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Accepted Version

Bye, A. P. ORCID: https://orcid.org/0000-0002-2061-2253, Unsworth, A. J., Vaiyapuri, S. ORCID: https://orcid.org/0000-0002-6006-6517, Stainer, A. R., Fry, M. J. and Gibbins, J. M. ORCID: https://orcid.org/0000-0002-0372-5352 (2015) Ibrutinib inhibits platelet integrin αIIbβ3 outside-in signaling and thrombus stability but not adhesion to collagen. Arteriosclerosis Thrombosis and Vascular Biology, 35 (11). pp. 2326-2335. ISSN 1524-4636 doi: 10.1161/ATVBAHA.115.306130 Available at https://centaur.reading.ac.uk/45107/

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Published version at: http://atvb.ahajournals.org/content/35/11/2326.long
To link to this article DOI: http://dx.doi.org/10.1161/ATVBAHA.115.306130

Publisher: American Heart Association

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Ibrutinib inhibits platelet integrin $\alpha_{IIb}\beta_3$ outside-in signaling and thrombus stability but not adhesion to collagen

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Running Title: Ibrutinib causes unstable thrombus formation

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Text word count – 6470

Number of figures - 6

Abstract

Objective - Ibrutinib is an irreversible Btk inhibitor approved for treatment of Waldenstrom's 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 immobilised ligands we found that the collagen signaling deficiency caused by ibrutinib is milder during adhesion to immobilised 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 immobilised 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.

Introduction

Ibrutinib is an inhibitor of the Tec family kinase, Bruton's tyrosine kinase (Btk) approved for the treatment of chronic lymphocytic leukemia, mantle cell lymphoma and Waldenstrom's macroglobulinemia. Patients receiving ibrutinib exhibit deficiencies in hemostasis, resulting in incidents of bruising and petechiae in up to 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 GPVI-specific agonist, 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 appears to inhibit collagen-evoked signaling via inhibition of Btk.[4] However, the effects of ibrutinib have predominantly been investigated using platelet suspension assays, while the impact of ibrutinib on adhesion and signaling on immobilised 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 haemostatic dysfunction caused by ibrutinib.

The involvement of Btk in GPVI-evoked platelet signaling was first identified when patients suffering from 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. Following activation of the platelet GPVI receptor, Btk and Tec are activated downstream of Syk, Fyn and Lyn and regulate PLCγ2 activation, and thereby Ca^{2+} release and PKC activation which are critical events in platelet activation.[6, 7] In addition to GPVI, platelets also express $\alpha_2\beta_1$ and the GPIb receptor complex that can mediate adhesion to collagen and generate intracellular signaling that supports haemostatic platelet function.[8] Btk has an established role in the signaling pathway evoked by GPIb[9] and consequent inhibition of adhesion to 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 believed to share many features of the GPVI signaling pathway, although conflicting reports regarding the involvement of Btk in outside-in signaling have been published. A study reporting that phosphorylation of Btk occurs following 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 β_3, harbouring tyrosine substitutions in the cytoplasmic tale that ablate outside-in signaling, have a mild bleeding phenotype characterised by poor clot stability that results in re-bleeding.[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 immobilised 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 stabilisation, 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 up to 1 µM, 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 1µM 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 effect of ibrutinib upon 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 employed $[Ca^{2+}]_i$ 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 nM ± 10.7 while half-maximal inhibition of aggregation was only achieved at 186 nM ± 81.2 (Figure 2A-B). We found that the concentration-dependent component of the inhibition curves covered the range of peak serum concentrations measured clinically in patients receiving 560mg ibrutinib daily (mean ≈ 300 nM).[12] This suggests that variability in plasma concentration may cause large differences in platelet inhibition among patients.

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 more than one target with different affinities (Figure 2C-D). It is tempting to speculate that these targets may represent the two 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 two 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 (SFKs) 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 appear 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 µM ± 1.05 (Figure 3A-B), which was 20 fold higher than that required for half-maximal inhibition of $[Ca^{2+}]_i$ elevation (IC_{50} = 51 nM ± 10.7) evoked by the same concentration of CRP-XL (Figure 2A). Phosphorylation of Syk was not inhibited by 1 µM 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 SFKs. In contrast to measurements of Src and Syk phosphorylation, 1 µM ibrutinib was found to ablate PLCγ2 tyrosine phosphorylation as well as total ser/thr phosphorylation of all substrates of PKC (Figure 3E-F). Measurement of p-selectin exposure and fibrinogen binding that are representative of the downstream signalling events of granule secretion and integrin $\alpha_{IIb}\beta_3$ activation were also inhibited by 1 µM 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 1µM was used, to ensure that complete inhibition of platelet function mediated by GPVI was achieved.

Ca^{2+} signaling evoked by adhesion to immobilised 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 immobilised ligands. Platelet adhesion and activation on immobilised collagen is thought to be orchestrated by GPVI which triggers activation, integrin $\alpha_2\beta_1$ which supports adhesion, and GPIb which, via the interaction with vWF ensures initial adhesion to collagen under arterial shear.[8] While GPVI has the most established role in mediating 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 immobilised CRP-XL was inhibited by 70% ± 13.9 in the presence of 1 µM ibrutinib (Figure 4Ai-ii). 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 µM ibrutinib (Figure 4Aiii). The efficacy of ibrutinib as an inhibitor of platelet function mediated by GPVI therefore appeared to be maintained in spreading experiments. Adhesion to CRP-XL appeared to be dependent upon activation which was blocked by ibrutinib. CRP-XL is not an adhesive ligand and adhesion to CRP-XL may be therefore be reliant on deposition of adhesive proteins present in α-granules such as vWF and fibrinogen that are secreted upon activation.[15] In contrast, adhesion to type I collagen was not significantly inhibited by the presence of 1µM 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 to 33% ± 6.6 with 1 µM ibrutinib) but to a lesser extent than on CRP-XL (Figure 4Aiii). The differential effects of ibrutinib on adhesion and spreading on immobilised 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 immobilised or solubilised 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 SFKs 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 immobilised 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 (supplementary video 1) more commonly associated with weaker agonists such as ADP (Figure 4Ci).[17] In contrast, the SFK inhibitor dasatinib caused complete inhibition of $[Ca^{2+}]_i$ elevation evoked by immobilised 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 following stimulation with collagen in suspension observed in this study (Figure 1-2) and others.[3, 4] We suggest that signalling 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 following treatment with ibrutinib

