SUPPLEMENTARY APPENDIX

Antiplatelet properties of Pim kinase inhibition are mediated through disruption of thromboxane A2 receptor signaling

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 doi:10.3324/haematol.2019.223529

Received: April 1, 2019.

Accepted: May 27, 2020.

Pre-published: May 28, 2020.

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ONLINE DATA SUPPLEMENT

Anti-platelet properties of Pim kinase inhibition is mediated through disruption of thromboxane A2 receptor signalling.

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MATERIALS AND METHODS

Reagents

AZD1208, CX-6258, LGH447, SGI-1776 and SMI-4a were purchased from SelleckChem. U46619 was purchased from Enzo Life Sciences (Exeter, UK). Bovine thrombin and ADP were purchased from Sigma Aldrich (Poole, UK). Horm collagen was purchased from Nycomed, Austria, CRP-XL from Prof. R Farndale (University of Cambridge, UK). Primary anti- TxA_2R (P20), Pim-1 and actin (C11) antibodies were purchased from Santa Cruz Biotechnology (Calne, UK). Primary antibodies for Pim-1, Pim-2 and Pim-3 (cat. Log #9779), anti-Phospho–PKC substrate antibody (#2261), Caspase-3 (#9662), cleaved caspase 3 (#9661) and phospho-Ser19 Myosin light chain (#3671) antibodies were purchased from New England Biosciences (Cell Signalling Hitchin, UK), anti-phospho-Ser 4A4 antibody was purchased from Millipore (Watford, UK), anti-TP receptor (ab137607) and DOK6 (ab72730) antibodies were purchased from Abcam (Cambridge, UK). Fluorophore conjugated secondary antibodies, Fluo-4 calcium indicator dye and Alexa-488 conjugated phalloidin were purchased from Life Technologies (Paisley, UK). All other reagents were from previously described sources.

Mice

Global *Pim1*$^{-/-}$ mice were described previously [1,2]. Genotyping was confirmed by PCR of tail tissue DNA using primers: AAGCACGTGGAGAAGGACCG (WT forward), GACTGTGTCCTTGAGCAGCG (WT reverse; *Pim1*$^{+}$ 487bp), and CGTCCTGCAGTTCATTCAGG (Neo reverse; *Pim1*$^{-}$ ~360bp). Due to breeding strategy age and litter matched Pim-1$^{+/-}$ mice were used as controls. Pim-1$^{+/-}$ mice were found to show no difference in platelet function to C57BL/6 WT mice (data not shown). Mice used were C57BL/6 of both sexes, aged 7-10 weeks, and were treated according to the National Institutes of Health and Medical College of Wisconsin Institutional Animal Care and Use Committee guidelines and following procedures approved by the University of Reading Research Ethics Committee.

Platelet preparation

For mouse experiments, blood was collected by mouse retro-orbital plexus bleeding and was anticoagulated in acid-citrate-dextrose [3]. Platelet-rich plasma was obtained by centrifugation of the blood at 100 g for 8 min, followed by centrifugation of the supernatant and buffy coat at 100 g for 6 min. After washing twice in washing buffer (140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, and 12.5 mM sucrose, pH 6.0), platelets were resuspended at 4 x 10^8 platelets/ml in resuspension buffer (140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 5 mM $NaHCO_3$, 10 mM glucose, 10 mM HEPES, pH 7.4) and were allowed to rest for 30 min before use.

For human experiments, blood was obtained from consenting aspirin-free healthy volunteers following procedures approved by the University of Reading Research Ethics Committee.

Blood was collected into 4% (w/v) sodium citrate and then mixed with acid citrate dextrose (29.9 mM trisodium citrate, 113.8 mM glucose and 2.9 mM citric acid [pH 6.4]) if washed platelets were prepared. Platelet rich plasma (PRP) was prepared by centrifugation at 100 g for 20 minutes at room temperature. ADP sensitive washed platelets were prepared from PRP (containing ACD) by centrifugation at 350 g for 20 minutes followed by resuspension in modified Tyrode's-HEPES buffer (134mM NaCl, 0.34mM Na_2HPO_4, 2.9mM KCl, 12mM $NaHCO_3$, 20mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 5mM glucose and 1mM $MgCl_2$, pH 7.3) and used immediately.

