Materials
ABT-737 and ABT-263 were purchased from Selleck Chemicals LLC (Houston TX, USA; distributed by Life Research Pty Ltd, Victoria, Australia). DiIC\textsubscript{12} was purchased from BD Biosciences (NSW, Australia). Alexa Fluor 488 was from Invitrogen Australia Pty Ltd (Mt Waverley, VIC, Australia). All other reagents were from sources previously described\textsuperscript{1-4}.

Antibodies
FITC-labeled PAC-1 monoclonal antibody (which recognises activated integrin $\alpha_{\text{IIb}\beta3}$), FITC-conjugated rat anti-mouse P-selectin antibody (CD62-P) and PE-conjugated mouse anti-human CD41$\alpha$ were purchased from BD Biosciences (NSW, Australia). Anti-ROCK1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GPIb$\alpha$ CD42b-PE was from Beckman Coulter. The anti-GPIb$\alpha$ monoclonal antibody WM23\textsuperscript{5}, anti-GPIb$\alpha$ polyclonal anti-glycocalcicin antibody\textsuperscript{6}, phycoerythrin (PE)-conjugated anti-GPVI 1G5, and anti-GPVI antibody (1A12) we have described previously\textsuperscript{7,8}. Anti-GPIb$\beta$ monoclonal antibody (RAM.1) was a generous gift from Dr Francois Lanza (INSERM, Strasbourg, France). Fluorescently-conjugated anti-GPIb$\alpha$ (Xia.G5), anti-GPIb$\beta$ (Xia.C3), anti-GPVI (JAQ-1) and activated integrin $\alpha_{\text{IIb}\beta3}$ (JON/A) antibodies were purchased from EMFRET Analytics GmbH & Co.KG (Germany).

Collection of blood and preparation of PRP and washed platelets
All procedures involving collection of human and mouse blood were approved by the Monash University Standing Committee on Ethics in Research involving Humans (Project number CF07/0125 – 2007/0005), and the AMREP AEC (Standard Operating Procedure 19 – collection of whole blood from mice), respectively. Isolation of human platelet-rich plasma (PRP), and preparation of washed platelets were performed essentially as we have described previously\textsuperscript{4,9}.

Measurement of integrin $\alpha_{\text{IIb}\beta3}$ activation and GPIb/V/IX and GPVI surface levels
Washed platelet studies – In washed human platelets ($3 \times 10^8$/ml), integrin $\alpha_{\text{IIb}\beta3}$ activation was assessed by measurement of binding of OG-FGN or PAC-1 binding, as described
previously\textsuperscript{1}. GPIb\textalpha, GPIb\textbeta, GPIX and GPVI expression was quantified using an anti-GPIb\textalpha monoclonal antibody (WM23)\textsuperscript{5}, an anti-GPIb\textbeta monoclonal antibody (RAM.1), an anti-GPIX monoclonal antibody (FMC25) and an anti-GPVI antibody (12H1) respectively.

**Whole blood studies** - GPIb\textalpha, GPIb\textbeta and GPVI expression on mouse platelets in whole blood was quantified using Xia.G5, Xia.C3 and JAQ-1, respectively. In human whole blood, GPIb\textalpha, GPIib\textIIIa and GPVI expression on platelets was quantified using CD42b-PE, PE-mouse anti-human CD41\textalpha and PE-anti GPVI 1G5, respectively. P-selectin expression and integrin \(\alpha_{\mu}\beta_3\) activation were quantified in response to CRP (10 \(\mu\)g/ml) and PAR4 peptide (300 \(\mu\)M), using FITC-CD62-P and FITC-JON/A, respectively. Antibody binding was measured by flow cytometry in a FACScaliber (Becton Dickinson, San Jose, CA, USA) and analysed using CellQuest and FLOWJO software as previously described\textsuperscript{16}.

**Measurement of PS exposure**

Surface exposure of PS in either washed human platelets or mouse whole blood was assessed by measuring the binding of Alexa488-labeled Annexin V, and quantified by flow cytometry as described previously\textsuperscript{4}.

**SDS-PAGE and western blot analysis**

For analysis of cleavage of platelet glycoproteins, platelet suspensions treated with various reagents were mixed with 10 mM (final concentration) ethylenediaminetetraacetic acid (EDTA), then lysed in SDS-sample loading buffer in the presence (reducing conditions) or absence (non-reducing conditions) of \(\beta\)-mercaptoethanol and heated to 100°C for 5 min. Equal volumes of platelet lysates were subjected to SDS-PAGE on either standard 7.5\% (v/v) polyacrylamide gels (for detection of ROCK1) or 5-20\% (v/v) acrylamide gradient gels for detection of GPIb\textalpha and GPVI protein and fragments using anti-GPIb\textalpha− or anti-GPVI cytoplasmic tail antibodies\textsuperscript{10} Separated proteins were analysed by western blot, as described previously\textsuperscript{9}.

