SUPPLEMENTARY APPENDIX

Inhibition of Btk by Btk-specific concentrations of ibrutinib and acalabrutinib delays but does not block platelet aggregation mediated by glycoprotein VI

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Supplementary Data

Methods

Reagents

The α-phosphotyrosine (4G10) monoclonal antibody (mAb) was from Millipore (Abingdon, UK). The α-Syk pAb (SC-1077) was from Santa Cruz Biotechnology (Dallas, USA). The α-Btk pAb (SAB3500372) was from Sigma-Aldrich (Poole, UK). Phosphospecific pAbs against Syk pY525/6, PLCɣ2 pY759 and pY1217, were from Cell Signalling Technology (Hitchin, UK) and against LAT pY200, SLP-76 PY145, PLCɣ2 Y753, Btk pY223 and pY551 were from Abcam (Cambridge, UK). Ibrutinib (PCI-32765) and acalabrutinib (ACP-196) were from Selleckchem (Munich, Germany). Eptifibatide was from GSK (Brentford, UK). The α-Tec and α-Btk pAbs (BL17 and BL19 respectively) have been described[22]. HRP-conjugated secondary pAbs and Hyperfilm ECL autoradiography film were from Amersham Biosciences (GE Healthcare, Bucks, UK). ECL reagent and the Ca^{2+} sensitive report precursor dye Fura-2-AM were from ThermoFisher (Waltham, MA). CRP was from Richard Farndale (Cambridge, UK). Collagen was made from equine tendon and sourced from Takeda (Linz, Austria). ChronoLume® and ATP standard were from ChronoLog Corporation (Havertown, PA). All other reagents were purchased from Sigma-Aldrich (Poole, UK).

Chemical analysis of inhibitors

Purity assessment of individual batches of ibrutinib and acalabrutinib was performed by High Performance Liquid Chromatography and Liquid Chromatograpy Mass Spectrometry. Individual batches of inhibitor were compared to each other using lumiaggregometry to CRP in washed platelets as a bioassay. Concentration calculations for each batch of inhibitor were adjusted to reflect their differences in potency in this bioassay when compared to the measured chemical concentration.

Platelet rich plasma (PRP) and washed platelet preparation

Blood was taken from consenting patients or healthy, drug-free volunteers, into 4% sodium citrate. Blood from patients, who were not taking concomitant antiplatelet medication, was taken at 2-3 hours after the ingestion of Btk inhibitor on the final day of a treatment cycle. PRP was obtained by centrifugation at 200 *g* for 20 min at room temperature. Washed platelets were obtained by centrifugation at 1,000 *g* for 10 min using 0.2 µg/mL prostacyclin and resuspended in modified-Tyrode's-HEPES buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$; pH 7.3) as previously described[39]. Platelets were used at 4×10^8/mL for aggregation and biochemistry unless otherwise stated.

Granule Release

During LTA, 5 µL ChronoLume® (a commercial reagent containing a D-luciferin-luciferase mixture) was added 1 min prior to insertion of glass vials into the

measurement chamber of the aggregometer. Light production by luciferase was measured by the Model 700 aggregometer. Calibration was performed by adding 5 µL of 2 µM ATP standard at the end of the experiment. Results were averaged and IC_{50} values were calculated from these data.

Measurement of $[Ca^{2+}]_i$

Platelets were loaded with the Ca^{2+} sensitive dye Fura-2 by incubation of PRP with 2 µM Fura-2-AM for 1 hour at 30°C. Platelets were then washed by centrifugation at 350 *g* for 20 min and resuspended in modified-Tyrode-HEPES buffer. Fura-2-loaded platelets were incubated with inhibitors or vehicle (DMSO) for 5 min 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 min using a NOVOstar plate reader (BMG Labtech) for experiments with ibrutinib or a FlexStation (Molecular Devices) for experiments with acalabrutinib. $[Ca^{2+}]_i$ was calculated using the ratio of the 340 and 380 nm excitation signals according to the method of Grynkiewicz *et al*[40]. Results were averaged and IC_{50} values were calculated from these data.