Thrombus formation on collagen under flow *in vitro*

Thrombus formation on and platelet interaction with immobilized type I collagen was performed as described previously [4] using the VenaFlux Platform and Vena8Fluor+ Biochips (Cellix) [5]. Channels were coated overnight at 4°C with collagen, then blocked with 1% BSA/PBS for 1 h and replaced with PBS. Mouse blood was collected by retro-orbital plexus bleeding and was anticoagulated in 25 mM PPACK. Human blood was collected into sodium citrate (4% w/v). Platelets in whole blood were incubated with 10 mM DiOC6 (Thermo Fischer Scientific) for 10 min. Perfusion was performed for either 4 min (mouse) or 12 minutes (human) at 37°C with an arterial shear rate of 1500 s^{-1}. For mouse, platelet adhesion to the collagen-coated chip was monitored with an Axioscope A.1 Inverted Microscope (Carl Zeiss) at 20x magnification through a Digital CCD Camera C10600-10B (Hamamatsu Photonics). Human thrombus formation was visualised using a 20 x magnification lens on a Nikon A1-R confocal microscope. Images were brightness corrected and converted to a binary mask with software provided auto-thresholds. Binary Mask Particles were then analysed to determine thrombus count and surface area.

$FeCl_3$ injury thrombosis model

Male C57BL/6 mice (8 – 10 weeks of age) were anesthetized through an intraperitoneal. injection of ketamine (100 mg/kg), metedor (20 mg/kg) and atropine (0.25 mg/kg) and injected with DIOC6 to fluorescently label platelets (0.28 µL of a 1mg/mL solution per body weight). AZD1208 (0.16 µL of a 100 mM solution per body weight) or vehicle control were injected immediately before isolation of mesenteric arteries. Vascular injury was induced by applying a 1 mm^2 Whatmann filter paper saturated with 10% $FeCl_3$ for 90 seconds and images recorded for at least 20 minutes. Images were acquired using a Hamamatsu digital camera C9300 (Hamamatsu Photonics UK Ltd) and Olympus BX61W1 microscope (Olympus Imaging Ltd, UK) and analysed using Slidebook5 (Intelligent Imaging Innovations, USA).

Tail bleeding

Bleeding time in Pim-$1^{-/-}$ and Pim-$1^{+/-}$ mice was determined by transecting 2 mm of distal mouse tail and immediately immersing the tail in 37°C isotonic saline [3]. A complete cessation of bleeding was defined as the bleeding time. Measurements exceeding 10 min were stopped by cauterization of the tail.

Tail bleeding experiments with Pim kinase inhibitors were performed on 20–35 g male mice, anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) injected intraperitoneally. AZD1208 (100 µM) or vehicle control (DMSO 0.1% v/v) was injected into the femoral vein 10 minutes prior to removal of the tip of the tail. The tail tip was then placed in sterile saline (37 ºC) and time to cessation of bleeding (secs) measured.

Platelet aggregation

Aggregation of human washed platelets was measured by optical aggregometry (Helena Biosciences, Gateshead, UK) as described previously [6]. Endpoint aggregation measurements were also taken using a plate based assay in which PRP or washed platelets at a concentration of $4x10^8$ cells/ml were loaded onto 96-well half-area plates (Greiner) and treated with inhibitors or vehicle for 10 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) and absorption of 405 nm light measured using a NOVOstar plate reader (BMG Labtech).