As we had identified key differences between the effects of ibrutinib on platelet function in suspension and during adhesion to immobilised 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. Following treatment with 1 µM ibrutinib levels of thrombus formation after 10 minutes were significantly inhibited relative to control (39% ± 9.2 of vehicle-treated controls, Figure 5Bi-ii). 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 following initial stable adhesion of platelets to collagen (Supplementary video 2). 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 in the same thrombus formation assay. When platelets were treated with 1 µM 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, while cangrelor treatment resulted in embolization of 'clumps' of platelets from growing thrombi, ibrutinib caused disaggregation of individual platelets (Supplementary video 2). 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, one-way ANOVA with Bonferroni post test, $p < 0.05$ after 7 minutes) while 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 two 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 immobilised 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 immobilised 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 following adhesion to fibrinogen in the presence of 1 µM 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 (supplementary video 3). The sustained $[Ca^{2+}]_i$ oscillations observed in the majority of adhered platelets was strongly inhibited by 1µM 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 coverglass and found that adhesion was inhibited by 48% ± 5.4 in the presence of 1 µM ibrutinib and the proportion of platelets forming lamellipodia was reduced (36% ± 5.6 compared to 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 FDA to treat mantle cell lymphoma, chronic lymphocytic leukemia and Waldenstrom's macroglobulinemia. However, ibrutinib treatment is associated with increased risk of bleeding among patients. Until the present 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 utility 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 employed 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 the present study the effects of ibrutinib in assays performed in suspension and during adhesion to immobilised collagen were compared to provide a better understanding of how ibrutinib causes platelet signaling deficiencies and how these may impact upon 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 μM) that exceeds plasma concentrations measured clinically, ibrutinib did not completely inhibit adhesion to or signaling evoked by immobilised 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 activate PKC and release Ca^{2+}. Furthermore, initial adhesion to collagen under arterial shear stress was not significantly inhibited by ibrutinib, while 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 μM 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 $\alpha_{IIb}\beta_3$ following 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 employed a mouse model lacking integrin $\alpha_{IIb}\beta_3$ outside-in signaling due to substitution of critical tyrosine residues in the β3 cytoplasmic tail, the outside-in signaling deficiency was found to cause re-bleeding 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 appeared 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 support for this hypothesis is found by comparing the bleeding phenotype of patients receiving ibrutinib to those of patients with disorders affecting Btk or GPVI function. Patients suffering from 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 two targets with distinct potencies downstream of GPVI that may represent the two 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 immobilised collagen,[21] whereas this was not the case following ibrutinib treatment in the present study. This suggests that components of the GPVI signaling pathway that are not dependent upon Btk may be able to contribute toward adhesion to immobilised 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 immobilised 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 while 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 believed 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 immobilised collagen and not in suspension either *in vitro*[14] or *in vivo* following 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 immobilised 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, due to the critical differences between the contribution of some signaling pathways in suspension and during adhesion to immobilised substrates. By utilising 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 stabilisation. 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, like 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 like ibrutinib cause disruption of hemostasis may come the ability to improve the safety profile of future cancer drugs, predict contraindications or even investigate new potential anti-platelet drug targets.

Acknowledgments

None

Sources of Funding

This work was supported by grants from the British Heart Foundation (RG/09/011/28094 & PG/13/93/30593).

Disclosure

The authors declare no competing financial interests

References

1. Byrd JC, Furman RR, Coutre SE, et al. *Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia.* N Engl J Med, 2013; 369: 32-42.
2. Rushworth SA, MacEwan DJ, and Bowles KM. *Ibrutinib in relapsed chronic lymphocytic leukemia.* N Engl J Med, 2013; 369: 1277-1278.
3. Kamel S, Horton L, Ysebaert L, et al. *Ibrutinib inhibits collagen-mediated but not ADP-mediated platelet aggregation.* Leukemia, 2015; 29: 783-787.
4. Levade M, David E, Garcia C, Laurent PA, Cadot S, Michallet AS, Bordet JC, Tam C, Sie P, Ysebaert L, and Payrastre B. *Ibrutinib treatment affects collagen and von Willebrand Factor-dependent platelet functions.* Blood, 2014; 124: 3991-3995.
5. Quek LS, Bolen J, and Watson SP. *A role for Bruton's tyrosine kinase (Btk) in platelet activation by collagen.* Curr Biol, 1998; 8: 1137-1140.
6. Atkinson BT, Ellmeier W, and Watson SP. *Tec regulates platelet activation by GPVI in the absence of Btk.* Blood, 2003; 102: 3592-3599.
7. Poenie M. *Alteration of intracellular Fura-2 fluorescence by viscosity: a simple correction.* Cell Calcium, 1990; 11: 85-91.
8. Nieswandt B and Watson SP. *Platelet-collagen interaction: is GPVI the central receptor?* Blood, 2003; 102: 449-461.
9. Liu J, Fitzgerald ME, Berndt MC, Jackson CW, and Gartner TK. *Bruton tyrosine kinase is essential for botrocetin/VWF-induced signaling and GPIb-dependent thrombus formation in vivo.* Blood, 2006; 108: 2596-2603.
10. Soriani A, Moran B, de Virgilio M, Kawakami T, Altman A, Lowell C, Eto K, and Shattil SJ. *A role for PKCtheta in outside-in alpha(IIb)beta3 signaling.* J Thromb Haemost, 2006; 4: 648-655.
11. Law DA, DeGuzman FR, Heiser P, Ministri-Madrid K, Killeen N, and Phillips DR. *Integrin cytoplasmic tyrosine motif is required for outside-in alphaIIbbeta3 signalling and platelet function.* Nature, 1999; 401: 808-811.
12. Advani RH, Buggy JJ, Sharman JP, et al. *Bruton tyrosine kinase inhibitor ibrutinib (PCI-32765) has significant activity in patients with relapsed/refractory B-cell malignancies.* J Clin Oncol, 2013; 31: 88-94.
13. Honigberg LA, Smith AM, Sirisawad M, Verner E, Loury D, Chang B, Li S, Pan Z, Thamm DH, Miller RA, and Buggy JJ. *The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy.* Proc Natl Acad Sci U S A, 2010; 107: 13075-13080.
14. Inoue O, Suzuki-Inoue K, Dean WL, Frampton J, and Watson SP. *Integrin alpha2beta1 mediates outside-in regulation of platelet spreading on collagen through activation of Src kinases and PLCgamma2.* J Cell Biol, 2003; 160: 769-780.

