y_{12}$ antagonists, which also inhibit thrombus stability, may have a detrimental effect on hemostasis. **(*Arterioscler Thromb Vasc Biol*. 2015;35:2326-2335. DOI: 10.1161/ATVBAHA.115.306130.)**

**Key Words:** collagen ■ fibrinogen ■ hemostasis ■ ligands ■ Waldenstrom macroglobulinemia



Ibrutinib is an inhibitor of the Tec family kinase, Bruton tyrosine kinase (Btk) approved for the treatment of chronic lymphocytic leukemia, mantle cell lymphoma, and Waldenstrom macroglobulinemia. Patients receiving ibrutinib exhibit deficiencies in hemostasis, resulting in incidents of bruising and petechiae in ≤48% of patients and grade 3 or greater bleeding in 5% of patients.[1] Platelets from patients receiving ibrutinib exhibit deficient responses to collagen and the glycoprotein VI (GPVI)-specific agonist, collagen-related peptide (CRP)-XL.[2–4] However, responses to other platelet agonists such as ADP and U46619 are unaffected or only mildly inhibited.[2] Btk is a critical component of the signaling pathway of the GPVI collagen receptor and ibrutinib seems to inhibit collagen-evoked signaling via inhibition of Btk.[4] However, the effects of ibrutinib have predominantly been investigated using platelet suspension assays, whereas the impact of ibrutinib on adhesion and signaling on immobilized ligands has not been explored but may be more representative of the platelet environment in vivo. The distinction between suspension and adhesion may be critical to understanding the underlying mechanism of hemostatic dysfunction caused by ibrutinib.

The involvement of Btk in GPVI-evoked platelet signaling was first identified when patients having X-linked agammaglobulinemia, caused by a deficiency in Btk, were found to have platelets with impaired responses to collagen.[5] However, the contribution of Btk to the GPVI signaling pathway is partially redundant to the closely related kinase, Tec as only simultaneous knockout of both Tec and Btk renders mouse platelets insensitive to CRP-XL.[6] X-linked agammaglobulinemia patients do not exhibit a platelet-dependent bleeding phenotype, which contrasts with the enhanced bleeding risk among patients receiving ibrutinib and suggests that Tec may also be inhibited by clinically relevant concentrations of Ibrutinib. After activation of the platelet GPVI receptor, Btk and Tec are activated downstream of Syk, Fyn, and Lyn and

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| Nonstandard Abbreviations and Acronyms | |
|---|---|
| **Btk** | Bruton tyrosine kinase |
| **CRP** | collagen-related peptide |
| **vWF** | Von Willebrand factor |

regulate phospholipase Cγ2 (PLCγ2) activation, and thereby $Ca^{2+}$ release and protein kinase C (PKC) activation, which are critical events in platelet activation.[6,7] In addition to GPVI, platelets also express $\alpha_2\beta_1$ and the glycoprotein Ib (GPIb) receptor complex that can mediate adhesion to collagen and generate intracellular signaling that supports hemostatic platelet function.[8] Btk has an established role in the signaling pathway evoked by GPIb[9] and consequent inhibition of adhesion to Von Willebrand factor (vWF) in the presence of ibrutinib has been reported.[4] The role of Btk in $\alpha_2\beta_1$-mediated signaling is less clear, as is the ability for $\alpha_2\beta_1$ to function in the absence of signaling evoked by GPVI,[8] making the effects of ibrutinib on collagen-evoked adhesion and signaling under shear difficult to predict.

The role of Btk may not be limited to collagen and vWF-evoked pathways, as integrin $\alpha_{IIb}\beta_3$-evoked outside-in signaling is thought to share many features of the GPVI signaling pathway, although conflicting reports about the involvement of Btk in outside-in signaling have been published. A study reporting that phosphorylation of Btk occurs after direct activation of integrin $\alpha_{IIb}\beta_3$ with $MnCl_2$ in mouse and human platelets suggests the involvement of Btk in outside-in signaling.[10] However, it has also been reported that mouse platelets with simultaneous deficiency of both Btk and Tec adhere and spread normally on fibrinogen.[6] Overall, the role of Btk in integrin $\alpha_{IIb}\beta_3$ outside-in signaling is unclear. The effect of Ibrutinib on integrin outside-in signaling has not previously been explored but offers an opportunity to investigate the role of Tec family kinases in such signaling in human platelets. It is possible that inhibition of integrin $\alpha_{IIb}\beta_3$ outside-in signaling may play a role in bleeding risk as mice with genetically modified $\beta_3$, harboring tyrosine substitutions in the cytoplasmic tale that ablate outside-in signaling, have a mild bleeding phenotype characterized by poor clot stability that results in rebleeding.[11]

To improve understanding of the effects of ibrutinib on platelet function, we performed a comparison of the effects of ibrutinib on platelet signaling in suspension and during adhesion to immobilized ligands. Using this strategy, we identified key differences that suggested that the previously reported loss of platelet collagen sensitivity may not cause a deficiency in adhesion to immobile collagen. Further investigation revealed that initial adhesion to collagen under arterial shear was not inhibited by ibrutinib; however, ibrutinib caused instability and disaggregation of thrombi. This lead us to investigate positive feedback signaling that supports thrombus stability and the subsequent finding that ibrutinib causes inhibition of outside-in signaling evoked by integrin $\alpha_{IIb}\beta_3$. Because of the important role of integrin $\alpha_{IIb}\beta_3$ outside-in signaling in clot stabilization, inhibition of this pathway in addition to GPVI signaling may be an important factor in the bleeding caused by ibrutinib.



## Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

## Results

### Ibrutinib Inhibits CRP and Collagen-Evoked $[Ca^{2+}]_i$ Elevation and Aggregation in Suspension

Platelet function has been measured in blood taken from patients receiving 560 mg of ibrutinib daily and responses to collagen and CRP were found to be inhibited; however, the effect of ibrutinib on platelet signaling and adhesion to immobile collagen has not been measured.[3,4] To provide a comparison of the effects of ibrutinib on platelet signaling in suspension and during adhesion we first investigated the effects of ibrutinib on elevation of $[Ca^{2+}]_i$ and aggregation in evoked by collagen, CRP-XL, ADP, and U46619 in suspension. At concentrations of ≤1 µmol/L, ibrutinib inhibited $Ca^{2+}$ elevation evoked by collagen and the GPVI agonist, CRP-XL but not ADP or U46619 (Figure 1A). Aggregation evoked by collagen or CRP-XL was completely inhibited by 1-µmol/L ibrutinib (Figure 1B). However, aggregation evoked by ADP was only mildly inhibited (12.7±5.5% inhibition) and the kinetics of U46619-evoked aggregation was often slowed and appeared biphasic although the trend did not reach statistical significance at 5 minutes (Figure 1B). The lack of