Alpha granule secretion and fibrinogen binding by flow cytometry

Flow cytometry was used to examine alpha granule secretion and affinity up-regulation of the integrin $\alpha_{IIb}\beta_3$ by detecting levels of P-selectin exposure and fibrinogen binding respectively . Following inhibitor or vehicle treatment for 10 minutes and stimulation with U46619, platelets were incubated at room temperature for 20 minutes with PE/Cy5 anti-human CD62P (P-selectin) and fluorescein isothiocyanate-labelled (FITC) anti-fibrinogen antibody. Reactions were stopped and platelets fixed by addition of 0.2% (v/v) paraformaldehyde. Data for 5000 events were collected using a BD Accuri C6 flow cytometer and analysed using the CFlow Sampler software as described previously [4].

TxB_2 generation assay

PRP was stimulated with either 3 µg/mL Collagen or 100 µM Arachidonic acid, both in the presence and absence of 100 µM AZD1208 or indomethacin (10 µM) at 37 °C with shaking at 1200 rpm on a plate shaker. The reaction was stopped after 5 min by the addition of 50 µM indomethacin and 2 mM EDTA, and the cell debris removed by centrifugation. Samples were diluted 1/100 in assay buffer provided prior to testing. The TxB_2 assay was performed using an assay kit (Enzo Life Sciences (UK) Ltd, Exeter UK) according to the manufacturer's specification and the 96-well ELISA plate read on a FlexStation microplate reader (Molecular Devices). Data analysis was subsequently conducted using a standard curve constructed from a serial dilution of working standard samples to calculate TxB_2 production.

Mobilisation of intracellular calcium

PRP was loaded with Fura-2 AM (2 µM) for 1h at 30°C and then washed by centrifugation at 350 g for 20 mins and resuspended in Tyrode's-HEPES buffer containing 0.4 U/ml apyrase. Fura-2 loaded platelets were incubated with inhibitors or vehicle 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 and the method of Grynkiewicz *et al* was utilised [7]. The maximum fluorescence ratio was measured following treatment with 50 µM digitonin and the minimum fluorescence ratio measured following treatment with 10 mM EGTA. Autofluorescence was also corrected for, using platelets which had not been loaded with Fura-2. $[Ca^{2+}]_i$ concentrations were calculated as described previously [8,9].

Immunoblotting and immunoprecipitation

Washed platelets (4×10^8 cells/mL) were lysed in an equal volume of NP40 buffer (300 mM NaCl, 20 mM Tris base, 2 mM EGTA, 2 mM EDTA, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 0.7 µg/ml pepstatin A, 2 mM sodium orthovanadate, 2% NP-40, pH 7.3) for lysate

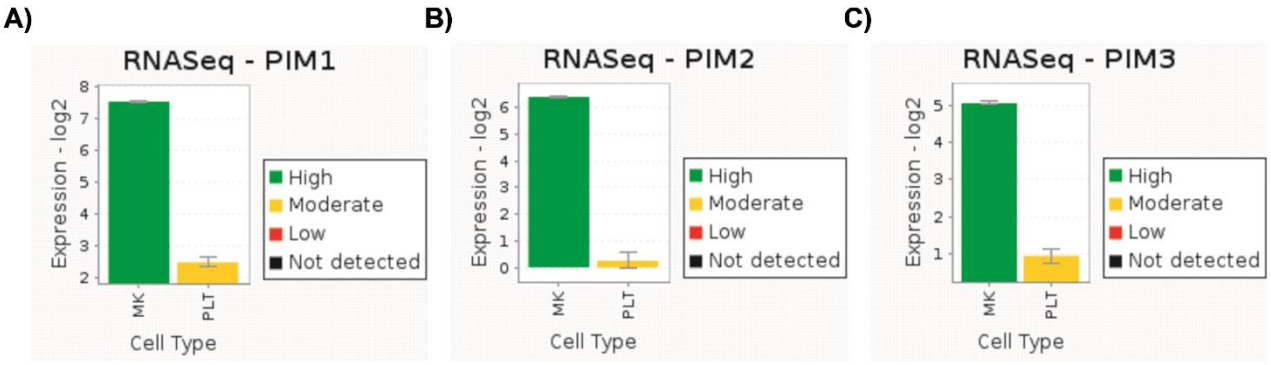
preparation for immunoprecipitation or lysed with 6 x Laemlli sample buffer for directanalysis by western blotting. For immunoprecipitation proteins of interest were isolated using 1 µg of appropriate antibodies as described previously [6]. Immunoblotting was performed using standard techniques as described previously [10].
Proteins were detected using fluorophore conjugated secondary antibodies and visualised using a Typhoon Fluorimager and Image Quant software (GE Healthcare). Band intensities were quantified and levels of the immunoprecipitated protein were used to normalize the phosphorylation data using Image Quant software.