**Scanning electron microscopy**

Washed platelets were fixed in suspension with paraformaldehyde (2\% final, w/v), applied to 3–aminopropyltriethoxysilane treated glass coverslips, and processed for scanning electron microscopy (SEM) as described previously\textsuperscript{3} with minor modifications. Briefly, adherent
platelets were incubated with 1% osmium tetroxide (30 min), rinsed in MQ water and dehydrated by successive immersions in increasing concentrations of ethanol. Samples were then dried in increasing concentrations of hexamethyldisilazane (HMDS), mounted onto stubs and coated with gold, prior to imaging on a Hitachi SS70 electron microscope (accelerating voltage 20 kV, working distance 6 mm). Morphological change was quantified and expressed as the percentage of platelets per field demonstrating loss of discoid shape. All images are taken from one experiment representative of at least 3 independent experiments, with histograms depicting the mean ± SEM (n=3).

**Platelet aggregation studies**

For aggregation studies, washed human platelets (3.0 x 10⁸/ml) in Tyrode’s buffer were preincubated (10 min) with vehicle alone, Q-VD-Oph (50 µM), calpeptin (100 µg/ml) or GM6001 (100 µM) prior to treatment with ABT-737 (1 µM) for up to 180 min. All aggregation studies were initiated by addition of the indicated concentrations of adenosine diphosphate (ADP), thrombin or CRP to platelet suspensions stirred at 800 rpm for 10 mins at 37°C in a four-channel automated platelet analyser (AggRAM, Helena Laboratories, Tyne and Wear, UK) in the presence of 1 mM calcium and 0.5 mg/ml fibrinogen. The extent of platelet aggregation was defined as the percentage change in optical density as measured by the automated platelet analyser.

**Thrombus formation in vitro under flow**

Flow-based platelet adhesion and thrombus formation assays were performed at 37°C as described previously.² Briefly, hirudin-anticoagulated (800 U/ml Lepirudin) human whole blood, or washed platelets reconstituted with 50% (v/v) red blood cells, were perfused through microcapillary tubes coated with fibrillar type I collagen (2.0 mg/ml) at 1800 s⁻¹. Thrombus formation was observed using an inverted Leica DMIRB microscope (Leica Microsystems, Wetzlar, Germany) with a 63X water objective (1.2 numeric aperture), and recorded in real-time using a Dage-MTI charge-coupled device (CCD) camera 300 ETRCX (Dage-MTI, Michigan City, IN).

**Quantification of platelet recruitment to thrombi**

Quantification of vehicle or ABT-737-treated platelet recruitment to thrombi was performed by ‘spiking’ whole blood with 7% (v/v) DilC₁₂-labeled platelets prior to perfusion, as
described previously. Spiked whole blood was then perfused over collagen matrices as described above, and DIC/fluorescence images were recorded in real-time as described above, for off-line analysis.

**Serotonin release**
Dense granule release was measured in $^{14}$C-serotonin labeled platelets, as previously described.

**Tail bleeding studies**
Haemostasis was measured in anesthetized mice (sodium pentobarbitone 60 mg/kg i.p.) administered either vehicle alone, or the indicated doses of ABT-263 or ABT-737, and assessed using two different methods.

1) **Template tail bleeding method:** Incisions 5 mm long and 1 mm deep were made in the tail using a template device and bleeding was monitored by carefully blotting with filter paper every 30 sec, until which time no more blood was absorbed (= tail bleeding time). During this time, care was taken not to directly touch the cut area of the tail.

2) **Tail transaction method:** Tail transection was also performed according to a modified method of Dejana et al. The mouse tail was transected at 3 mm from the tip and immediately immersed into warmed (37 °C) saline. The bleeding time was determined as the time from the tail transection to the moment the blood flow stopped for more than 120 s. A bleeding time beyond 30 min was considered as the cut-off time for the purpose of statistical analysis. Red blood cells were pelleted and lysed in 1 ml H$_2$O. Haemoglobin was quantified by absorbance at 575 nm (Beckman DU®530 Life Science UV/Vis Spectrophotometer).