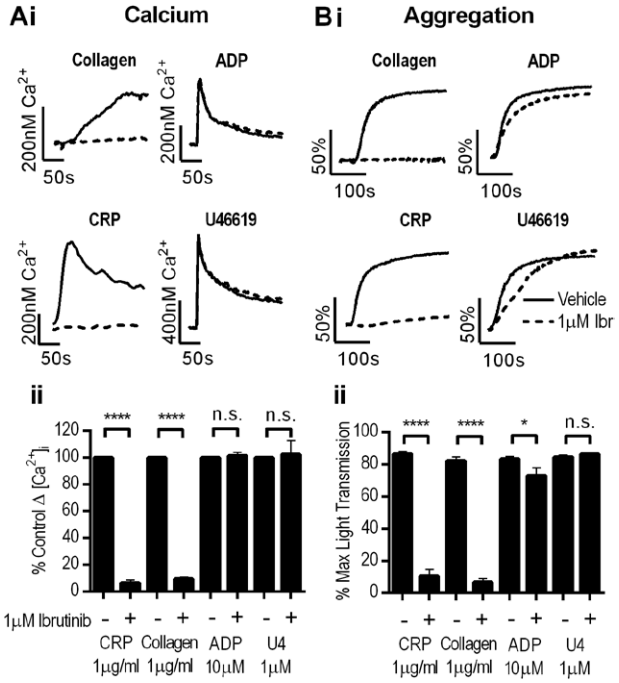


**Figure 1.** Ibrutinib inhibits $Ca^{2+}$ elevation and aggregation evoked by collagen-related peptide (CRP)-XL and collagen. For $[Ca^{2+}]_i$, measurements and aggregometry washed platelets (loaded with fura-2 for $[Ca^{2+}]_i$ measurements) were pretreated with 1-µmol/L ibrutinib or vehicle for 5 minutes and stimulated with 1-µg/mL type I collagen, 1-µg/mL CRP-XL, 10-µmol/L ADP, or 1-µmol/L U46619. **Ai**, Representative $[Ca^{2+}]_i$ traces and (**Aii**) mean peak $[Ca^{2+}]_i$ increases normalized to % of vehicle response±SEM (n=4). **Bi**, Representative aggregation traces and (**Bii**) mean light transmission 5 minutes after addition of agonist±SEM (n=4). *$P$<0.05, ****$P$<0.0001 using 1-way ANOVA with a Bonferroni post test.

effect of ibrutinib on ADP and U46619-evoked $[Ca^{2+}]_i$ elevation suggests that the observed trend toward mild inhibition of aggregation, which has also been noted in other studies,[2,4] may indicate inhibition of positive feedback signaling, such as integrin $\alpha_{IIb}\beta_3$ outside-in signaling.

## Inhibition of GPVI-Evoked Signaling Caused by Ibrutinib Is Concentration-Dependent Within the Range of Clinically Relevant Concentrations

We used $[Ca^{2+}]_i$ quantification and plate-based aggregometry to define the concentration dependency of platelet inhibition caused by ibrutinib (Figure 2). Ibrutinib inhibited $[Ca^{2+}]_i$ elevation evoked by 1 µg/mL CRP-XL with an $IC_{50}$ of 51 nmol/L±10.7, whereas half-maximal inhibition of aggregation was only achieved at 186 nmol/L±81.2 (Figure 2A and 2B). We found that the concentration-dependent component of the inhibition curves covered the range of peak serum concentrations measured clinically in patients receiving 560 mg ibrutinib daily (mean, 300 nmol/L).[12] This suggests that variability in plasma concentration may cause large differences in platelet inhibition among patients.

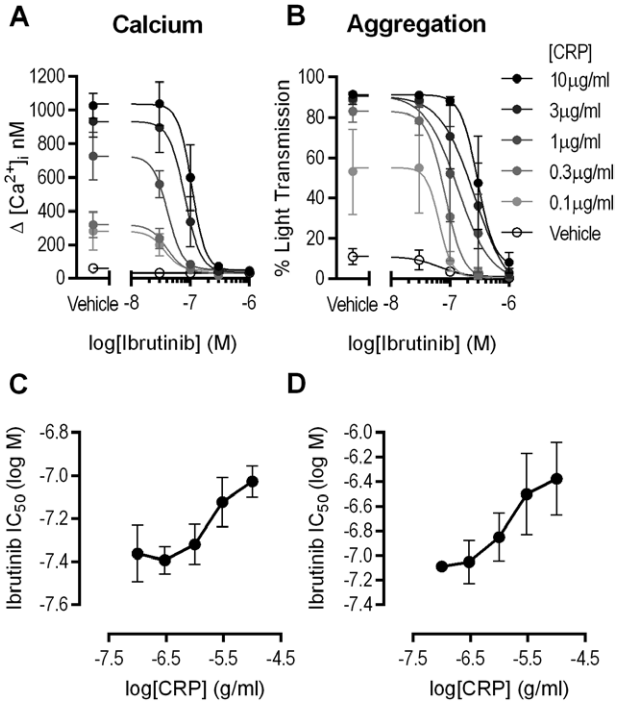


**Figure 2.** Ibrutinib inhibits GPVI-evoked signaling with potency that is partially dependent on stimulus strength. $[Ca^{2+}]_i$ measurements and aggregations were performed using washed platelets (loaded with fura-2 for $[Ca^{2+}]_i$ measurements) pretreated for 5 minutes with ibrutinib at the indicated concentrations and stimulated with a range of collagen-related peptide (CRP)-XL concentrations between 10 and 10 ng/mL CRP-XL or vehicle only. Average concentration responses (**A**) expressed as the peak increase in $[Ca^{2+}]_i$ or (**B**) % light transmission after 5 minutes of platelet aggregation are mean±SEM (n=4). The relationship between the stimulating (CRP-XL) and apparent $IC_{50}$ of ibrutinib in $Ca^{2+}$ (**C**) and aggregation (**D**) assays are derived from the curves presented in (**A**) and (**B**), respectively and are mean±SEM (n=4). Correlation between the apparent $IC_{50}$ of ibrutinib and CRP-XL concentration was tested using Pearson correlation test and found to be significant ($P<0.05$) for both $[Ca^{2+}]_i$ increase ($r=0.93$) and aggregation ($r=0.97$).

Ibrutinib is an irreversible inhibitor of Btk,[13] and therefore, expected to exhibit insurmountable inhibition with limited or no dependence on stimulus strength. However, a positive correlation between log[CRP-XL] and $\log IC_{50}$ was identified, and may be indicative of ibrutinib interacting with >1 target with different affinities (Figure 2C and 2D). It is tempting to speculate that these targets may represent the 2 Tec family kinases expressed by platelets, Btk and Tec, both of which are inhibited by ibrutinib.[13] However, we were unable to blot to determine the relative phosphorylation of these 2 Tec family kinases to investigate this further.