15. Sakurai Y, Fitch-Tewfik JL, Qiu Y, Ahn B, Myers DR, Tran R, Fay ME, Ding L, Spearman PW, Michelson AD, Flaumenhaft R, and Lam WA. *Platelet geometry sensing spatially regulates alpha-granule secretion to enable matrix self-deposition.* Blood, 2015; 126: 531-538.
16. Quintas-Cardama A, Kantarjian H, Ravandi F, O'Brien S, Thomas D, Vidal-Senmache G, Wierda W, Kornblau S, and Cortes J. *Bleeding diathesis in patients with chronic myelogenous leukemia receiving dasatinib therapy.* Cancer, 2009; 115: 2482-2490.
17. Heemskerk JW, Willems GM, Rook MB, and Sage SO. *Ragged spiking of free calcium in ADP-stimulated human platelets: regulation of puff-like calcium signals in vitro and ex vivo.* J Physiol, 2001; 535: 625-635.
18. Harrison P, Mackie I, Mumford A, Briggs C, Liesner R, Winter M, Machin S, and British Committee for Standards in H. *Guidelines for the laboratory investigation of heritable disorders of platelet function.* Br J Haematol, 2011; 155: 30-44.
19. de Witt SM, Swieringa F, Cavill R, et al. *Identification of platelet function defects by multi-parameter assessment of thrombus formation.* Nat Commun, 2014; 5: 4257.
20. Oda A, Ikeda Y, Ochs HD, Druker BJ, Ozaki K, Handa M, Ariga T, Sakiyama Y, Witte ON, and Wahl MI. *Rapid tyrosine phosphorylation and activation of Bruton's tyrosine/Tec kinases in platelets induced by collagen binding or CD32 cross-linking.* Blood, 2000; 95: 1663-1670.
21. Arthur JF, Dunkley S, and Andrews RK. *Platelet glycoprotein VI-related clinical defects.* Br J Haematol, 2007; 139: 363-372.
22. Moroi M, Jung SM, Okuma M, and Shinmyozu K. *A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion.* J Clin Invest, 1989; 84: 1440-1445.
23. Takahashi H and Moroi M. *Antibody against platelet membrane glycoprotein VI in a patient with systemic lupus erythematosus.* Am J Hematol, 2001; 67: 262-267.
24. Kojima H, Moroi M, Jung SM, Goto S, Tamura N, Kozuma Y, Suzukawa K, and Nagasawa T. *Characterization of a patient with glycoprotein (GP) VI deficiency possessing neither anti-GPVI autoantibody nor genetic aberration.* J Thromb Haemost, 2006; 4: 2433-2442.
25. Marjoram RJ, Voss B, Pan Y, Dickeson SK, Zutter MM, Hamm HE, and Santoro SA. *Suboptimal activation of protease-activated receptors enhances alpha2beta1 integrin-mediated platelet adhesion to collagen.* J Biol Chem, 2009; 284: 34640-34647.
26. Marjoram RJ, Li Z, He L, Tollefsen DM, Kunicki TJ, Dickeson SK, Santoro SA, and Zutter MM. *alpha2beta1 integrin, GPVI receptor, and common FcRgamma chain on mouse platelets mediate distinct responses to collagen in models of thrombosis.* PLoS One, 2014; 9: e114035.

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 signalling pathways causing both a partial inhibition of collagen-evoked signalling 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.

Figure Legends

Figure 1: Ibrutinib inhibits Ca^{2+} elevation and aggregation evoked by CRP-XL and collagen. For $[Ca^{2+}]_i$ measurements and aggregometry washed platelets (loaded with fura-2 for $[Ca^{2+}]_i$ measurements) were pre-treated with 1 µM ibrutinib or vehicle for 5 minutes and stimulated with 1 µg/ml type I collagen, 1 µg/ml CRP-XL, 10 µM ADP or 1 µM U46619. (Ai) Shows representative $[Ca^{2+}]_i$ traces and (Aii) mean peak $[Ca^{2+}]_i$ increases normalised to % of vehicle response ± s.e.m (n=4). (Bi) Shows representative aggregation traces and (Bii) mean light transmission 5 minutes after addition of agonist ± s.e.m (n=4). * $p < 0.05$, **** $p < 0.0001$ using one-way ANOVA with a Bonferroni post test.

Figure 2: Ibrutinib inhibits GPVI-evoked signaling with potency that is partially dependent upon stimulus strength. $[Ca^{2+}]_i$ measurements and aggregations were performed using washed platelets (loaded with fura-2 for $[Ca^{2+}]_i$ measurements) pre-treated for 5 minutes with ibrutinib at the indicated concentrations and stimulated with a range of CRP-XL concentrations between 10 µg/ml 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 ± s.e.m. (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 ± s.e.m. (n=4). Correlation between the apparent IC_{50} of ibrutinib and CRP-XL concentration was tested using Pearson's correlation test and found to be significant ($p < 0.05$) for both $[Ca^{2+}]_i$ increase (r=0.93) and aggregation (r=0.97).

