SUPPLEMENTARY APPENDIX

Low-dose Btk inhibitors selectively block platelet activation by CLEC-2

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Supplementary Material

Methods

Reagents

The mouse α-human CLEC-2 antibody AYP1 has been previously described[29]. α-LAT pAb (06-807) was from Merck Millipore (Burlingon, MA). Rhodocytin was isolated according a published protocol[30], Human podoplanin-Fc has been previously described[31]. The Btk-deficient DT40 cells, plasmid constructs and rabbit α-Btk antibody were a kind gift from Dr Mike Tomlinson (University of Birmingham, UK). FITC-conjugated anti-mouse P-selectin and PE-conjugated anti-mouse activated αIIbβ3 antibodies were from Emfret (Eibelstadt, Germany). Erythrocyte lysing agent was from Dako (Carpinteria, CA). All other reagents are previously described[16] or are from Sigma-Aldrich (Poole, UK).

Flow cytometry

Heparinised whole mouse blood was obtained by femoral vein sampling and was diluted using phosphate buffered saline (PBS) in the presence or absence of fluorophore conjugated antibody before being stimulated with agonist. This was then incubated with the stated antibodies for 30 minutes at room temperature in the dark. Following this, erythrocytes were lysed and the samples were fixed. Samples were then analysed using an Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA).

Flow adhesion studies

$DiOC_6$ labelled whole blood was flowed at 125 s^{-1} across podoplanin-Fc (100 µg/ml) coated micro-capillaries within Cellix Vena8 Fluoro+ Biochips (Cellix Limited, Dublin) as previously described[31].

Imaging

Visualisation of platelet flow adhesion was performed with a 20X objective on a Zeiss Axio Observer 7 microscope (Carl Zeiss AG, Oberkocken, Germany).

Image analysis

Flow adhesion videos were analysed using the KNIME 3.4 analytics platform (KNIME.com AG, Konstanz, Germany)[32]. Ilastik 1.1.2 machine learning software (University of Heidelberg, Germany) was used to automatically and reproducibly identify platelets[33].

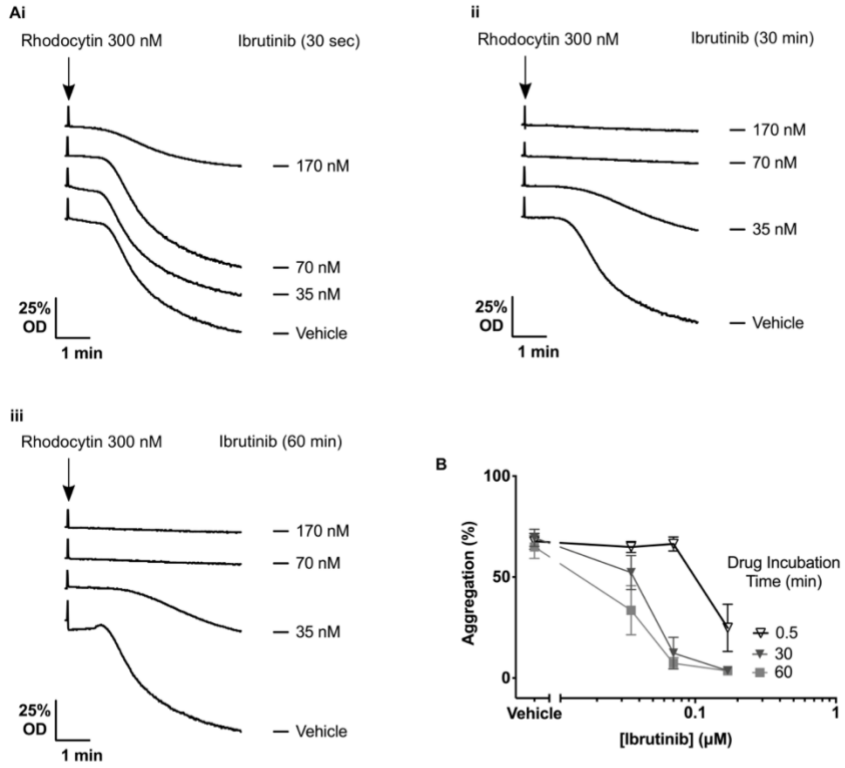
DT40 cell transfection and luciferase assay

DT40 cells were cultured and transfected with 10 µg of CLEC-2 plasmids as well as NFAT-luciferase and Btk plasmids as previously described[16]. Once transfected, cells were stimulated with rhodocytin 50 nM for 6 hours in the presence or absence of ibrutinib (0.5 – 10 µM), acalabrutinib (0.5 – 10 µM) or vehicle (DMSO) as stated. Luciferase activity was measured as previously described[16].