## Ibrutinib Inhibits Signaling Evoked by GPVI Downstream of PLCγ2

Ibrutinib is an inhibitor of other kinases in addition to Btk, including Src family kinases and Tec,[13] which could contribute toward the observed platelet inhibition. Although ibrutinib has been screened for activity against a panel of kinases, the screen was performed in a cell-free in vitro assay and the estimated $IC_{50}$ values do not seem to correlate closely with the potency of ibrutinib observed in vivo or ex vivo. We found that ibrutinib inhibits Src phosphorylation at Y418 with an $IC_{50}$ of 2.0 µmol/L±1.05 (Figure 3A and 3B), which was 20-fold higher than that required for half-maximal inhibition of $[Ca^{2+}]_i$ elevation ($IC_{50}$=51 nmol/L±10.7) evoked by the same concentration of CRP-XL (Figure 2A). Phosphorylation of Syk was not inhibited by 1-µmol/L ibrutinib, (174%±54 relative to control; Figure 3D) a concentration that ablated both $[Ca^{2+}]_i$ elevation and aggregation evoked by 1 µg/mL CRP-XL. Taken together, these observations suggest that the potent inhibition of GPVI signaling caused by ibrutinib is not attributable to inhibition of Src family kinases. In contrast to measurements of Src and Syk phosphorylation, 1-µmol/L ibrutinib was found to ablate PLCγ2 tyrosine phosphorylation as well as total ser/thr phosphorylation of all substrates of PKC (Figure 3E and 3F). Measurement of p-selectin exposure and fibrinogen binding that are representative of the downstream signaling events of granule secretion and integrin $\alpha_{IIb}\beta_3$ activation were also inhibited by 1-µmol/L ibrutinib (Figure 3G). Btk/Tec forms part of the PLCγ2 activation complex down stream of GPVI and, therefore, inhibition of PLCγ2, PKC, and $[Ca^{2+}]_i$ elevation are consistent with a Btk/Tec-dependent mode of action. To investigate the effects of ibrutinib in subsequent experiments, a concentration of 1 µmol/L was used to ensure that complete inhibition of platelet function mediated by GPVI was achieved.

## $Ca^{2+}$ Signaling Evoked by Adhesion to Immobilized Collagen Is Only Partially Inhibited by Ibrutinib

To understand the significance of the collagen and CRP-XL-specific inhibition caused by ibrutinib in suspension, we studied the effects of ibrutinib on platelet function during adhesion to immobilized ligands. Platelet adhesion and activation on immobilized collagen is thought to be orchestrated by GPVI, which triggers activation, integrin $\alpha_2\beta_1$ that supports adhesion, and GPIb, which via the interaction with vWF ensures initial adhesion to collagen under arterial shear.[8] Although GPVI has the most established role in mediating