Surface expression of TPαR by flow cytometry
Flow cytometry was used to determine surface expression levels of the TP receptor. Following inhibitor or vehicle treatment for 10 minutes and stimulation by U46619. Platelets were incubated at room temperature for 20 minutes with anti-human TP receptor (Abcam) that recognises the extracellular portion of the TP receptor and Alexa647 conjugated anti-rabbit secondary antibody. Anti-DOK6 antibody (Abcam) was used as a negative control. Reactions were stopped by dilution in HEPES- buffered saline and analysed immediately. Data for 10,000 events were acquired using a BD Accuri C6 flow cytometer and analysed using the CFlow Sampler software.

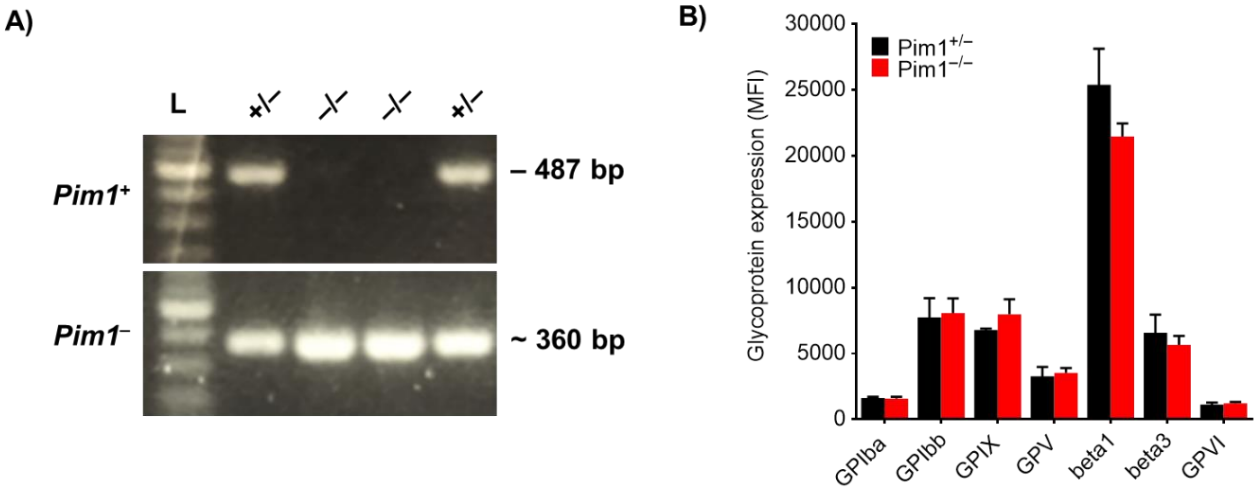
Statistics
All experiments were performed at least in triplicate. Statistical analyses of the data were carried out using GraphPad prism software. When comparing two sets of data, an unpaired, 2-tailed Student's t test (simple) statistical analysis was used. If more than two means were present, significance was determined by one-way or two-way ANOVA followed by Bonferroni correction (multiple) or the Kaplan-Meier analysis (time-to-event). Where data was normalised, statistical analysis was performed prior to normalisation and also using the non-parametric Wilcoxon signed-rank test. $P \leq 0.05$ was considered statistically significant. Unless stated otherwise, values were expressed as mean ±SEM.