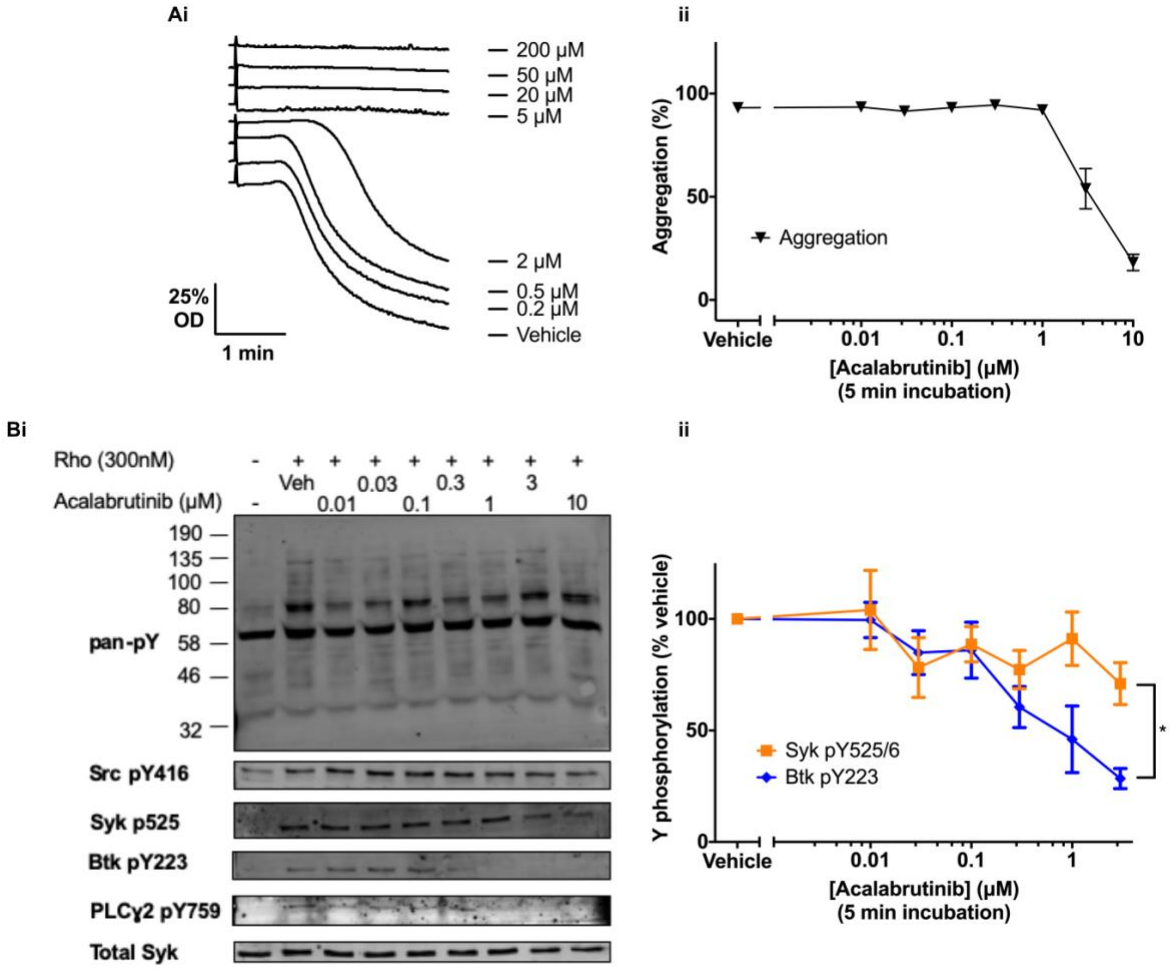
To confirm expression of CLEC-2, cells were pelleted and resuspended in 10 µg/mL AYP1. Cells were washed and resuspended in AF 488-conjugated goat α-mouse antibody before washing. Cells were resuspended and stained with propidium iodide before flow cytometry analysis using a BD Accuri C6 flow cytometer.