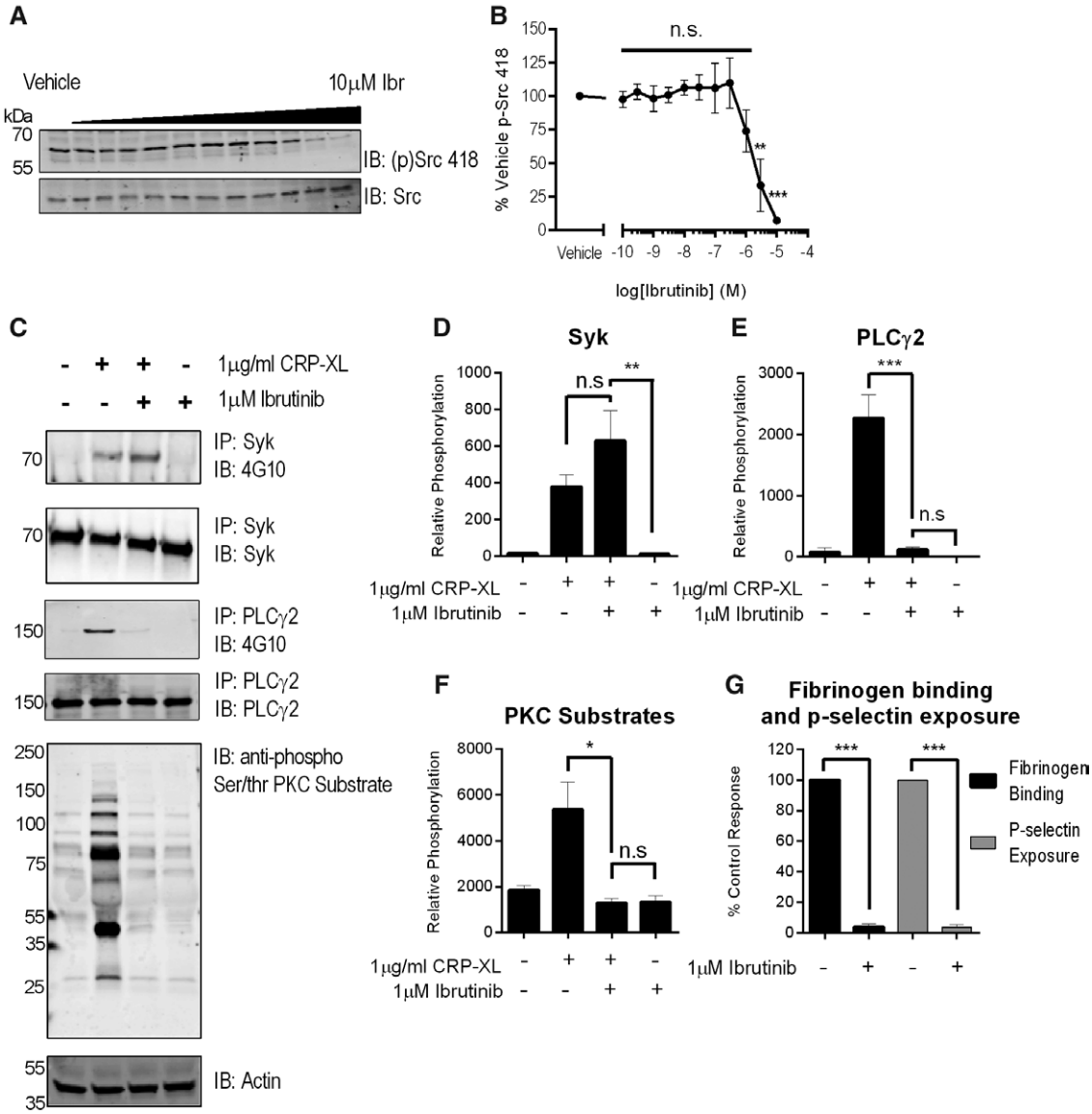


**Figure 3.** Inhibition of GPVI-evoked signaling occurs at the level of phospholipase Cγ2 (PLCγ2) activation. **A**, Washed platelets were pretreated for 5 minutes with a range of concentrations of ibrutinib or vehicle before stimulation with 1 μg/mL collagen-related peptide (CRP)-XL for 1 minute before immunoblotting for Src Y418. **B**, The mean relative phosphorylation levels of Src Y418±SEM (normalized to total Src; n=4). **C**, Washed platelets were pretreated for 5 minutes with 1-μmol/L ibrutinib or vehicle before stimulation with 1-μg/mL CRP-XL for 1 minute, samples were blotted for Ser/Thr phosphorylation of protein kinase C (PKC) substrates or used for immunoprecipitation of Syk or PLCγ2 and blotted for tyrosine phosphorylation (using 4G10 antibody). Bar charts are mean normalized phosphorylation values±SEM for (**D**) tyrosine phosphorylation of Syk normalized to total Syk (**E**) tyrosine phosphorylation PLCγ2 relative to total PLCγ2 and (**F**) total serine/threonine phosphorylation of substrates of PKC relative to actin (n=3). **G**, Flow cytometry measurements of fibrinogen binding and p-selectin exposure after treatment of platelets for minutes with 1-μg/mL CRP-XL in the presence or absence of 1-μmol/L ibrutinib (n=4). *$P<0.05$, **$P<0.01$, ***$P<0.001$ using 1-way ANOVA with a Bonferroni post test.



collagen-evoked intracellular signaling, integrin $\alpha_2\beta_1$[14] may also be capable of initiating signaling. The effects of ibrutinib on adhesion to immobile collagen were, therefore, difficult to predict, but may be significant in understanding the mechanism, by which it causes bleeding. We found that adhesion of platelets to immobilized CRP-XL was inhibited by 70%±13.9 in the presence of 1-μmol/L ibrutinib (Figure 4Ai and 4Aii). Spreading under these conditions was also strongly inhibited where the proportion of platelets exhibiting lamellipodia was reduced from 69%±5.3 to 11%±4.8 in the presence of 1-μmol/L ibrutinib (Figure 4Aiii). The efficacy of ibrutinib as an inhibitor of platelet function mediated by GPVI, therefore, seemed to be maintained in spreading experiments. Adhesion to CRP-XL seemed to be dependent on activation, which was blocked by ibrutinib. CRP-XL is not an adhesive ligand and adhesion to CRP-XL may be, therefore, reliant on deposition of adhesive proteins present in α-granules, such as vWF and fibrinogen that are secreted on activation.[15] In contrast, adhesion to type I collagen was not significantly inhibited by the presence of 1-μmol/L ibrutinib relative to control (100%±2.5; Figure 4Aii). Spreading and formation of lamellipodia on collagen was significantly inhibited in the presence of ibrutinib (48%±8.9 expressing lamellipodia in the presence of vehicle compared with

