Ibrutinib Inhibits Platelet Integrin α_{IIb}β_{3} Outside-In Signaling and Thrombus Stability But Not Adhesion to Collagen

Alexander P. Bye, Amanda J. Unsworth, Sakthivel Vaiyapuri, Alexander R. Stainer, Michael J. Fry, Jonathan M. Gibbins

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 immobilized 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 α_{IIb}β_{3} outside-in signaling is also affected 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 α_{IIb}β_{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. Platelets from patients receiving ibrutinib exhibit deficient responses to collagen and the glycoprotein VI (GPVI)-specific agonist, collagen-related peptide (CRP)-XL. However, responses to other platelet agonists such as ADP and U46619 are unaffected or only mildly inhibited. 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. 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. 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. 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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From the Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, University of Reading, Reading, United Kingdom.
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Correspondence to Jonathan Gibbins, PhD, Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, University of Reading, Harborne Bldg, Reading, RG6 6AS, United Kingdom. E-mail j.m.gibbins@reading.ac.uk.
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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 platelet function, we performed a comparison of the effects of ibrutinib on platelet signaling and adhesion to immobilized ligands. 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 led us to investigate positive feedback signaling that supports thrombus stability. To investigate this pathway in addition to GPVI signaling may be an important factor in the bleeding caused by ibrutinib.

Table 1. Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Btk</td>
<td>Bruton tyrosine kinase</td>
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<tr>
<td>CRP</td>
<td>collagen-related peptide</td>
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<tr>
<td>vWF</td>
<td>Von Willebrand factor</td>
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Results

Ibrutinib Inhibits CRP and Collagen-Evoked [Ca2+]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. 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 [Ca2+]i and aggregation evoked by collagen, CRP-XL, ADP, and U46619. At concentrations of ≤1 µmol/L, ibrutinib inhibited Ca2+ 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).

Figure 1. Ibrutinib inhibits Ca2+ elevation and aggregation evoked by collagen-related peptide (CRP)-XL and collagen. For [Ca2+]i, measurements and aggregometry washed platelets loaded with fura-2 for [Ca2+]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 [Ca2+]i traces and (Ai) mean peak [Ca2+]i Elevation and Aggregation in Suspension. Bi, Representative aggregation traces and (Bi) mean light transmission. *P<0.05, ****P<0.0001 using 1-way ANOVA with a Bonferroni post test.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.
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 logIC\(_{50}\) 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 GPV1 Downstream of PLC\(_{\gamma2}\)**

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 \(\mu\text{mol/L} \pm 1.05\) (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\(\pm 10.7\)) evoked by the same concentration of CRP-XL (Figure 2A). Phosphorylation of Syk was not inhibited by 1-\(\mu\text{mol/L}\) ibrutinib, (174\%\(\pm 54\) relative to control; Figure 3D) a concentration that ablated both [Ca\(^{2+}\)]\(_{i}\) elevation and aggregation evoked by 1 \(\mu\text{g/mL}\) 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-\(\mu\text{mol/L}\) ibrutinib was found to ablate PLC\(_{\gamma2}\) tyrosine phosphorylation as well as total ser/th phosphophorylation 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 \(\alpha\_\text{IIb}\beta\_3\) activation were also inhibited by 1-\(\mu\text{mol/L}\) ibrutinib (Figure 3G). Btk/Tec forms part of the PLC\(_{\gamma2}\) activation complex down stream of GPVI and, therefore, inhibition of PLC\(_{\gamma2}\), 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 \(\mu\text{mol/L}\) 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 \(\alpha\_\text{IIb}\beta\_3\), that supports adhesion, and GP Ib, which via the interaction with vWF ensures initial adhesion to collagen under arterial shear.\(^{8}\) Although GPVI has the most established role in mediating

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**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 \(\mu\text{g/mL}\) CRP-XL with an IC\(_{50}\) of 51 nmol/L\(\pm 10.7\), whereas half-maximal inhibition of aggregation was only achieved at 186 nmol/L\(\pm 81.2\) (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.

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**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 means\(\pm\)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 means\(\pm\)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 (\(r<0.05\)) for both [Ca\(^{2+}\)]\(_{i}\) increase (\(r=0.93\)) and aggregation (\(r=0.97\)).
collagen-evoked intracellular signaling, integrin α2β1 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. 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 3](http://atvb.ahajournals.org/)
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...
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(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 [Ca²⁺] 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), [Ca²⁺] 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 [Ca²⁺] elevation evoked by collagen in the majority of platelets was replaced by ragged [Ca²⁺] spiking (Movie I in the online-only Data Supplement) more commonly associated with weaker agonists, such as ADP (Figure 4Ci). In contrast, the Src family kinase inhibitor dasatinib caused complete inhibition of [Ca²⁺] 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 cangrelor 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.
This suggested that early stages in the outside-in signaling pathway are unaffected by ibrutinib. We measured Ca\(^{2+}\) signaling evoked by integrin \(\alpha_{\text{IIb}}\beta_3\), 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\(^{2+}\)] oscillations observed in the majority of adhered platelets was strongly inhibited by 1-\(\mu\)mol/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-\(\mu\)mol/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 \(\alpha_{\text{IIb}}\beta_3\) 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 \(\alpha_{\text{IIb}}\beta_3\), resulting in inhibition of [Ca\(^{2+}\)] 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\(^\text{18}\) and consequently more sophisticated in vitro thrombus formation assays have been developed to enhance diagnosis of platelet disorders.\(^\text{19}\) 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.\(^\text{2,4}\) We found that although ibrutinib caused potent inhibition of aggregation and [Ca\(^{2+}\)] elevation in suspension, that even at a concentration (1 \(\mu\)mol/L) that exceeds plasma concentrations measured clinically, ibrutinib did not completely inhibit adhesion to or signaling evoked by immobilized collagen. Although PLC\(\gamma_2\) 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 PLC\(\beta\) to
Figure 6. Signaling and adhesion mediated by integrin α<sub>IIb</sub>β<sub>3</sub> and clot retraction are inhibited by ibrutinib. 