To examine Btk expression, cells were washed in PBS before being pelleted. Pellets were resuspended in 1X RIPA buffer. 6X sample buffer was added to the sample and boiled. Proteins were then separated by SDS -PAGE before western blotting for Btk.

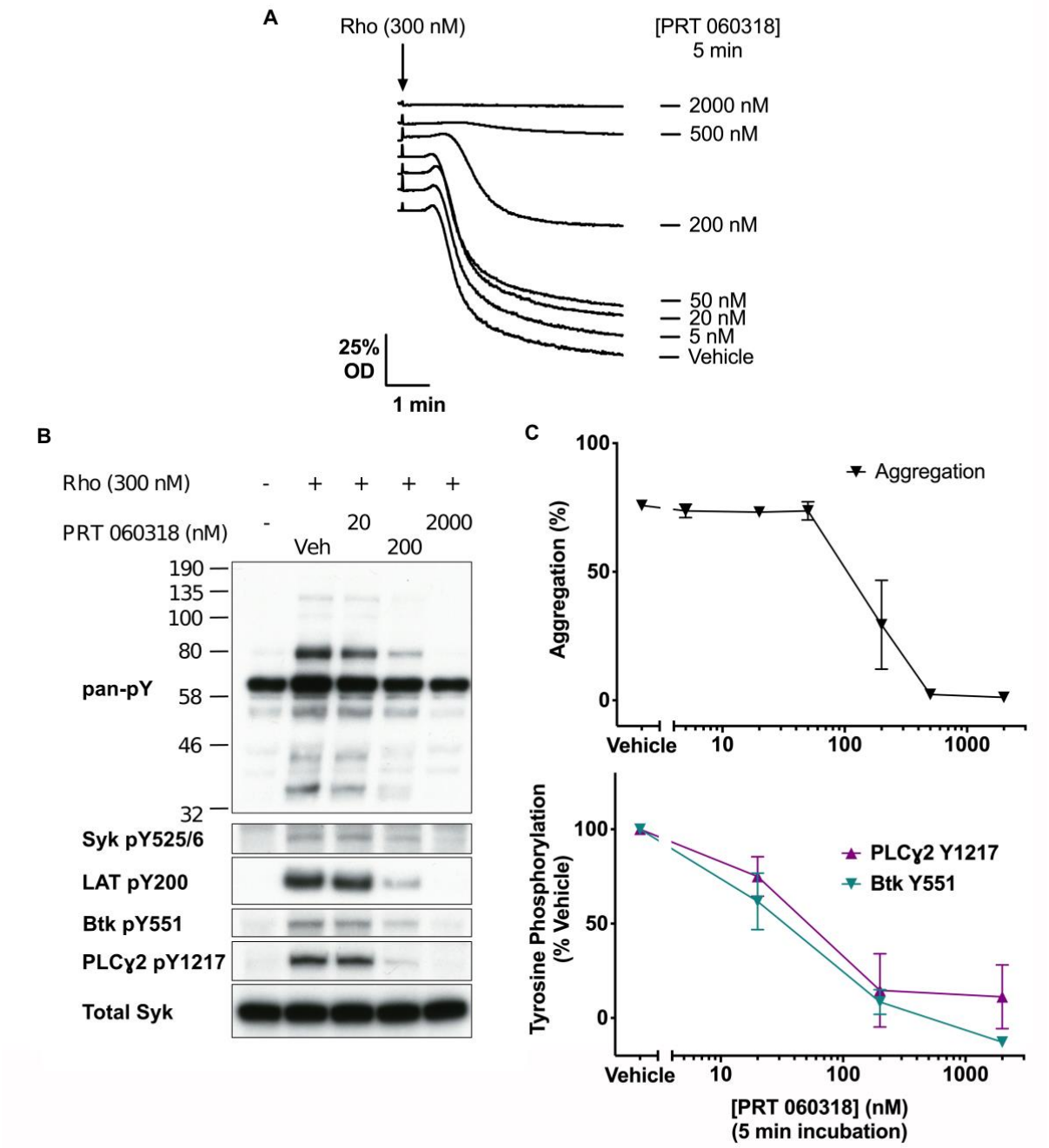
Supplementary Figure 1



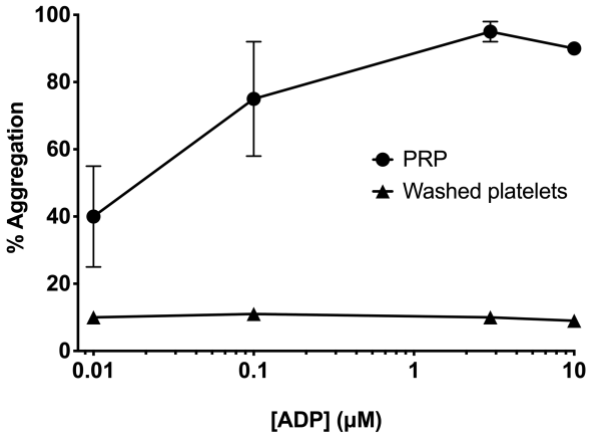
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 1: Ibrutinib blocks CLEC-2 mediated platelet aggregation in a time-dependent manner. Human healthy donor washed platelets at $4x10_8$/ml were incubated with ibrutinib or vehicle for 30 seconds – 60 minutes before being stimulated with rhodocytin 300 nM for 5 minutes. (A) Representative aggregation traces from one of the five identical experiments. (B) Mean data ± SEM (n=5) showing the effect of ibrutinib incubation time on CLEC-2-mediated platelet aggregation.

Supplementary Figure 2: Acalabrutinib blocks CLEC-2 mediated platelet aggregation and tyrosine phosphorylation in a dose dependent manner. (A) Healthy donor washed human platelets at $4x10_8$/ml were incubated with acalabrutinib or vehicle (DMSO) for 5 minutes prior to stimulation with 300 nM rhodocytin. LTA measurements were then undertaken for 5 minutes. (i) Representative trace. (ii) Mean data ± SEM are shown (n=3). (B) Healthy donor washed human platelets at $4x10_8$/ml in the presence of eptifibatide 9 µM were incubated with acalabrutinib or vehicle (DMSO) for 5 minutes prior to stimulation with 300 nM rhodocytin. Platelets were then lysed with 5X reducing sample buffer 5 minutes after addition of agonist. Whole cell lysates were then separated by SDS-PAGE and western blots were probed for whole cell phosphorylation