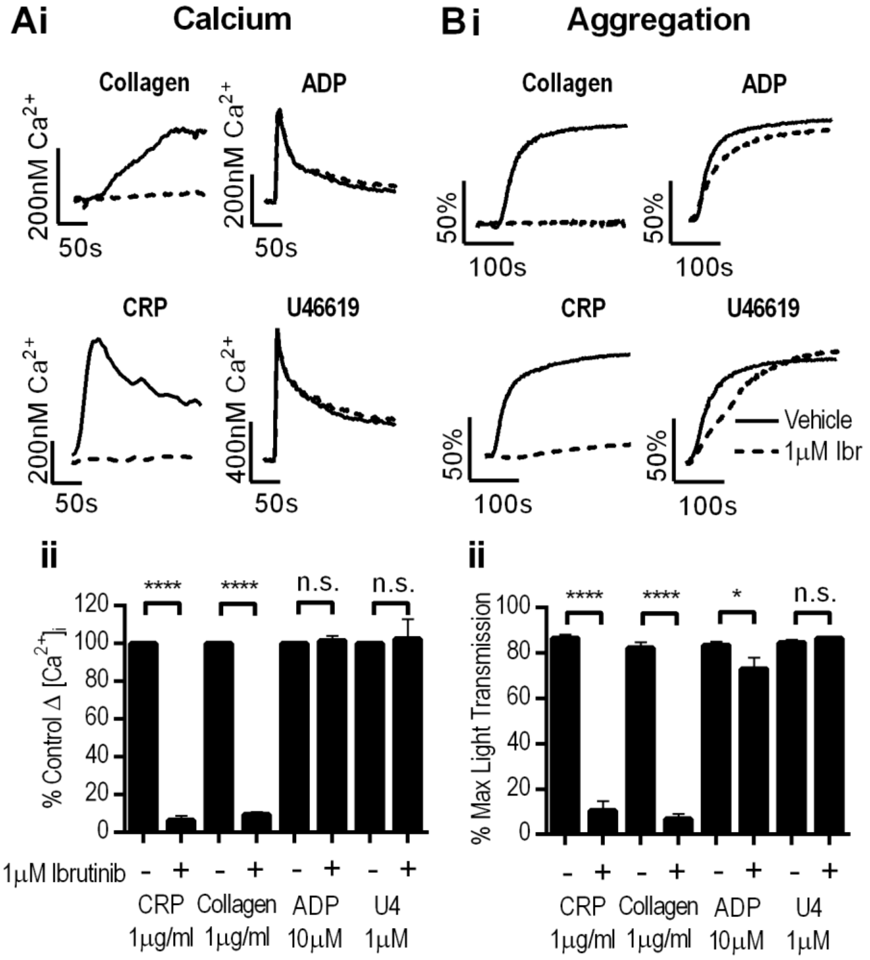
Figure 3: Inhibition of GPVI-evoked signaling occurs at the level of PLCγ2 activation. (A) Washed platelets were pre-treated for 5 minutes with a range of concentrations of ibrutinib or vehicle before stimulation with 1 µg/ml CRP-XL for one minute before immunoblotting for Src Y418. (B) The mean relative phosphorylation levels of Src Y418 ± s.e.m. (normalised to total Src) (n=4). (C) Washed platelets were pre-treated for 5 minutes with 1 µM ibrutinib or vehicle before stimulation with 1 µg/ml CRP-XL for one minute, samples were blotted for Ser/Thr phosphorylation of PKC substrates or used for immunoprecipitation of Syk or PLCγ2 and blotted for tyrosine phosphorylation (using 4G10 antibody). Bar charts are mean normalised phosphorylation values ± s.e.m for (D) tyrosine phosphorylation of Syk normalised 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 following treatment of platelets for minutes with 1µg/ml CRP-XL in the presence or absence of 1µM ibrutinib (n=4). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using one-way ANOVA with a Bonferroni post test.

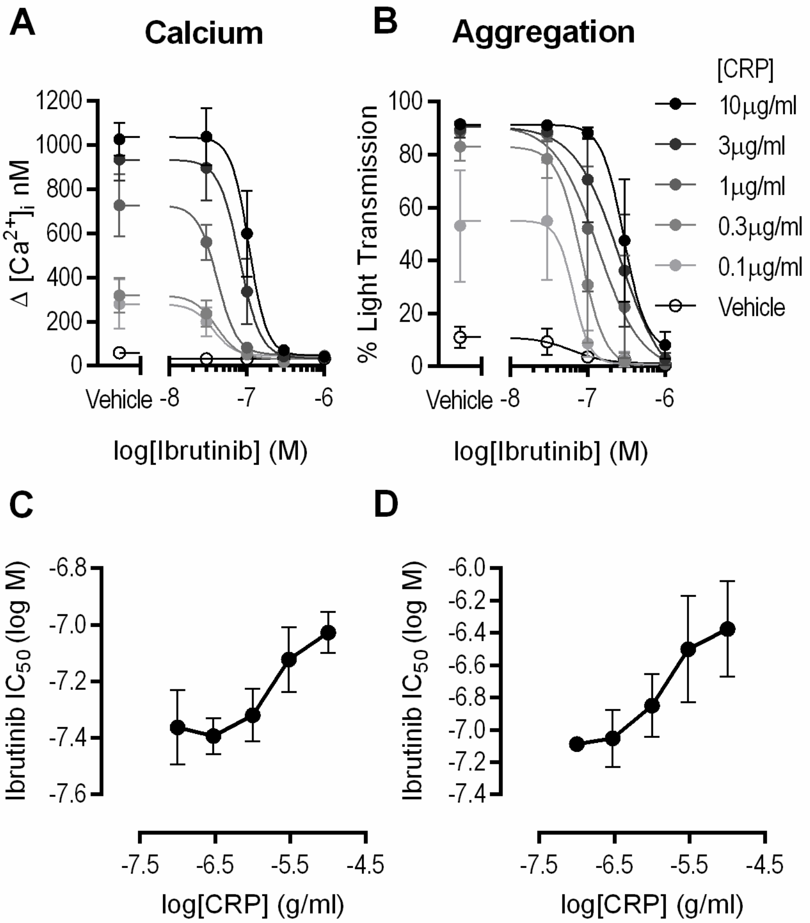
Figure 4: Signaling and adhesion evoked by immobilised collagen is only partially inhibited by ibrutinib and ablated by dasatinib. (Ai) Washed platelets were pre-treated for 5 minutes with 1µM ibrutinib or vehicle and allowed to adhere to type I collagen or CRP coated cover glass for 45 minutes. (Aii)The relative percentage of adhered platelets relative to vehicle treated (Aiii) and the percentage of platelets that had formed lamellapodia were quantified as mean % ± s.e.m (n=5). (Bi) Washed platelets were pre-treated for 5 minutes with 1 µM ibrutinib or vehicle and allowed to adhere to type I collagen-coated plates for 30 mins before platelets were lysed and probed for PLCγ2 phosphorylation and phosphorylation of PKC substrates. Bar chats are mean normalised phosphorylation values ± s.e.m for (Bii) tyrosine phosphorylation of PLCγ2 relative to total PLCγ2 and