Measurement of platelet adhesion under flow

Washed platelets were incubated with inhibitor or vehicle for 5 min. Platelets were added back to non-ACD treated red blood cells and Platelet Poor Plasma (PPP) to a final concentration of $4x10^8$/mL. Platelets were incubated with 4 µM $DiOC_6$ for 5 min to aid visualisation. Flow adhesion using a Cellix microfluidic system was performed as described[13]. Microcapillaries were subsequently fixed with 10% neutral buffered Formalin solution and platelet adhesion was viewed using a Zeiss Axio Observer 7 microscope at 20X objective using fluorescence intensity emitted at 520 nm. Platelet surface coverage on flow adhesion microcapillary images were measured using ImageJ 1.5.

Cells and plasmids

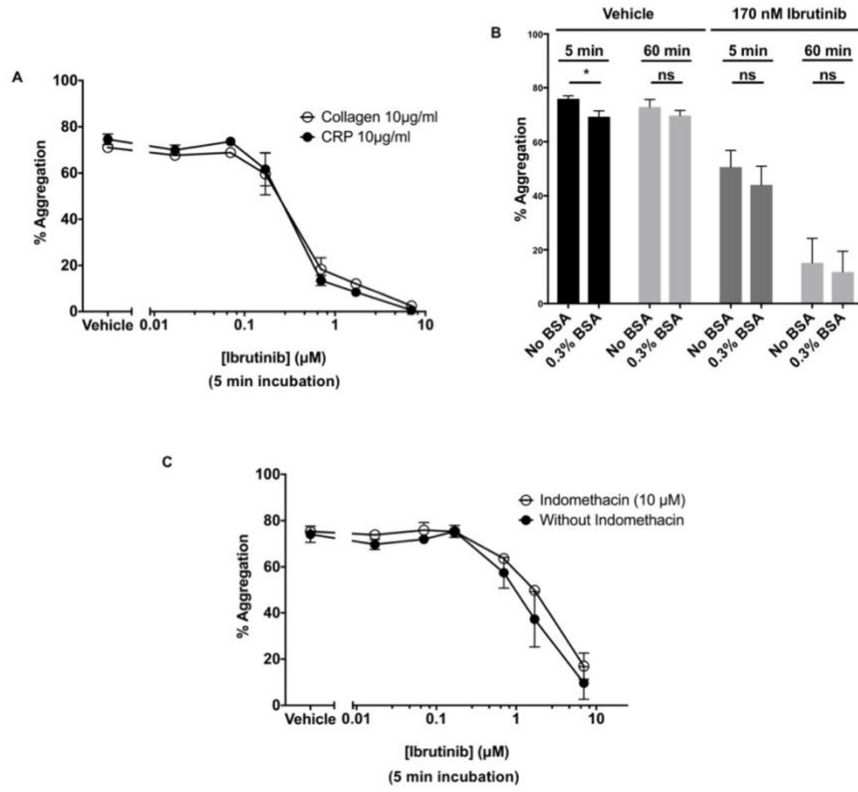
The DT40 chicken lymphoma cell line rendered deficient in Btk[41] was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% chicken serum, 100 U/ml penicillin, 100 µg/mL streptomycin, 20 mM glutamine and 50 µM 2β-mercaptoethanol. GPVI, FcRɣ and NFAT-luciferase plasmids have been described previously[42]. The WT and K430E Btk plasmids have been previously described[26].