Study approval
For experiments using human blood, procedures and experiments were approved by the University of Reading and Manchester Metropolitan University Research Ethics Committees. Mice used were treated according to the National Institutes of Health and Medical College of Wisconsin Institutional Animal Care and Use Committee guidelines and as following procedures approved by the University of Reading Research Ethics Committee.

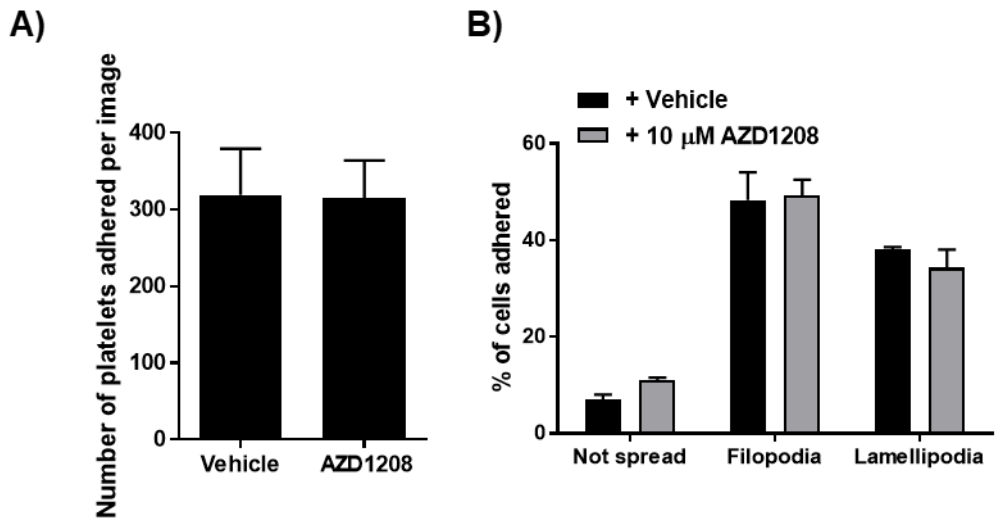
SUPPLEMENTAL DATA



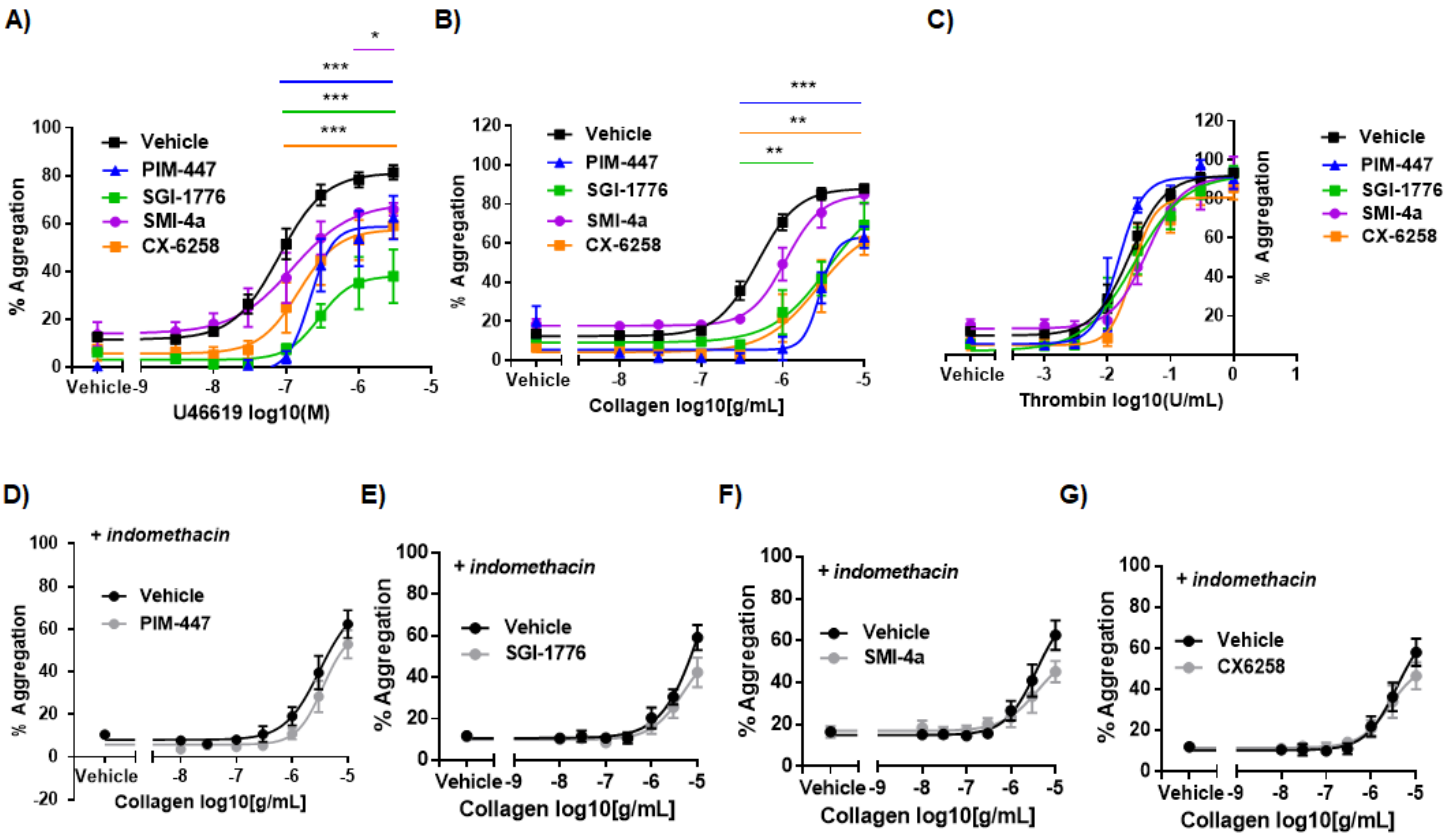
Supplemental Figure 1. Expression of Pim kinase in human megakaryocytes and platelets. RNA seq analysis of A) Pim-1, B) Pim-2 and C) Pim-3 mRNA-seq expression in megakaryocytes and platelets from 5 different data sets, taken from the HAEMGEN Tools portal Expressed! https://haemgen.haem.cam.ac.uk/expressed



