Supplemental Materials

Supplementary Methods

Platelet preparation

For human platelets, blood was obtained from healthy donors who had not ingested aspirin or NSAIDs in the 2 weeks prior to donation. Platelets were isolated by centrifugation followed by isolation of washed platelets from platelet-rich plasma. For single cell amperometry experiments, blood was drawn from the midear artery of New Zealand rabbits (Bakkom Rabbitry) according to approved University of Minnesota IACUC protocol #0802A27063. Isolation of washed rabbit platelets in Tyrode's buffer (NaCl, 137 mM; KCl, 2.6 mM; $MgCl_2$, 1.0 mM; D-glucose, 5.6 mM; N-2-hydroxyethylpiperazine- N'-2-ethanesulfonic acid (HEPES) 5.0 mM; and $NaHCO_3$, 12.1 mM with pH adjusted to 7.3) was previously described.[1]

Immunogold Electron Microscopy

For preparation of cryosections, isolated human platelets were fixed with 4% paraformaldehyde in 0.1 M Na phosphate buffer, pH 7.4. After 2 hours of fixation at room temperature, the cell pellets were washed with PBS containing 0.2 M glycine. Before freezing in liquid nitrogen, cell pellets were infiltrated with 2.3 M sucrose in PBS for 15 minutes. Frozen samples were sectioned at 120°C, and the sections were transferred to formvar-carbon coated copper grids and floated on PBS until the immunogold labeling was carried out. The gold labeling was carried out at room temperature on a piece of parafilm. Anti-Drp1 antibody (Abcam) and Protein A gold were diluted with 1% BSA. The anti-Drp1 antibody recognized a single band of the appropriate molecular weight in immunoblot staining of platelet lysates (Fig. 1A,B). Grids were floated on drops of 1% BSA for 10 minutes to block for nonspecific labeling, transferred to primary antibody, and incubated for 30 minutes. The grids were then washed, transferred to Protein A gold for 20 minutes, and washed in PBS followed by double distilled water. Contrasting/embedding of the labeled grids was carried out on ice in 0.3% uranyl acetate in 2%

methyl cellulose for 10 minutes. The grids were examined in a Tecnai G2 Spirit BioTWIN transmission electron microscope (Hillsboro, OR) at 18 500 magnification at an accelerating voltage of 80 kV. Images were recorded with anAMT 2k CCD camera.[2] Control samples using a non-immune antibody demonstrated no staining.

Immunoblot analysis

Platelet lysates were prepared by lysis of human platelets (2 x 10^8/ml) in sample buffer. To prepare cytosol and membranes, platelets were incubated with 15 U/mL streptolysin-O (Sigma, MO) overnight and subsequently pelleted at 1000*g* for 15 minutes. The cytosol fraction was collected and the pellet was washed in PIPES/EGTA/KCl buffer (25 mM PIPES, 2 mM EGTA, 137 mM KCl, 4 mM NaCl, 0.1% glucose, pH 6.4). Proteins in cytosol and membranes were then solubilized in sample buffer and separated by SDS-PAGE. Immunoblotting was performed using antibodies directed against dynamin 1, dynamin 2, Drp1 (Abcam), phospho-Drp1 serine 616 (Cell Signaling Technology), and phospho-Drp1 serine 637 (Cell Signaling Technology) and FITC-labeled or HRP-labeled secondary antibodies (Jackson Immunorearch Laboratories). FITC-labeled or HRP-labeled secondary antibodies were visualized using fluorescence detection on a Typhoon 9400 Molecular Imager (GE Healthcare) or chemiluminescence using an X-OMAT 2000A Processor (Kodak), respectively.

Flow cytometry

Gel-filtered platelets (10 μL; [0.5-1 x 10^8/mL]) were incubated with the indicated concentrations of inhibitor for 20 minutes. Samples were then exposed to 5 μM SFLLRN and analyzed by flow cytometry using a Becton Dickinson FACSCalibur flow cytometer as previously described.[3] PE-conjugated antihuman P-selectin (BD Biosciences) was used to detect P-selectin exposure. For evaluation of mitochondrial membrane potential, platelets were incubated with 1 μM JC-1

(Invitrogen) for 10 minutes and either buffer alone, mdivi-1, or actimycin A (Sigma) for 20 minutes. Platelets were then exposed to either buffer or SFLLRN for 20 minutes and evaluated by flow cytometry. Fluorescent channels were set at logarithmic gain and 1 x 10^4 particles were acquired for each sample. A 530/30 band pass filter was used to measure FL1 fluorescence and a 585/42 band pass filter was used to measure FL-2 fluorescence. Data were analyzed using CellQuest software (BD Biosciences) on a MacIntosh PowerPC (Apple). Data using JC-1 are represented using the ratio of FL2/FL1 as previously reported.[4]

Detection of adenine nucleotide release

A luciferin-luciferase detection system was used to quantify ADP/ATP release to monitor bulk dense granule secretion.[5] For these experiments, 9 µL human platelets (0.5-1 x 10^8/mL) were incubated in the presence or absence of mdivi-1 and then stimulated with SFLLRN. Samples were then incubated with 1 µL luciferin-luciferase (final concentration of 3 mg/mL) at the indicated time following addition of agonists. Chemoluminescence was quantified using a luminometer (TD 20/20; Turner Design).