**Analysis of rebleeding events:** Following cessation of bleeding, the incision was monitored for a further 120 s, and further bleeding within this period classified as ‘re-bleeding’. The duration of rebleeding was monitored as described above.

**Quantification of thrombin generation**
Thrombin generation *in vivo* was measured in anesthetized mice (sodium pentobarbitone 60 mg/kg i.p.) administered either vehicle alone, or the indicated doses of ABT-263 or ABT-737. Hirudinated whole blood was collected via the inferior vena cava, and PPP collected via centrifugation at 3000 x g. Thrombin generation was measured through quantification of
thrombin-antithrombin complex using Enzygnost® TAT micro kit (Dade Behring) according to the manufacturer’s instructions.
REFERENCES


Figure S1. ABT-737 induces morphological changes associated with platelet apoptosis, independent of platelet activation. (A,B) Washed human platelets (3.0 x 10^8/ml) were resuspended in Tyrodes buffer in the presence of BSA (5 mg/ml), then incubated with vehicle (DMSO) or ABT-737 (1 µM) for the indicated times. (B) In some experiments, platelets were preincubated with Q-VD-Oph (50 µM) or Y27632 (30 µM) prior to treatment with ABT-737. At the indicated time point platelets were either fixed in suspension with paraformaldehyde (2% final) and processed for SEM. These images, depict a lower magnification of the images in Figure 1, and demonstrate morphology of a platelet population in one field of view. Images are taken from 1 experiment representative of 3 independent experiments. (C) Washed platelets resuspended in Platelet Washing Buffer (pH 6.5, containing 10 mM Theophylline and 5 mg/ml BSA) were preincubated with inhibitors of platelet activation [inhibitors: apyrase (0.02 U/ml), MRS-187 (100 µM), 2MeSAMP (10 µM) and indomethacin (10 µM)], prior to treatment with ABT-737 (1 µM). At the indicated time points, platelets were imaged by DIC and shape changed quantified (expressed as percent shape change per field), where histogram depicts the mean ± SEM (n=4).
Figure S2. Effect of ABT-737 on platelet glycoprotein receptor expression and platelet activation potential in residual circulating platelets in vivo. C57BL6 mice were administered ABT-737 (75 mg/kg) (open bars) or an equal volume of DMSO (filled bars) i.p, and whole blood sampled over a 24 hour time period, as described under 'Methods'. Whole blood was analyzed for (A) platelet count, (B) PS exposure, (C) GPIbα and (D) GPVI surface expression. (E) Tail bleeding time was determined using a template bleeding method, as described under 'Supplementary Methods'. (F) Post-transfusion recovery of ABT-737 treated mouse platelets - Washed platelets were labeled with CMFDA followed by pretreatment with vehicle (untreated) or ABT-737 (0.1 - 1.0 μM). Recipient mice were intravenously injected with 10⁶ CMFDA labeled platelets (arrow) and peripheral blood sampled and analysed as described under ‘Methods’, at the indicate time-points. Results depict the mean ± SEM of 4-6 mice per treatment group.
Figure S3. Effect of ABT-263 on washed platelet PS expression in vitro and in vivo. (A,B) Washed mouse platelets (1.0 x 10⁸/ml) were resuspended in Tyrodes buffer, and incubated with vehicle ABT-263 (500 nM; closed circles) or ABT-737 (1 µM; closed square) for the indicated time periods, or with ionophore A23187 (1 µM, 30 min, open circle). PS surface exposure was quantified by flow cytometry, as described under ‘Methods’. These results depict the mean ± SEM of 3 independent experiments. The FACS profile is taken from 1 experiment representative of 4 independent experiments. (C) C57BL6 mice were administered ABT-263 (25 mg/kg, red) or an equal volume of vehicle (black) by oral gavage, and whole blood sampled at 2 hours, as described under “Methods”. The effect of ABT-263 administration on exposure of PS was assessed as described under “Methods” and in Figure 5B. This FACS profile is taken from 1 experiment representative of 4 independent experiments.
Figure S4. Effect of BH3 mimetics on thrombin generation *in vivo*

C57BL6 mice were administered (A) ABT-737 (75 mg/kg) (open bars) or an equal volume of DMSO (filled bars) *i.p* (A), or (B) ABT-263 (25 mg/kg) or an equal volume of vehicle by oral gavage. (A,B) Whole blood was sampled at 4 hours, or the indicated time point and analyzed for plasma levels of thrombin-anti-thrombin (TAT) using an ELISA, as described under ‘Methods’. Histograms depict the data collected from 5-6 independent experiments (mean ± SEM).