Ai. Washed platelets were pre-treated 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 β<sub>3</sub> Y773 and Src Y418 phosphorylation. 

Aii. Bar charts are mean normalized phosphorylation values for β<sub>3</sub> Y773 (relative to total β<sub>3</sub>) and Src Y418 (relative to 14-3-3)±SEM (n=3). 

Bi. 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. 

Bii. The traces are fluorescence intensity plots representative of 10 to 60 adhered platelets measured in each of 3 donors. 

Biii. 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 Ca²⁺. 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 others²⁻⁴ have demonstrated that signaling evoked by secondary mediators is largely unaffected by ibrutinib, and we found that signaling via the ADP receptor, P2Y₁₁, 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 α₃β₁, was inhibited by ibrutinib. Ibrutinib inhibited integrin α₃β₁ outside-in signaling via a similar mechanism to GPVI signaling where it prevented PLCγ₂ activation and Ca²⁺ elevation. This is consistent with studies that have shown that Btk is activated downstream of α₃β₁ after direct activation with MnCl₂.¹⁰ 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 immobilized fibrinogen. In a study that used a mouse model lacking integrin α₃β₁, 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 α₃β₁ 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 potency¹³ 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, 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 GPIbα and integrin α₃β₁ mediate adhesion to collagen via vWF. The integrin α₃β₁ 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 α₃β₁ to collagen-evoked platelet activation is only apparent during adhesion to immobilized collagen and not in suspension either in vitro 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 α₃β₁ inside-out signaling. In addition, integrin α₃β₁ 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 α₃β₁ 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 investi-
gate new potential antiplatelet drug targets.

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Disclosures
None.

References

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- and ADP-evoked signaling during adhesion but also a strong inhibition of integrin alpha2beta3 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.
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Materials and Methods

Materials and methods

Materials and reagents

Type I Collagen was obtained from Nycomed (Munich, Germany) and collagen-related peptide (CRP-XL) from Professor Richard Farndale (University of Cambridge, Cambridge, UK). ADP, U46619, aspirin and thrombin was from Sigma (Gillingham, UK). Ibrutinib and dasatinib were from Selleckchem (Munich, Germany). Cangrelor was kindly provided by The Medicines Company (Parsippany, NJ). Antiphosphotyrosine antibody 4G10 was obtained from Millipore (Massachusetts, USA). PLCγ2 Q20, Syk 4D10, β3 N20, actin C11 and 14-3-3 antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany). PKC substrate antibody was from New England Biolabs (Hitchin, UK). P-Src 418 antibody and Fura-2 AM was from Life Technologies (Paisley, UK). Y773 β3 antibody was from Abcam (Cambridge, UK).

Platelet preparation

Blood was drawn from healthy donors that had given informed consent and using procedures approved by the University of Reading Research Ethics Committee and collected into 50 ml syringes containing 4% (w/v) sodium citrate and acid citrate dextrose (ACD, 2.5% sodium citrate, 2% D-glucose and 1.5% citric acid). PRP was prepared by centrifugation of whole blood at 100g for 20 mins. Washed platelets were prepared by centrifugation of PRP at 350g for 20 mins, the platelet pellet was resuspended in Tyrodes containing 0.4 U/ml apyrase.

Plate-based platelet aggregation

PRP or washed platelets at a concentration of 4x10^8 cells/ml were loaded onto 96-well plates (Greiner) and treated with inhibitors or vehicle for 5 mins at 37°C prior to addition of agonist. Plates were shaken at 1,200 rpm for 5 mins at 37°C using a plate shaker (Quantifoil Instruments) as described by Lordkipanidzé et al. and absorption of 405 nm light measured using a NOVOstar plate reader (BMG Labtech).

Light transmission aggregometry

Washed platelets at a concentration of 4x10^8 cells/ml were treated with inhibitors or vehicle for 5 mins at 37°C prior to addition of agonist. Light transmission as measured for 5 mins using an optical aggregometer (Chronolog).