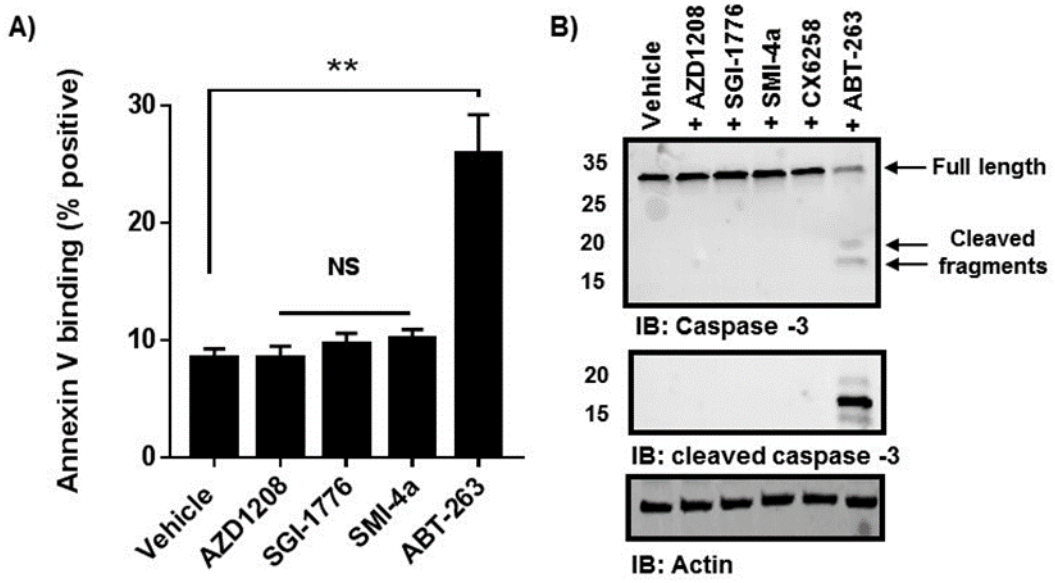
Supplemental Figure 2. Pim-1$^{-/-}$ deficient platelets have normal receptor expression levels. A) Global *Pim1*$^{-/-}$ mice genotyping was confirmed by PCR of tail tissue DNA using primers: AAGCACGTGGAGAAGGACCG (WT forward), GACTGTGTCCTTGAGCAGCG (WT reverse; *Pim1*$^{+}$ 487bp), and CGTCCTGCAGTTCATTCAGG (Neo reverse; *Pim1*$^{-}$ ~360bp). B) Expression levels of different platelet surface receptors, GPIba, GPIbb, GPIX, GPV, Beta1, Beta3 and GPVI were determined by flow cytometry in platelets from Pim-1$^{+/-}$ and Pim-1$^{-/-}$ mice. Results are mean ± S.E.M. for n≥3, * indicates $p<0.05$ in comparison to controls.