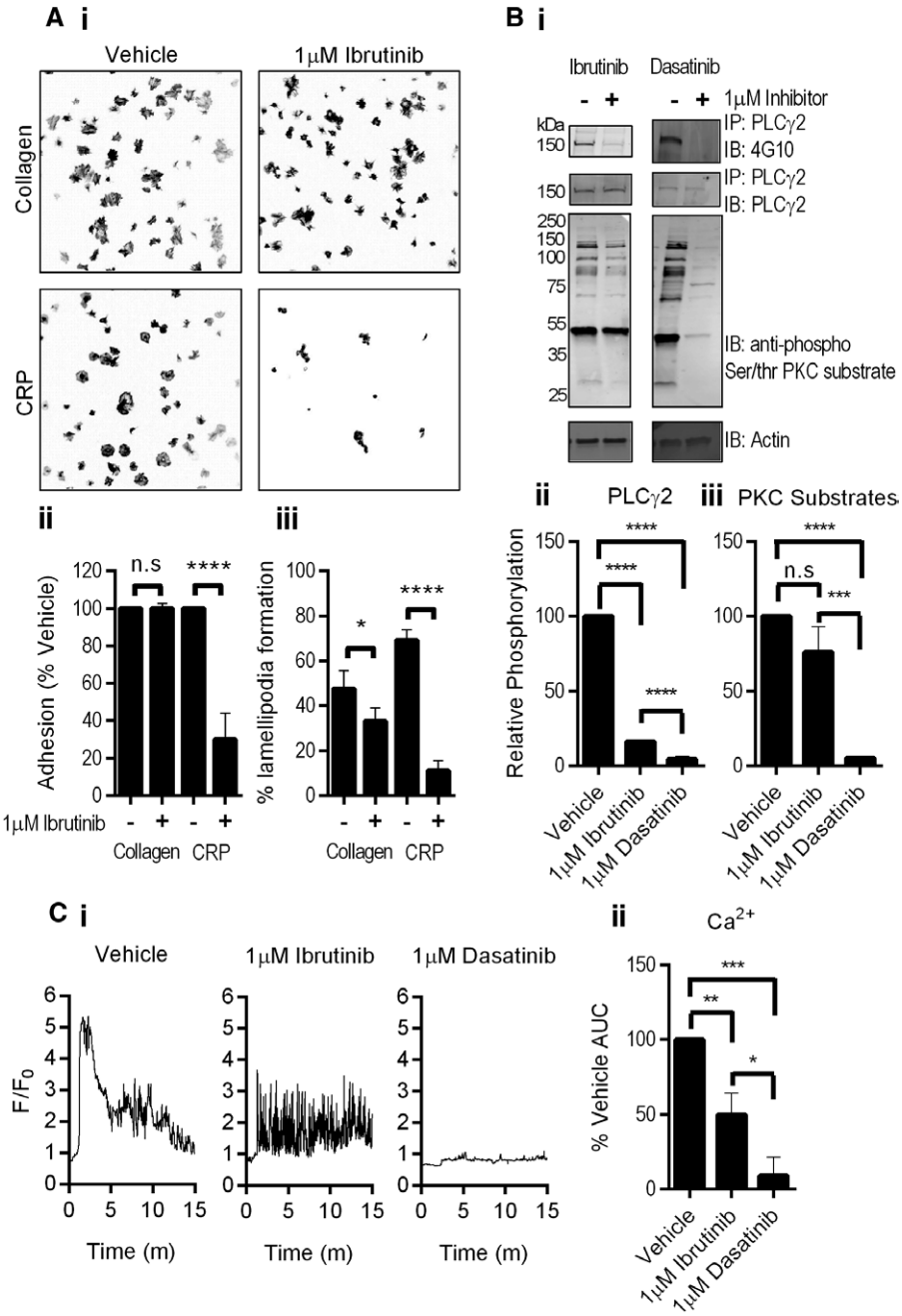


**Figure 4.** Signaling and adhesion evoked by immobilized collagen is only partially inhibited by ibrutinib and ablated by dasatinib. **Ai**, Washed platelets were pretreated for 5 minutes with 1-μmol/L ibrutinib or vehicle and allowed to adhere to type I collagen or collagen-related peptide (CRP)-coated cover glass for 45 minutes. **Aii**, The relative percentage of adhered platelets relative to vehicle treated (**Aiii**) and the percentage of platelets that had formed lamellapodia were quantified as mean %±SEM (n=5). **Bi**, Washed platelets were pretreated for 5 minutes with 1-μmol/L ibrutinib or vehicle and allowed to adhere to type I collagen-coated plates for 30 minutes before platelets were lysed and probed for phospholipase Cγ2 (PLCγ2) phosphorylation and phosphorylation of protein kinase C (PKC) substrates. Bar charts are mean normalized phosphorylation values±SEM for (**Bii**) tyrosine phosphorylation of PLCγ2 relative to total PLCγ2 and (**Biii**) serine/threonine phosphorylation of substrates of PKC relative to actin. (n=5). **C**, Platelet rich plasma was loaded with fluo-4 AM and imaged at 1 Hz during adhesion and spreading on type I collagen-coated cover glass under static conditions for 15 minutes in the presence of 1-μmol/L ibrutinib, 1-μmol/L dasatinib or vehicle only. **Ci**, The traces are fluorescence intensity plots representative of between 10 and 60 adhered platelets measured in each of 3 different donors, (**Cii**) bar charts are mean area under the curve (AUC) measurements±SEM (n=3). *$P$<0.05, **$P$<0.01, ***$P$<0.001, ****$P$<0.0001 using 1-way ANOVA with a Bonferroni post test.

33%±6.6 with 1-μmol/L ibrutinib) but to a lesser extent than on CRP-XL (Figure 4Aiii). The differential effects of ibrutinib on adhesion and spreading on immobilized collagen and CRP-XL suggest that Btk-independent signaling may be stimulated by adhesion to collagen.

To explore the apparent differences in the effects of ibrutinib during stimulation with immobilized or solubilized collagen, we investigated signaling evoked by adhesion to type I collagen. Ibrutinib reduced PLCγ2 phosphorylation by 84% relative to control, whereas it was ablated by dasatinib

(Figure 4B), a kinase inhibitor that targets Src family kinases and causing platelet inhibition and bleeding side effects in patients.[16] Total ser/thr phosphorylation of all PKC substrates, which lie downstream of PLCγ2 were also partially inhibited by ibrutinib (76%±9.8 relative to control) and strongly inhibited by dasatinib (5%±0.6 relative to control). We used single-cell imaging to measure $[Ca^{2+}]_i$ in individual platelets as they came into contact with type I collagen-coated cover glass in the presence or absence of ibrutinib or dasatinib. Unlike the complete ablation observed when platelets were stimulated with collagen in suspension (Figure 1A), $[Ca^{2+}]_i$ elevation was only partially inhibited by ibrutinib during adhesion to immobilized collagen (50%±8.1 of vehicle response; Figure 4Cii). In the presence of ibrutinib, the sustained $[Ca^{2+}]_i$ elevation evoked by collagen in the majority of platelets was replaced by ragged $[Ca^{2+}]_i$ spiking (Movie I in the online-only Data Supplement) more commonly associated with weaker agonists, such as ADP (Figure 4Ci).[17] In contrast, the Src family kinase inhibitor dasatinib caused complete inhibition of $[Ca^{2+}]_i$ elevation evoked by immobilized collagen (9%±8.7 of vehicle response; Figure 4Cii). Taken together, this suggests that signaling stimulated by adhesion to collagen is not potently inhibited by ibrutinib. This contrasts directly with the total inhibition caused by ibrutinib after stimulation with collagen in suspension observed in this study (Figures 1 and 2) and in other studies.[3,4] We suggest that signaling initiated by adhesion to collagen differs fundamentally to that in suspension and the role of Btk and the effects of ibrutinib also differ under these conditions.

## Platelets Adhere to Collagen But Do Not Form Stable Thrombi Under Arterial Shear After Treatment With Ibrutinib

Because we had identified key differences between the effects of ibrutinib on platelet function in suspension and during adhesion to immobilized collagen, we investigated further using an in vitro thrombus formation assay to assess whether the remaining collagen-mediated signaling of ibrutinib-treated whole blood could support thrombus formation on type I collagen under arterial shear conditions. After treatment with 1-μmol/L ibrutinib levels of thrombus formation after 10 minutes were significantly inhibited relative to control