Clot retraction
The retraction of clots formed in PRP pre-incubated for 5 minutes with 1 μM ibrutinib or vehicle and stimulated with 1 U/mL thrombin for 2 h at room temperature was assayed by measurement of clot weight.

**Immunoprecipitation and phosphorylation studies**

Washed platelets were incubated with ibrutinib or vehicle for 5 mins at 37°C prior to addition of 1 μg/ml CRP. Samples were shaken at 1,200 rpm for 1 min at 37°C using a plate shaker (Quantifoil Instruments) prior to addition of reducing Laemmli sample treatment buffer.

**Single platelet Ca²⁺ imaging**

Platelets were loaded with Fluo 4 by incubating human PRP for 1h at room temperature with 2μM Fluo 4 AM. The platelets were then spun at 350 g for 10 minutes and resuspended in Tyrodes at a concentration of 4x10⁸ cells/ml. The washed platelet suspension was then seeded onto fibrinogen or type I collagen coated coverslips (100μg/ml) at a density of 4x10⁷ cells/ml and imaged at a rate of 1 Hz using the 60 x oil immersion lens and 488nm laser of a Nikon A1-R Confocal microscope.

**FACs**

Flow cytometry was performed in 96-well platelets. Platelets at 2x10⁷ cells/ml were stimulated with 1μg/ml CRP-XL at room temperature for 20 minutes in the presence of fluorescein isothiocyanate-labelled (FITC) anti-fibrinogen antibody and PE/Cy5 anti-human CD62P (P-selectin). Reactions were stopped by with 0.2% (v/v) paraformaldehyde. Flow cytometric acquisition was performed using a BD Accuri C6 flow cytometer (BD Biosciences, Oxford, UK), and data were collected from 10,000 events and analysed using the CFlow Sampler software as described previously.

**Spreading studies**

Washed platelets were exposed to fibrinogen (100 μg/ml) or type I collagen (100 μg/ml) coated coverslips and left to incubate for 45 minutes at 37°C. Non-adherent platelets were removed, and the coverslips washed three times with PBS, before fixing using 0.2% paraformaldehyde solution for 10 minutes. The coverslips were then washed and treated with 0.1% (v/v) Triton-X to permeabilise the platelets. The Triton-X was then removed and coverslips washed three times before staining with phalloidin-Alexa Fluor 488 for 1 hr in the dark at room temperature. Coverslips were then further washed in PBS and mounted onto microscope slides. Adherent platelets were then imaged with a 100x oil immersion lens on a Nikon A1-R Confocal microscope.
Adhesion data in each experiment was obtained by counting the number of platelets on 5 images of each coverslip that were chosen at random. The platelets scored as being non-spread, spreading (extending filopodia) and fully spread (formation of lamellipodia) and the relative frequency of these were determined.

In vitro thrombus formation

Thrombus formation was studied in vitro using microfluidic flow cells (Vena8, CellixLtd, Dublin, Ireland) and type I collagen as described previously.²

Measurement of [Ca²⁺],

Platelets were loaded with Fura-2 by incubating PRP with 2 µM Fura-2 AM for 1h at 30°C. Platelets were then washed by centrifugation at 350 g for 20 mins and resuspended in Tyrodes containing 0.4 U/ml apyrase. Fura-2 loaded platelets were incubated with inhibitors or vehicle for 5 mins at 37°C prior to addition of agonists. Fluorescence measurements with excitation at 340 and 380 nm and emission at 510 nm were recorded over a period of 5 mins using a NOVOstar plate reader (BMG Labtech). [Ca²⁺], was estimated using the ratio of the 340 and 380 nm excited signals, the method of Grynkiewicz et al was utilised.³ The maximum fluorescence ratio was measured by lysing the cells with 50 µM digitonin which released the Fura-2 into the saline containing 2 mM CaCl₂. The minimum fluorescence ratio was measured by chelating Ca²⁺ ions with 10 mM EGTA and 10 mM TRIS base to ensure that the pH remained alkaline for optimum Ca²⁺ buffering by EGTA. The autofluorescence was measured using cells at the same final concentration which had not been loaded with fura-2. The following equation was then used to calculate experimental [Ca²⁺], concentrations using the calibration values acquired as described above:

$$[Ca^{2+}]_i = K_d \times S_f \times \frac{R - R_{\text{min}}}{R_{\text{max}} - R}$$

Where $K_d$ is the dissociation constant of Fura-2 (224 nM). $S_f$ and $S_b$ are the background-corrected values of the fluorescence at 380 nm excitation, with zero or saturating Ca²⁺ respectively. $R$ is the background-corrected 340/380 nm fluorescence ratio and $R_{\text{min}}$ and $R_{\text{max}}$ are the ratio limits at zero or saturating Ca²⁺ respectively, which have been adjusted using a viscosity constant of 0.85 which corrects for the effects of the cellular environment upon Fura-2 fluorescence.⁴

Statistical analysis
Prism v.6 (Graphpad, La Jolla, CA) was used to perform statistical analysis and to generate graphs. Statistical comparisons were tested using one-way analysis of variance (ANOVA) and p-values were generated using the Bonferroni multiple comparisons test.