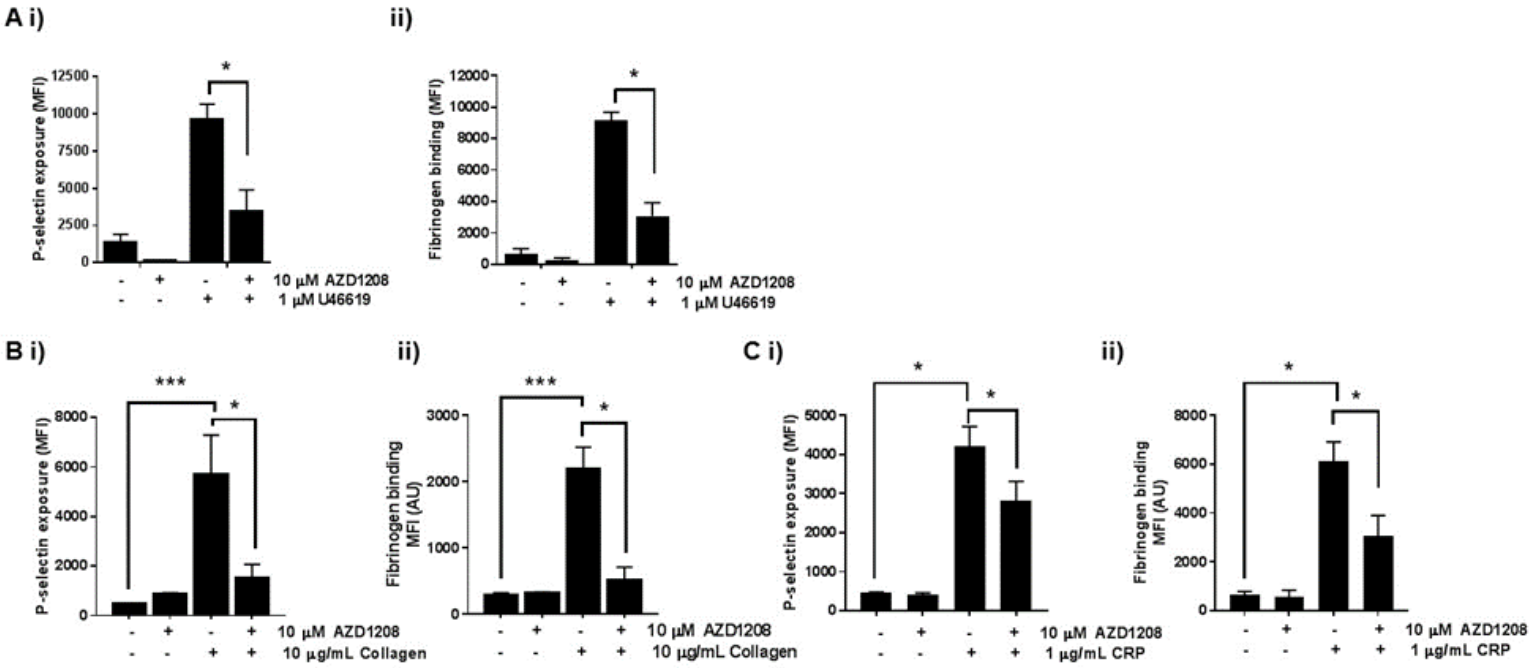
Supplemental Figure 3. Pim kinase inhibition does not alter adhesion to collagen. . Human washed platelets pretreated with 10 µM AZD1208 or vehicle control were exposed to collagen (100 µg/mL) coated coverslips and left to adhere and spread for 1 hour at room temperature. A) Adhesion, number of platelets adhered were counted in 5 randomly selected fields of view and expressed as the average number of cells per image. B) Spreading, platelets were classified into 3 different categories to determine the extent of their spreading (Adhered but not spread, Filopodia: platelets in the process of extending filopodia and Lamellipodia: platelets in the process of extending lamellipodia including those fully spread). Results expressed as relative frequency, as a percentage of the total number of platelets adhered. n≥3.