Confocal Microscopy

For colocalization measurements, platelet mitochondria were labeled by incubation of the PRP with 100 nM Mitotracker Red (Life Technologies) for 45 min at 37 $^{\circ}$C. PRP with a platelet count of 10^7platelets/mL was then transferred to poly-L-lysine containing coverslips. Adhered platelets were fixed and permeabilized with 4% formaldehyde (in Tyrode's buffer) and 0.2% Triton X-100, respectively. After incubating with IF blocking buffer (10% FCS, 1% BSA and 0.05% sodium azide in PBS) overnight, platelets were labeled with mouse anti-human Drp-1 antibody (Abcam). FITC-conjugated goat anti-mouse IgG antibody (Abcam) used as a secondary antibody for imaging the Drp-1.[6] An Olympus FluoView F1000 upright confocal microscope was used for capturing images. Sequential scanning with 488 and 543 nm lasers enabled selective imaging

of the two fluorescent labels (mitotracker red and FITC) without spectral crosstalk. ImageJ, with the Mander's coefficients plugin, was used for colocalization analysis.

Supplementary Figures

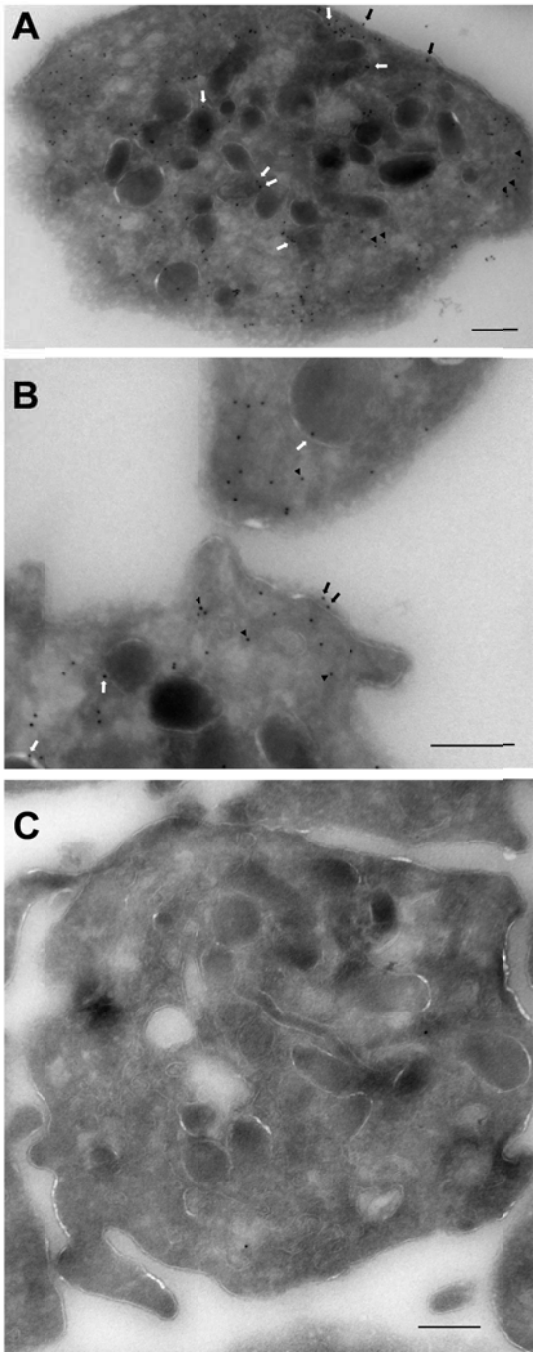

Figure I. Localization of Drp1 in resting platelets. (A) Immunogold staining of resting platelets processed with anti-Drp1 antibody demonstrates Drp1 associated with plasma membrane (*black arrow*s), granule membranes (*white arrows*), and cytosol (*black arrowheads*). Scale bar, 250 nm. (B) Immunogold staining using anti-Drp1 antibody as described in A. Scale bar, 400 nm. (C) Immunogold staining processed using a non-immune antibody instead of anti-Drp1antibody. Staining is nearly absent. Scale bar, 300 nm.