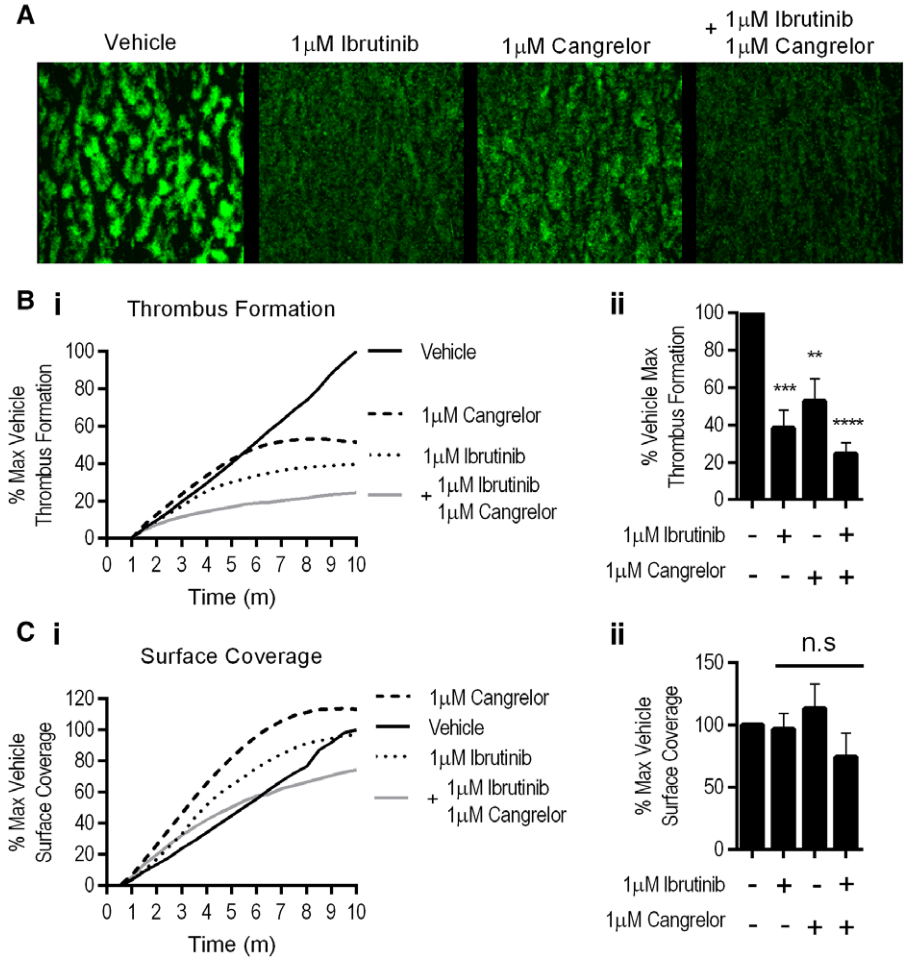


**Figure 5.** Ibrutinib inhibits stable thrombus formation but not adhesion to immobilized collagen. **A**, Thrombus formation was measured using whole blood in preincubated for 5 minutes with vehicle, 1-μmol/L ibrutinib or 1-μmol/L cangrlor or both before perfusion through a type I collagen-coated channel for 8 minutes. Images are representative fluorescence intensity plots after 7 minutes and traces are (**Bi**) mean fluorescence intensity of thrombi with (**Bii**) bar charts of mean fluorescence intensity±SEM after 10 minutes and (**Ci**) traces of mean surface coverage with (**Cii**) bar charts of mean surface coverage±SEM after 10 minutes (n=4). $^{**}P<0.01$, $^{***}P>0.001$, $^{****}P<0.0001$ using 1-way ANOVA with a Bonferroni post test.

(39%±9.2 of vehicle-treated controls; Figure 5Bi and 5Bii). However, surface coverage was not significantly reduced by ibrutinib (96%±12.2 of vehicle-treated controls; Figure 5C). This suggests that although collagen-evoked signaling via GPVI is effectively ablated by this concentration of ibrutinib in other assays, the remaining signaling evoked by collagen is sufficient to support adhesion. Despite this, thrombus stability appeared to be affected by ibrutinib resulting in disaggregation of growing thrombi after initial stable adhesion of platelets to collagen (Movie II in the online-only Data Supplement). To test whether ibrutinib might be inhibiting secretion of secondary mediators such as ADP, the $P2Y_{12}$ inhibitor cangrelor was tested alone and in combination with the same thrombus formation assay. When platelets were treated with 1-µmol/L cangrelor, thrombus formation was inhibited (51%±12.9 of vehicle-treated controls; Figure 5B), whereas surface coverage was not (113%±19.6 of vehicle-treated controls; Figure 5C). Similar to the effect of ibrutinib, cangrelor did not affect initial adhesion to collagen but did inhibit stable thrombus formation. However, although cangrelor treatment resulted in embolization of clumps of platelets from growing thrombi, ibrutinib caused disaggregation of individual platelets (Movie II in the online-only Data Supplement). When ibrutinib and cangrelor treatments were combined, thrombus formation was more strongly inhibited (24%±6.0 of vehicle-treated controls; Figure 5B) than by cangrelor treatment alone (although the difference was only significant at intermediate time points, 1-way ANOVA with Bonferroni post test; $P<0.05$ after 7 minutes), whereas surface coverage was not significantly reduced (79% 18.9 of vehicle-treated controls; Figure 5C). These findings suggested that stimulation of $P2Y_{12}$ by secreted ADP was still occurring in the presence of ibrutinib and that blockade using cangrelor resulted in further inhibition of thrombus stability. The comparison with cangrelor also highlighted that the effects of the 2 inhibitors were similar and both caused decreased thrombus stability rather than a defect in initial adhesion to collagen.

### Ibrutinib Inhibits Outside-In Signaling Mediated by Integrin $\alpha_{IIb}\beta_3$

It has already been established that ibrutinib does not inhibit signaling evoked by secreted secondary mediators that support thrombus growth and stability, therefore, to explore the unexplained effects of ibrutinib on thrombus stability, we investigated integrin $\alpha_{IIb}\beta_3$ outside-in signaling. Binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ evokes outside-in signaling that supports adhesion and spreading on immobilized fibrinogen. The physiological role of outside-in signaling is to provide positive feedback for platelet-activating stimuli and support clot retraction. We investigated outside-in signaling evoked during adhesion and spreading on immobilized fibrinogen to explore whether the inhibitory mechanism of ibrutinib lies in the disruption of the outside-in signaling pathway, which has been reported to involve Btk.[10] Phosphorylation of β3 Y773 (83%±14.26), one of the critical tyrosine residues in the human β3 cytoplasmic domain for initiation of outside-in signaling,[11] and Src Y418 (77%±20.3) were not significantly altered relative to vehicle-treated control after adhesion to fibrinogen in the presence of 1-µmol/L ibrutinib (Figure 6A). This suggested that early stages in the outside-in signaling pathway are unaffected by ibrutinib. We measured $Ca^{2+}$ signaling evoked by integrin $\alpha_{IIb}\beta_3$ using live imaging of intracellular fluo-4 in individual platelets adhering to and spreading on fibrinogen (Movie III in the online-only Data Supplement). The sustained $[Ca^{2+}]_i$ oscillations observed in the majority of adhered platelets was strongly inhibited by 1-µmol/L ibrutinib resulting in significantly lower area under the curve measurements relative to control (Figure 6B). We also measured adhesion and spreading of platelets on fibrinogen-coated cover glass and found that adhesion was inhibited by 48%±5.4 in the presence of 1-µmol/L ibrutinib and the proportion of platelets forming lamellipodia was reduced (36%±5.6 compared with 79%±8.9 in the presence of vehicle; Figure 6C). Finally, ibrutinib also inhibited the process of thrombin-stimulated clot retraction, which is stimulated by integrin $\alpha_{IIb}\beta_3$ outside-in signaling, by 110%±32.4 (relative to vehicle-treated mean clot weight measured after 2 hours; Figure 6D). Taken together, these experiments provide evidence that ibrutinib affects outside-in signaling evoked by integrin $\alpha_{IIb}\beta_3$ resulting in inhibition of $[Ca^{2+}]_i$ elevation and the processes of platelet spreading and clot retraction.

## Discussion

Pharmacological inhibition of the kinase, Btk, has proven to be a successful therapeutic strategy for the treatment of B-cell based cancers and the irreversible Btk inhibitor ibrutinib has been approved by the Food and Drug Administration to treat mantle cell lymphoma, chronic lymphocytic leukemia, and Waldenstrom macroglobulinemia. However, ibrutinib treatment is associated with increased risk of bleeding among patients. Until this study, the effects of ibrutinib on platelet function have predominantly been investigated using aggregation assays and other techniques performed in suspension. Aggregation assays constitute a critical clinical and diagnostic tool that aid investigation of platelet-based bleeding disorders. However, aggregometry has limited use in that it does not replicate platelet thrombus formation on immobile surfaces in vivo. Adhesion-based platelet function assays such as the PFA-100 are frequently used, but have limited sensitivity[18] and consequently more sophisticated in vitro thrombus formation assays have been developed to enhance diagnosis of platelet disorders.[19] In this study, the effects of ibrutinib in assays performed in suspension and during adhesion to immobilized collagen were compared to provide a better understanding of how ibrutinib causes platelet signaling deficiencies and how these may affect hemostasis.