Transfections and luciferase assay

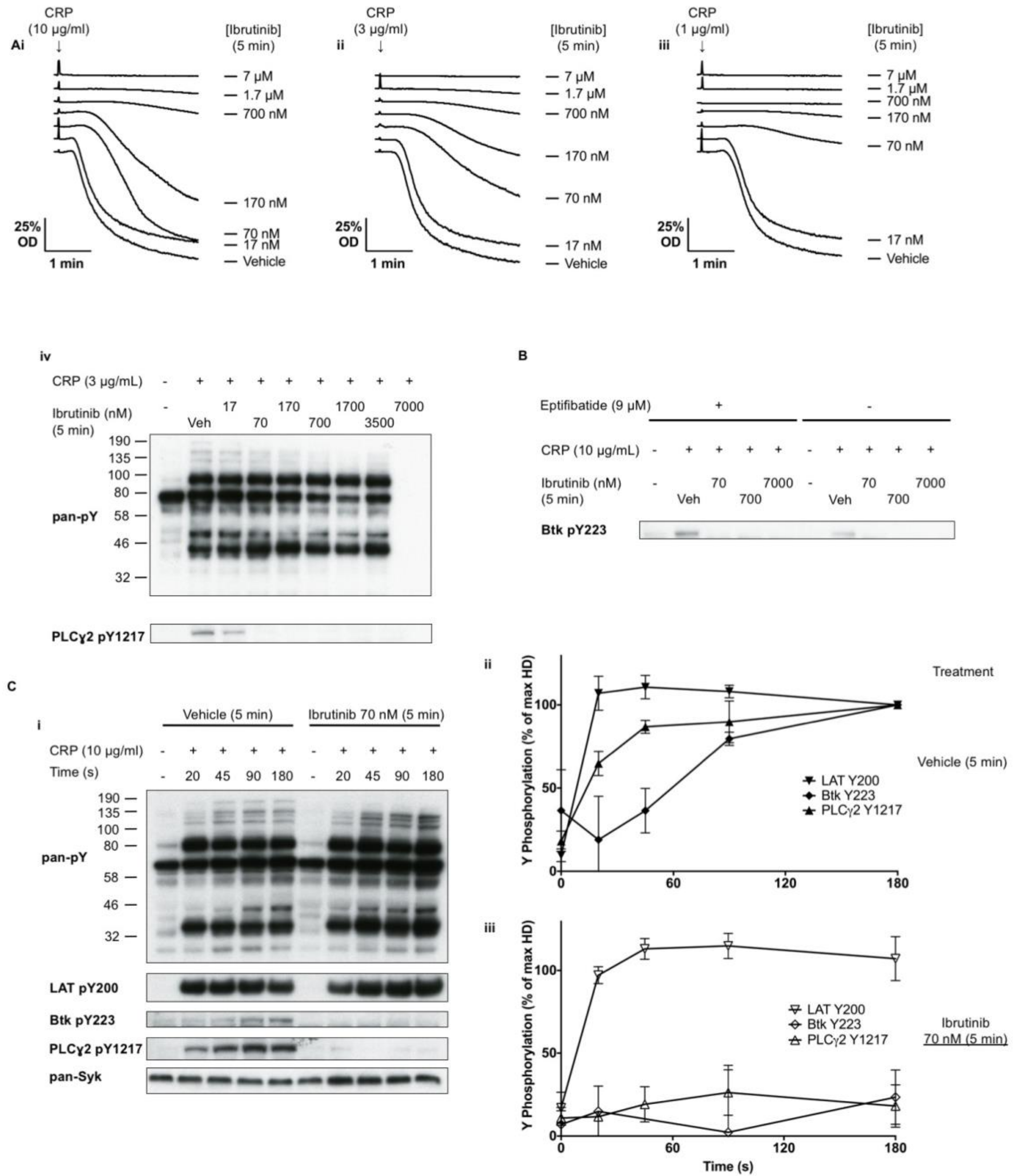
Cells were transfected in a volume of 0.4 mL of serum free RPMI using a Gene Pulser II Electroporator (Bio-Rad) set at 350 V and 500 µF. Cells were transfected with 2 µg of both GPVI and FcRɣ, and 7.5 µg of NFAT-luciferase plasmids and where stated, either 5 µg WT or KD Btk plasmids. Twenty hours after transfection, live cells were counted by Trypan blue exclusion and diluted to $2x10^6$/mL. Cells (50 µL) were stimulated with 50 µL Horm collagen (10 µg/mL final concentration) (Nycomed, Germany) for 6 hours. In experiments involving ibrutinib and acalabrutinib, cells were

stimulated with collagen (10 µg/mL final) in the presence of ibrutinib (0.5-10 µM), acalabrutinib (0.5-10 µM) or vehicle (0.2% DMSO) for 6 hours in the absence of serum as these drugs have a high degree of plasma binding{Honigberg:2010hh}. Cells were then lysed with 11 µL of lysis buffer (10% Triton X-100, 200 mM $NaPO_4$ (pH 7.8), and 4 mM dithiothreitol) and added to an equal volume of assay buffer (200 mM $NaPO_4$, 20 mM $MgCl_2$ and 10 mM ATP). Luciferase activity was measured using a NOVOstar plate reader (BMGLabtech) following the addition of 50 µL of 1 mM D-luciferin. Luciferase assay values were recorded in triplicate and averaged. The luciferase assay data were normalized to basal wild-type Btk values.