or kinase phosphorylation with the stated antibodies downstream of the platelet CLEC-2 receptor. (i) Representative blot from 4 identical experiments. (ii) Mean tyrosine levels phosphorylation level ± SEM of 4 identical experiments for Syk pY525/6 and Btk pY223. Statistical analysis with two-way ANOVA with Sidak's correction for multiple comparisons. *P<0.05.

Supplementary Figure 3: The Syk inhibitor PRT 060318 blocks CLEC-2 mediated platelet aggregation and Btk Y551 phosphorylation. Healthy donor washed human platelets at $4x10_8$/ml were incubated with PRT 060318 or vehicle (DMSO) for 5 minutes prior to stimulation with 300 nM rhodocytin. LTA measurements were then undertaken for 5 minutes. (A) Representative trace and (C) mean data ± SEM are shown. (B&D) ADP-sensitive healthy donor washed platelets at $4x10_8$/ml were incubated, in the presence of eptifibatide (9 µM), with PRT 060318 or vehicle for 5 minutes prior to stimulation with 300 nM rhodocytin. Platelets were then lysed with 5X reducing sample buffer 5 minutes after addition of agonist. Whole cell lysates were then separated by SDS-PAGE and western blots were probed for whole cell phosphorylation or kinase phosphorylation with the stated antibodies downstream of the platelet CLEC-2 receptor. (B) Representative blot from 3 identical experiments. (D) Mean tyrosine levels phosphorylation level ± SEM (n=3) for Btk pY551 and PLCγ2 pY1217.

Supplementary Figure 4: Washed platelets do not aggregate in response to ADP. PRP and washed platelets ($4x10^8$mL) isolated from healthy donors were stimulated with 0.1 – 10 µM ADP and LTA measurements were then undertaken for 5 minutes. Dose response curves (n=3) are shown as mean ± SEM.