The bleeding associated with ibrutinib has been correlated with its inhibitory effects on collagen-mediated platelet aggregation.[2–4] We found that although ibrutinib caused potent inhibition of aggregation and $[Ca^{2+}]_i$ elevation in suspension, that even at a concentration (1 µmol/L) that exceeds plasma concentrations measured clinically, ibrutinib did not completely inhibit adhesion to or signaling evoked by immobilized collagen. Although PLCγ2 activation in the presence of ibrutinib was markedly reduced, $Ca^{2+}$ signaling and PKC substrate phosphorylation was less strongly inhibited. This may be indicative of signal amplification mediated by secretion of secondary mediators that act via PLCβ to

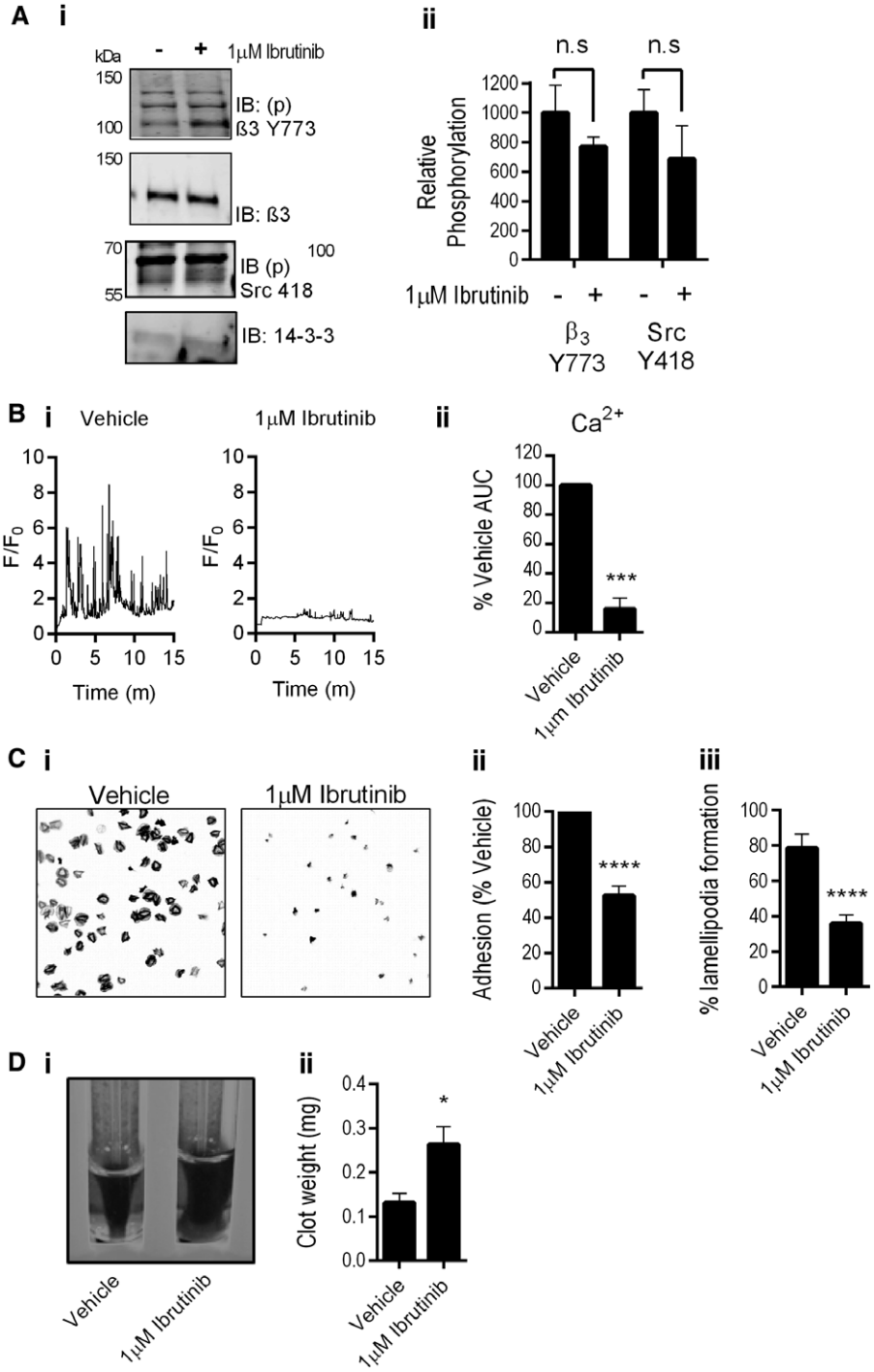


**Figure 6.** Signaling and adhesion mediated by integrin $\alpha_{IIb}\beta_3$ and clot retraction are inhibited by ibrutinib. **Ai**, Washed platelets were pretreated for 5 minutes with 1-μmol/L ibrutinib or vehicle and allowed to adhere to fibrinogen-coated plates for 30 minutes before platelets were lysed and probed for $\beta_3$ Y773 and Src Y418 phosphorylation. **Aii**, Bar charts are mean normalized phosphorylation values for β3 Y773 (relative to total β3) and Src Y418 (relative to 14-3-3)±SEM (n=3). **B**, Platelet rich plasma (PRP) was loaded with fluo-4 AM and imaged at 1 Hz during adhesion and spreading on fibrinogen-coated cover glass under static conditions for 25 minutes in the presence of 1-μmol/L ibrutinib or vehicle only. **Bi**, The traces are fluorescence intensity plots representative of 10 to 60 adhered platelets measured in each of 3 donors. **Bii**, Bar charts are mean area under the curve (AUC) measurements±SEM. **Ci**, Washed platelets were pretreated for 5 minutes with 1-μmol/L ibrutinib or vehicle as indicated in the Figure and allowed to adhere to fibrinogen-coated cover glass for 45 minutes. **Cii**, The relative percentage of adhered platelets relative to vehicle treated and (**Ciii**) the percentage of platelets that had formed lamellipodia were quantified as mean %±SEM (n=5). **Di**, A representative image of clot retraction assessed using PRP treated with vehicle or 1-μmol/L ibrutinib 2 hours after addition of 1-U/mL thrombin, (**Dii**) bars represent mean clot weight±SEM (n=6). *$P$<0.05, ***$P$<0.001, ****$P$<0.0001 using 1-way ANOVA with a Bonferroni post test.