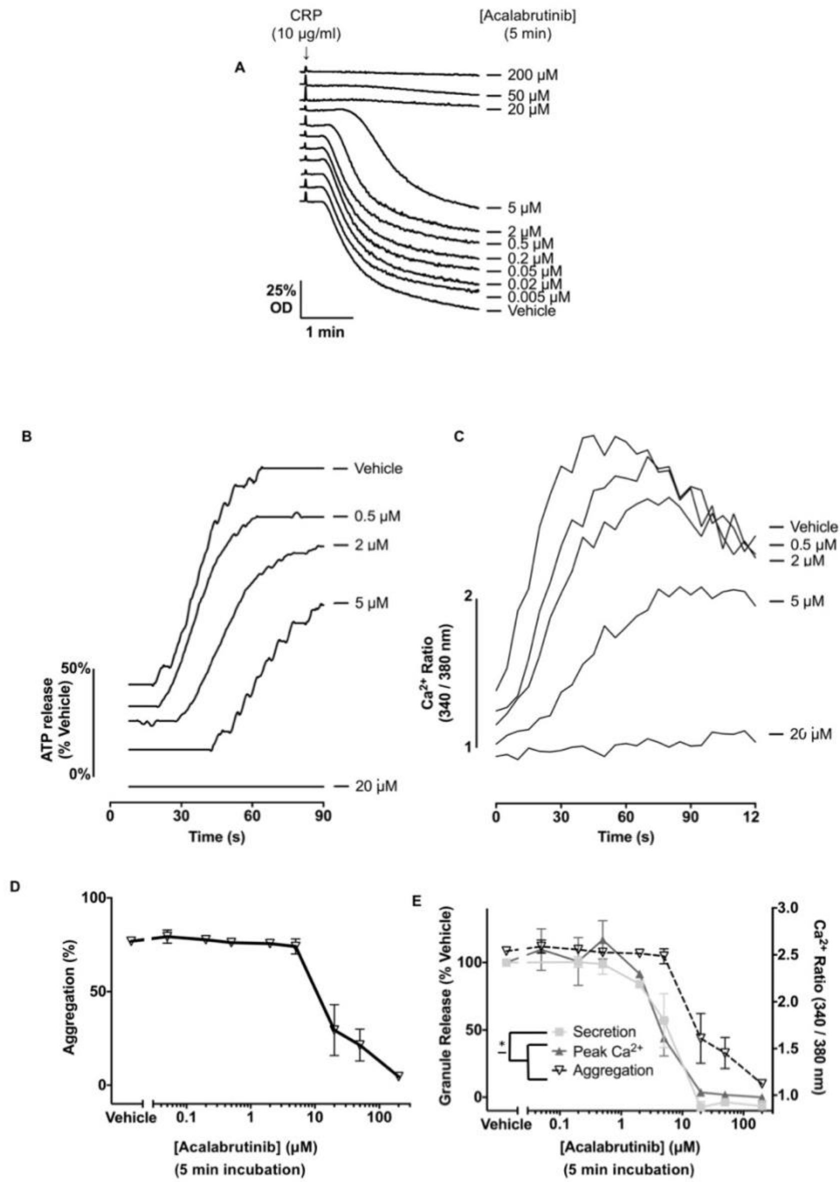
Figures



Supplementary Figure 2



Supplementary Figure 3



Supplementary Table 1. IC_{50} values for dose response curves (all numbers in μM)

Ibrutinib containing figures	**Name of Curve**	**IC_{50} (μM)**	**95% C.I.**
2Aiii	HD Aggregation	1.19	0.73 – 1.95
2B	HD Secretion	0.25	0.121 – 0.548
2B	HD Ca^{2+} Mobilisation	1.88	0.445 – 22.18
3Aii	HD Btk pY223	0.023	0.014 – 0.036
3Aii	HD PLCγ2 pY1217	0.035	0.019 – 0.065
3Aii	HD PLCγ2 pY759	0.048	0.026 – 0.088
3Aii	HD PLCγ2 pY753	0.055	0.029 – 0.105
3Aiii	HD Syk pY525/6	-	-
3Aiii	HD LAT pY200	-	-
3Aiii	HD SLP-76 pY145	-	-
3Aiii	HD Btk pY551	-	-
3Aiv	HD Src pY418	2.1	1.24 – 3.7
5Aiii	XLA Aggregation	0.086	0.037 – 0.212
5Aiv	XLA PLCγ2 pY1217	0.035	0.014 – 0.087
Acalabrutinib containing figures			
7Aii	HD Btk pY223	0.308	0.177 – 0.549
7Aii	HD PLCγ2 pY1217	0.88	0.393 – 2.07
7Aii	HD PLCγ2 pY759	1.211	0.479 – 3.244
7Aii	HD PLCγ2 pY753	2.75	1.20 – 6.59
7Aiii	HD Syk pY525/6	-	-
7Aiii	HD LAT pY200	-	-
7Aiii	HD SLP-76 pY145	-	-
7Aiii	HD Btk pY551	-	-
7Aiv	HD Src pY418	-	-
Supp Fig 3D	HD Aggregation	21.25	12.27 – 37.11
Supp Fig 3E	HD Secretion	6.37	3.706 – 11.02
Supp Fig 3E	HD Ca^{2+} Mobilisation	5.317	2.843 – 10.15