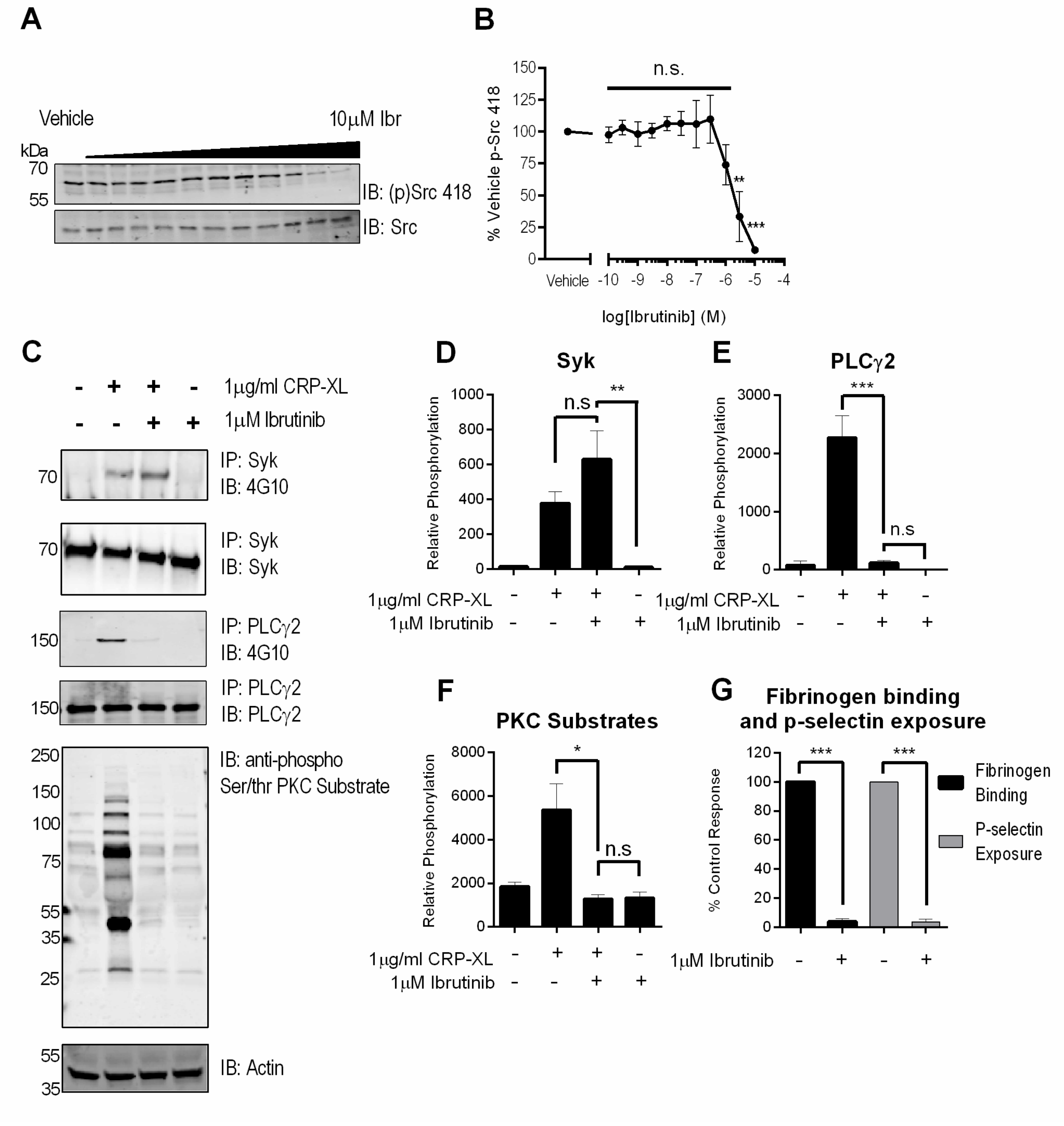
(Biii) serine /threonine phosphorylation of substrates of PKC relative to actin. (n=5). (C) PRP was loaded with fluo 4-AM and imaged at 1Hz during adhesion and spreading on type I collagen-coated cover glass under static conditions for 15 minutes in the presence of 1 µM ibrutinib, 1 µM dasatinib or vehicle only. (Ci) The traces are fluorescence intensity plots representative of between 10-60 adhered platelets measured in each of 3 different donors, (Cii) bar charts are mean area-under-the-curve (AUC) measurements ± s.e.m. (n=3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ using one-way ANOVA with a Bonferroni post test.

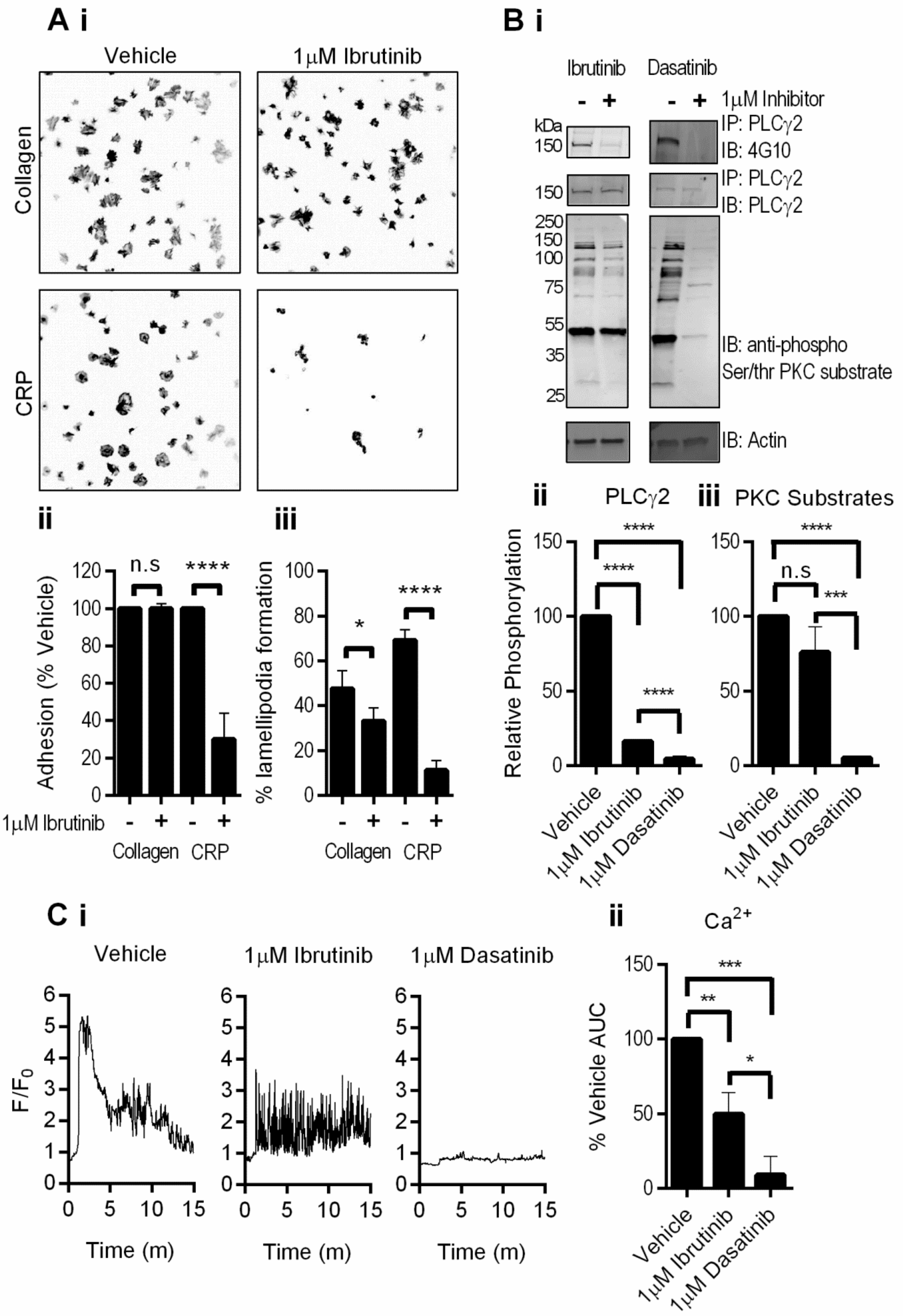
Figure 5: Ibrutinib inhibits stable thrombus formation but not adhesion to immobilised collagen. (A) Thrombus formation was measured using whole blood in pre-incubated for 5 minutes with 1 µM ibrutinib and / or 1 µM cangrlor or vehicle only before perfusion through a type I collagen-coated channel for 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 ± s.e.m after 10 minutes and (Ci) traces of mean surface coverage with (Cii) bar charts of mean surface coverage ± s.e.m after 10 minutes. (n=4). ** $p < 0.01$, *** $p > 0.001$, **** $p < 0.0001$ using one-way ANOVA with a Bonferroni post test.