Supplemental Figure 4. Structurally distinct Pim kinase inhibitors attenuate platelet activation by the TP receptor. (A-C) Human washed platelets were pre-treated with different PIM kinase inhibitors PIM-447 (LGH-447) (10 µM) (blue) SGI-1776 (10 µM) (green), SMI-4a (30 µM) (purple), CX-6258 (10 µM) (orange) or vehicle control (black) prior to stimulation with i) U46619 (3 nM- 3µM), ii) collagen (0.01-10 µg/mL) or iii) Thrombin (0.01-1 U/mL). (D-G) Human washed platelets were treated with A) 10 µM PIM447 (LGH447), B) 10 µM SGI-1776, C) 30 µM SMI-4a or D) 10 µM CX6258 (grey) in the presence of indomethacin prior to stimulation y collagen (0.01-10 µg/mL) and aggregation was monitored after 5 minutes using an optical light transmission plate based aggregometry assay, quantified data shown. Results are mean + S.E.M. for n≥3, * indicates $p<0.05$ in comparison to controls.



Supplemental Figure 5. AZD1208 does not cause apoptosis in platelets. Human washed platelets were treated for 2 hours with or without A) AZD1208 (10μM), SGI-1776 (10μM), SMI-4a (30μM), ABT-263 (10 μM) or vehicle control prior to analysis by flow cytometry for Annexin V binding which is a measure of phosphatidylserine exposure, a marker of apoptosis. Data are expressed as % positive events for Annexin V. B) Platelets were incubated with AZD1208 (10μM), SGI-1776 (10μM), SMI-4a (30μM) or CX6258 (10μM) for 2 hours and lysed in Laemmli sample buffer. Samples were then run on SDS PAGE gels, transferred to PVDF membrane before blotting for caspase-3 or cleaved caspase-3 (a marker of apoptosis). Actin was included as a loading control. ABT-263 (10 μM) a Bcl-2 inhibitor and known initiator of apoptosis in platelets was included as a positive control. Results are mean ± S.E.M. for n≥3, * indicates $p<0.05$ in comparison to vehicle controls.



Supplemental Figure 6. AZD1208 attenuates alpha granule secretion and integrin αIIbβ3 activation. Resting and stimulated human washed platelets were treated with 10 µM AZD1208 prior to stimulation with A) U46619 (1 µM), B) Collagen (10 µg/mL) or C) CRP (1 µg/mL) and i) Alpha granule secretion determined by monitoring P-selectin exposure and ii) Integrin activation measured as fibrinogen binding. Results are mean + S.E.M. for n≥3, * indicates $p<0.05$ in comparison to vehicle controls.