activate PKC and release $Ca^{2+}$. Furthermore, initial adhesion to collagen under arterial shear stress was not significantly inhibited by ibrutinib, whereas stable thrombus formation was strongly inhibited. This unexpected finding suggested that the mechanism underlying platelet dysfunction caused by ibrutinib might involve multiple signaling pathways. We and others[2–4] have demonstrated that signaling evoked by secondary mediators is largely unaffected by ibrutinib, and we found that signaling via the ADP receptor, $P2Y_{12}$ still occurs in the presence of 1-µmol/L ibrutinib. We therefore looked to other sources of positive feedback signaling that contribute toward stable thrombus formation and found that outside-in signaling evoked by integrin $\alpha_{IIb}\beta_3$ was inhibited by ibrutinib. Ibrutinib inhibited integrin $\alpha_{IIb}\beta_3$ outside-in signaling via a similar mechanism to GPVI signaling where it prevented PLCγ2 activation and $Ca^{2+}$ elevation. This is consistent with studies that have shown that Btk is activated downstream of $\alpha_{IIb}\beta_3$ after direct activation with $MnCl_2$.[10] Initial events in the outside-in signaling cascade such as phosphorylation of β3 (Y773) and Src were not significantly inhibited by ibrutinib suggesting they lie upstream of PLCγ2 activation when platelets are activated by contact with immobile fibrinogen. In a study that used a mouse model lacking integrin $\alpha_{IIb}\beta_3$ outside-in signaling because of substitution of critical tyrosine residues in the β3 cytoplasmic tail, the outside-in signaling deficiency was found to cause rebleeding in tail bleed experiments.[11] It is possible that inhibition of integrin $\alpha_{IIb}\beta_3$ outside-in signaling mediated by ibrutinib has similar effects in patients and, therefore, may have a role in causing bleeding. Interestingly, the effects of ibrutinib on thrombus formation under arterial shear were similar to the inhibition caused by cangrelor, which caused embolization of thrombi. However, ibrutinib seemed to cause disaggregation of individual platelets from growing thrombi rather than embolization and inhibited thrombus stability additively with cangrelor, suggesting that the effects were mediated via distinct mechanisms.

We suggest that ibrutinib causes a combination of platelet function defects via inhibition of Btk and Tec, and we support this hypothesis, which is found by comparing the bleeding phenotype of patients receiving ibrutinib with that of patients with disorders affecting Btk or GPVI function. Patients having the Btk deficiency disorder, X-linked agammaglobulinemia are not at increased risk of bleeding because platelet Tec expression, which is unaffected, contributes signaling that overlaps that of Btk.[6,20] Ibrutinib inhibits Tec in addition to Btk but with lower potency[13] and this may explain the difference in bleeding phenotype between X-linked agammaglobulinemia patients and patients treated with ibrutinib. We found evidence that ibrutinib inhibits at least 2 targets with distinct potencies downstream of GPVI that may represent the 2 Tec family kinases expressed by platelets. Despite strong evidence that ibrutinib causes a GPVI-specific platelet signaling defect, bleeding observed in patients does not correlate wholly with the reported bleeding phenotype of individuals lacking functional GPVI. Although patients with GPVI signaling deficiencies frequently present with petechiae,[21] which is also observed in patients receiving ibrutinib, lack of GPVI function is normally associated with only a mild bleeding defect.[22–24] Interestingly, platelets with dysfunctional GPVI often display markedly impaired adhesion to immobilized collagen,[21] whereas this was not the case after ibrutinib treatment in this study. This suggests that components of the GPVI signaling pathway that are not dependent on Btk may be able to contribute toward adhesion to immobilized collagen via synergy with signaling evoked by secondary mediators or other adhesive receptors.

The mechanism underlying adhesion, secretion of secondary mediators, and thrombus formation on immobilized collagen in the presence of ibrutinib is unclear given the critical role of GPVI-evoked signaling in this process. Multiple platelet membrane proteins underlie adhesion and signaling in response to collagen. The major role of GPVI lies in initiating intracellular signaling, whereas GPIb and integrin $\alpha_{IIb}\beta_3$ mediate adhesion to collagen via vWF. The integrin $\alpha_2\beta_1$ mediates adhesion directly to collagen and is thought to be capable of initiating intracellular signaling, although evidence also exists that the affinity for collagen must first be enhanced via intracellular signaling mediated by another receptor.[25] The contribution of $\alpha_2\beta_1$ to collagen-evoked platelet activation is only apparent during adhesion to immobilized collagen and not in suspension either in vitro[14] or in vivo after injection of mice with soluble collagen.[26] This correlates with the observed effect of ibrutinib, which inhibits collagen-mediated platelet signaling and aggregation in suspension but only partially inhibits signaling and thrombus formation on immobilized collagen. However, further investigation is required to identify the collagen receptor capable of initiating signaling in response to collagen in the presence of ibrutinib.

Our study has demonstrated that platelet aggregometry does not provide an accurate means of identifying the nature of platelet function deficiency caused by some drugs, such as ibrutinib, because of the critical differences between the contribution of some signaling pathways in suspension and during adhesion to immobilized substrates. By using techniques that enabled us to study platelet function during adhesion, we came to the conclusion that ibrutinib causes a combination of platelet functional defects, which result in unstable thrombus formation in vitro and may cause bleeding in vivo. The inhibition of GPVI-evoked signaling caused by ibrutinib does not ablate collagen-evoked signaling or adhesion but may reduce platelet activation and integrin $\alpha_{IIb}\beta_3$ inside–out signaling. In addition, integrin $\alpha_{IIb}\beta_3$ outside-in signaling is inhibited by ibrutinib, removing a critical source of positive feedback signaling that supports clot stabilization. The combined effects of ibrutinib on inside-out activation of integrin $\alpha_{IIb}\beta_3$ and outside-in signaling may, therefore, account for its effects on hemostasis. The success of ibrutinib may stimulate the development of other drugs that target kinases for treatment of cancers and these new drugs, such as ibrutinib and dasatinib, may have off-target effects on hemostasis. It is therefore critical to understand how new kinase inhibitors affect platelet function and hemostasis. With improved understanding of the mechanism by which new kinase inhibitors such as ibrutinib cause disruption of hemostasis may come with the ability to improve the safety profile

of future cancer drugs, predict contraindications or even investigate new potential antiplatelet drug targets.

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## Disclosures

None.

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## Significance

Ibrutinib is known to cause bleeding among patients receiving the drug but the role of platelet function deficiencies caused by ibrutinib downstream of GPVI are unclear. We have demonstrated that ibrutinib affects multiple platelet signaling pathways causing both a partial inhibition of collagen-evoked signaling during adhesion but also a strong inhibition of integrin $\alpha_{IIb}\beta_3$ outside-in signaling that results in the formation of unstable thrombi that disaggregate under shear. These defects are likely to play a role in bleeding risk among patients receiving ibrutinib and have implications for dosing and contraindications with drugs such as P2Y12 antagonists that we found to have an additive effect on thrombus stability. A better understanding of how new drugs that target kinases modulate platelet function and hemostasis is critical to improving the safety of current drug strategies and future drug development.