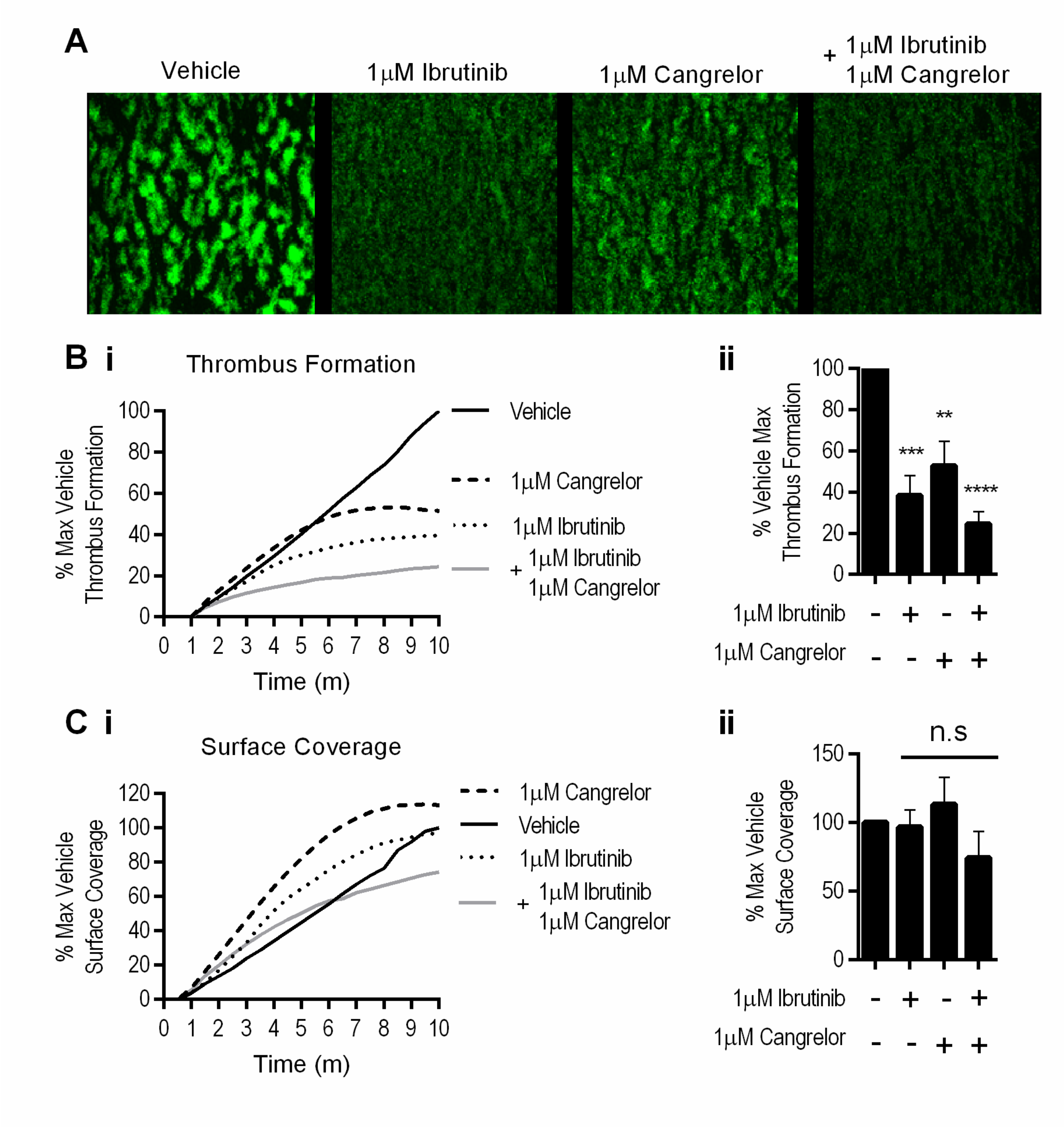
Figure 6: Signaling and adhesion mediated by integrin $\alpha_{IIb}\beta_3$ and clot retraction are inhibited by ibrutinib. (Ai) Washed platelets were pre-treated for 5 minutes with 1 µM ibrutinib or vehicle and allowed to adhere to fibrinogen-coated plates for 30 mins before platelets were lysed and probed for β_3 Y773 and Src Y418 phosphorylation. (Aii) Bar charts are mean normalised phosphorylation values for β3 Y773 (relative to total β3) and Src Y418 (relative to 14-3-3) ± s.e.m. (n=3). (B) PRP was loaded with fluo 4-AM and imaged at 1Hz during adhesion and spreading on fibrinogen-coated cover glass under static conditions for 25 minutes in the presence of 1 µM ibrutinib or vehicle only. (Bi) The traces are fluorescence intensity plots representative of 10-60 adhered platelets measured in each of 3 donors. (Bii) Bar charts are mean area-under-the-curve (AUC) measurements ± s.e.m. (Ci) Washed platelets were pre-treated for 5 minutes with 1 µM 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 % ± s.e.m (n=5). (Di) A representative image of clot retraction assessed using PRP treated with vehicle or 1µM ibrutinib 2h after addition of 1 U/ml thrombin, (Dii) bars represent mean clot weight ± s.e.m (n=6). * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ using one-way ANOVA with a Bonferroni post test.