Supplementary Table 2. Btk mutations of XLA patients

Patient	Mutation	Predicted effect
1	c. 1750+1G>A	5′ donor site of exon 17 abolished
2	c. 700C>T	Stop codon at Gln234
3	c. 710del	Frameshift with premature termination
4	c. 1820C>A	Mutation likely to affect the kinase domain

Figure Legends

Supplementary Figure 1. Ibrutinib dose dependently inhibits GPVI-mediated aggregation, ATP secretion and Ca^{2+}.
(A) Healthy donor washed platelets (4x10^{8}/mL) we incubated with ibrutinib or vehicle (DMSO) at the stated doses for 5 min. These were then stimulated with CRP (10 µg/mL) or collagen (10 µg/mL) for 180 sec. (B) Washed platelets in the presence or absence of 0.3% BSA were incubated with ibrutinib or vehicle (DMSO) at the stated doses in the for the stated time before being stimulated with CRP (10 µg/mL). Results shown are representative of three independent experiments. * $p < 0.05$, ns = not significant. (C) Healthy donor washed platelets (4x10^{8}/mL) we incubated with ibrutinib or vehicle (DMSO) at the stated doses for 5 min in the presence or absence of 10 µM indomethacin. These were then stimulated with CRP (10 µg/mL) for 180 sec. Data shown are mean ± SEM of three identical experiments.

Supplementary Figure 2. Reducing the concentration of the CRP or the presence or absence of eptifibatide does not change the dose range over which ibrutinib has its effects or the concentration at which it blocks tyrosine phosphorylation at and downstream of Btk. (A) Healthy donor washed platelets (4x10^{8}/mL) were incubated with ibrutinib or vehicle (DMSO) at the stated doses for 5 min. Representative traces of stimulation with CRP at (i) 10 µg/mL, (ii) 3 µg/mL or (iii) 1 µg/mL for 180 sec are shown. Washed platelets (4×10^{8}/mL) were stimulated for 180 sec with (iv) CRP (3 µg/mL) in the presence of eptifibatide (9 µM) or (B) CRP (10 µg/mL) in the presence or absence of eptifibatide (9 µM) followed by lysis with 5X SDS reducing sample buffer. Prior to addition of agonist, platelets were pre-incubated with either ibrutinib or vehicle (DMSO) for 5 min. Whole cell lysates were then separated by SDS-PAGE and Western blot with the stated antibodies for whole cell phosphorylation and kinases downstream of GPVI. (C) Whole cell lysates were made at the time points shown after stimulation with CRP (10 µg/ml) in the presence or absence of low dose (70 nM) ibrutinib incubated for 5 min. These were then separated by SDS-PAGE and western blot was performed with the stated antibodies. Representative blot (i), quantification of band intensity relative to healthy donor at 180 sec without (ii) and with (iii) low dose ibrutinib (n=3). Aggregation traces and blots are representative of three similar experiments.

Supplementary Figure 3. Acalabrutinib dose dependently inhibits GPVI-mediated platelet aggregation, ATP secretion and Ca^{2+} mobilisation. (A) Representative traces showing effect of increasing doses of *in vitro* acalabrutinib incubated for 5 min in washed platelets for (A) aggregometry, (B) ATP secretion and (C) and Ca^{2+} mobilisation. (D) Acalabrutinib dose response curve for aggregation in washed platelets (n=3). (E) Acalabrutinib dose response curves in washed platelets with ATP secretion (n=3) and Ca^{2+} mobilisation (n=3). The dose response curve for inhibition of washed platelet aggregation from (D) is shown as a dotted line to enable comparison. Results are shown as mean ± SEM. All experiments were stimulated with CRP (10 µg/mL).