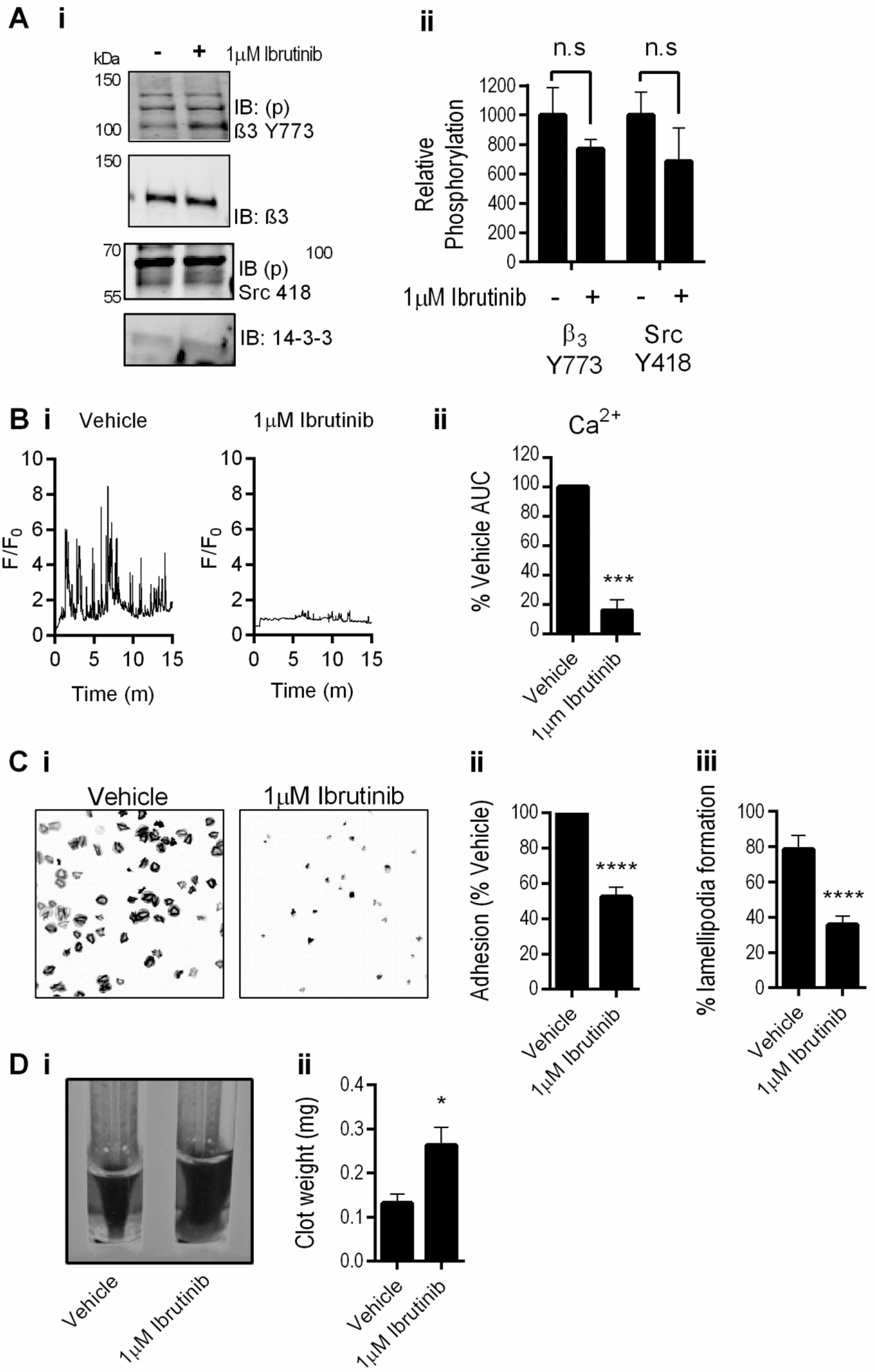

Ai
Calcium
Collagen
ADP
200nM Ca2+
50s
CRP
U46619
400nM Ca2+
Bi
Aggregation
50%
100s
Vehicle
1μM Ibr
ii
% Control Δ [Ca2+]i
% Max Light Transmission
****
n.s.
*
1μM Ibrutinib
- +
CRP 1μg/ml
Collagen 1μg/ml
ADP 10μM
U4 1μM


A
Calcium
B
Aggregation
[CRP]
10μg/ml
3μg/ml
1μg/ml
0.3μg/ml
0.1μg/ml
Vehicle
Δ [Ca2+]i nM
1200
1000
800
600
400
200
0
Vehicle
-8
-7
-6
log[Ibrutinib] (M)
% Light Transmission
100
80
60
40
20
0
Vehicle
-8
-7
-6
log[Ibrutinib] (M)
C
Ibrutinib IC50 (log M)
-6.8
-7.0
-7.2
-7.4
-7.6
-7.5
-6.5
-5.5
-4.5
log[CRP] (g/ml)
D
Ibrutinib IC50 (log M)
-6.0
-6.2
-6.4
-6.6
-6.8
-7.0
-7.2
-7.4
-7.5
-6.5
-5.5
-4.5
log[CRP] (g/ml)


A
Vehicle
10μM Ibr
kDa
70
55
IB: (p)Src 418
IB: Src
B
% Vehicle p-Src 418
150
125
100
75
50
25
0
n.s.
**
***
Vehicle
-10
-9
-8
-7
-6
-5
-4
log[Ibrutinib] (M)
C
1μg/ml CRP-XL
1μM Ibrutinib
IP: Syk
IB: 4G10
IP: Syk
IB: Syk
IP: PLCγ2
IB: 4G10
IP: PLCγ2
IB: PLCγ2
IB: anti-phospho Ser/thr PKC Substrate
IB: Actin
250
150
100
75
55
35
25
D
Syk
Relative Phosphorylation
1000
800
600
400
200
0
n.s
**
E
PLCγ2
3000
2000
1000
0
***
n.s
F
PKC Substrates
8000
6000
4000
2000
0
*
n.s
G
Fibrinogen binding and p-selectin exposure
% Control Response
120
100
80
60
40
20
0
***
***
Fibrinogen Binding
P-selectin Exposure
1μg/ml CRP-XL - + + -
1μM Ibrutinib - - + +


A i
Vehicle
1μM Ibrutinib
Collagen
CRP
ii
Adhesion (% Vehicle)
0
20
40
60
80
100
120
n.s
****
1μM Ibrutinib
- + - +
Collagen CRP
iii
% lamellipodia formation
0
20
40
60
80
100
*
****
- + - +
Collagen CRP
B i
Ibrutinib
Dasatinib
- + - +
1μM Inhibitor
kDa
150
IP: PLCγ2
IB: 4G10
150
IP: PLCγ2
IB: PLCγ2
250
150
100
75
55
35
25
IB: anti-phospho Ser/thr PKC substrate
IB: Actin
ii
PLCγ2
Relative Phosphorylation
0
50
100
150
****
****
****
Vehicle
1μM Ibrutinib
1μM Dasatinib
iii
PKC Substrates
0
50
100
150
****
n.s
***
Vehicle
1μM Ibrutinib
1μM Dasatinib
C i
Vehicle
1μM Ibrutinib
1μM Dasatinib
F/F0
0
1
2
3
4
5
6
0
5
10
15
Time (m)
ii
Ca2+
% Vehicle AUC
0
50
100
150
***
**
*
Vehicle
1μM Ibrutinib
1μM Dasatinib


A
Vehicle
1μM Ibrutinib
1μM Cangrelor
+ 1μM Ibrutinib
1μM Cangrelor
B i
Thrombus Formation
% Max Vehicle Thrombus Formation
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
Time (m)
Vehicle
1μM Cangrelor
1μM Ibrutinib
+ 1μM Ibrutinib
1μM Cangrelor
ii
% Vehicle Max Thrombus Formation
100
80
60
40
20
0
***
**
****
1μM Ibrutinib - + - +
1μM Cangrelor - - + +
C i
Surface Coverage
% Max Vehicle Surface Coverage
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
Time (m)
1μM Cangrelor
Vehicle
1μM Ibrutinib
+ 1μM Ibrutinib
1μM Cangrelor
ii
n.s
% Max Vehicle Surface Coverage
150
100
50
0
1μM Ibrutinib - + - +
1μM Cangrelor - - + +


A i
kDa
- + 1μM Ibrutinib
150
100
IB: (p) ß3 Y773
150
IB: ß3
70
100
55
IB (p) Src 418
IB: 14-3-3
ii
n.s
n.s
Relative Phosphorylation
1200
1000
800
600
400
200
0
1μM Ibrutinib - + - +
β_3 Y773
Src Y418
B i
Vehicle
1μM Ibrutinib
F/F$_0$
10
8
6
4
2
0
0 5 10 15
Time (m)
ii
Ca^{2+}
% Vehicle AUC
120
100
80
60
40
20
0
***
Vehicle
1μm Ibrutinib
C i
Vehicle
1μM Ibrutinib
ii
Adhesion (% Vehicle)
100
80
60
40
20
0
****
Vehicle
1μM Ibrutinib
iii
% lamellipodia formation
100
80
60
40
20
0
****
Vehicle
1μM Ibrutinib
D i
Vehicle
1μM Ibrutinib
ii
Clot weight (mg)
0.4
0.3
0.2
0.1
0.0
*
Vehicle
1μM Ibrutinib

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 4×10^8 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.* [1] and absorption of 405 nm light measured using a NOVOstar plate reader (BMG Labtech).

Light transmission aggregometry

Washed platelets at a concentration of 4×10^8 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^{o}C prior to addition of 1 ug/ml CRP. Samples were shaken at 1,200 rpm for 1 min at 37^{o}C using a plate shaker (Quantifoil Instruments) prior to addition of reducing Laemmli sample treatment buffer.

Single platelet Ca^{2+} 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 $4x10^8$ cells/ml. The washed platelet suspension was then seeded onto fibrinogen or type I collagen coated coverslips (100µg/ml) at a density of $4x10^7$ 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 $2x10^7$ 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 $[Ca^{2+}]_i$

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). $[Ca^{2+}]_i$ 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 $CaCl_2$. The minimum fluorescence ratio was measured by chelating Ca^{2+} ions with 10 mM EGTA and 10 mM TRIS base to ensure that the pH remained alkaline for optimum Ca^{2+} 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 $[Ca^{2+}]_i$ concentrations using the calibration values acquired as described above:

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

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 Ca^{2+} respectively. R is the background-corrected 340/380 nm fluorescence ratio and R_{min} and R_{max} are the ratio limits at zero or saturating Ca^{2+} 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.

1. Lordkipanidze M, Lowe GC, Kirkby NS, et al. *Characterization of multiple platelet activation pathways in patients with bleeding as a high-throughput screening option: use of 96-well Optimul assay.* Blood, 2014; 123: e11-22.
2. Vaiyapuri S, Jones CI, Sasikumar P, et al. *Gap junctions and connexin hemichannels underpin hemostasis and thrombosis.* Circulation, 2012; 125: 2479-2491.
3. Grynkiewicz G, Poenie M, and Tsien RY. *A new generation of Ca2+ indicators with greatly improved fluorescence properties.* J Biol Chem, 1985; 260: 3440-3450.
4. Poenie M. *Alteration of intracellular Fura-2 fluorescence by viscosity: a simple correction.* Cell Calcium, 1990; 11: 85-91.