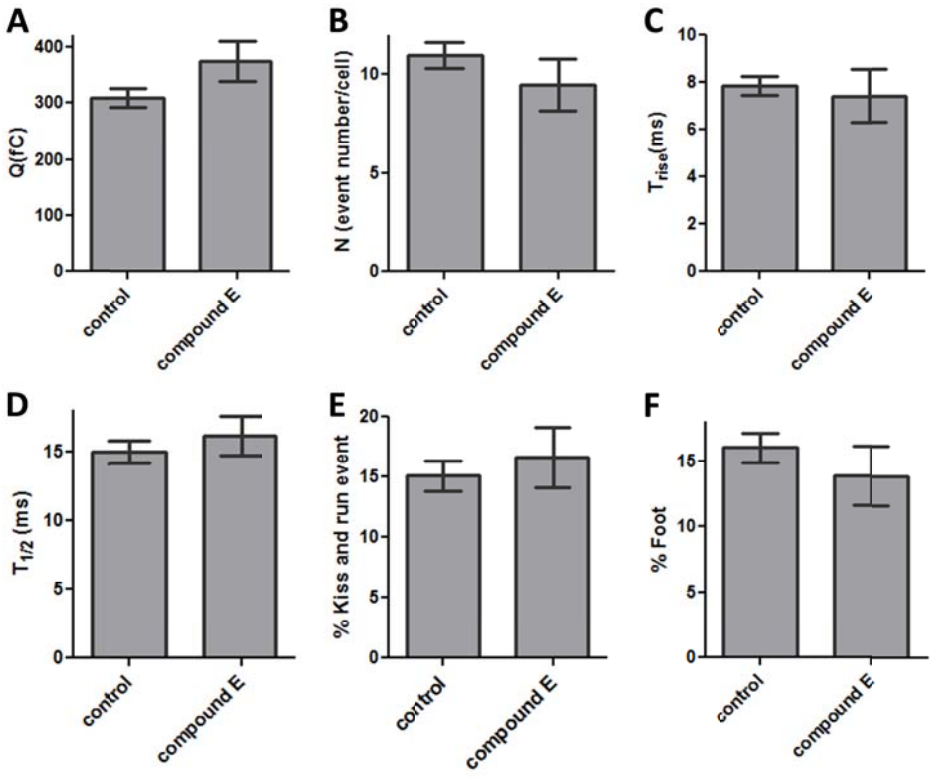

Fig. II. Effect of compound E on rabbit platelet pore formation as measured by single cell amperometry. Compound E (10 μM), an mdivi-1 analog that lacks activity against Drp1, did not significantly affect quantal release (A), number of granule released per cell (B), time required for transition from fusion pore to full fusion (C), total time for release (D), % kiss and run events (E), or % foot processes (F). Data represent the mean ± S.E.M of 107 control tracings and 31 tracings from platelets exposed to mdivi-1.

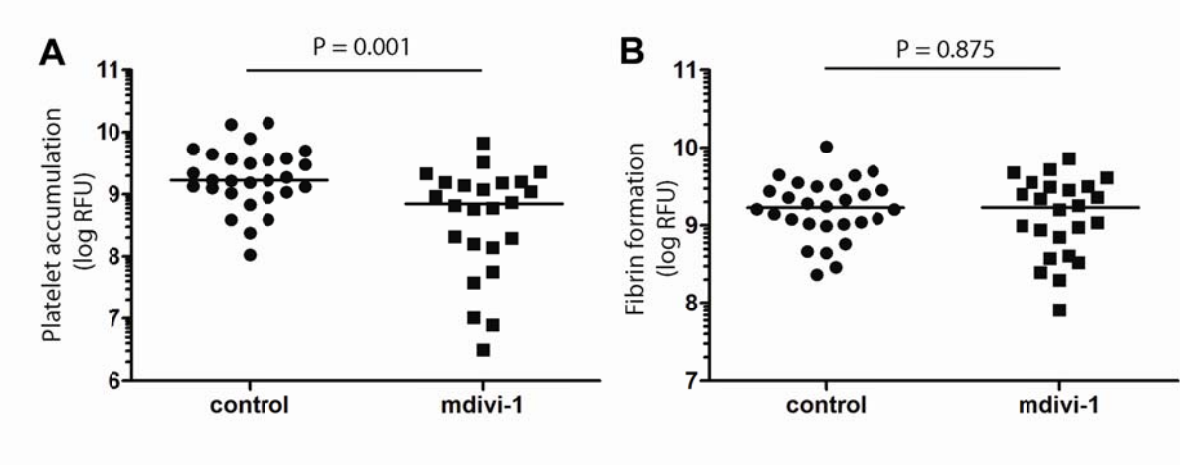

Figure III. Inhibition of platelet accumulation, but not fibrin generation, during thrombus formation following laser-induced injury of murine cremaster arterioles. The distribution of fluorescence intensities representing (A) platelet accumulation and (B) fibrin generation during thrombus formation is shown. Each point represents the area under the curve of platelet or fibrin fluorescence measured over the 3 minute interval following laser injury. A 59% decrease in platelet accumulation is observed in the presence of mdivi-1 ($p = 0.001$). In contrast, mdivi-1 has no effect on the fibrin generation.

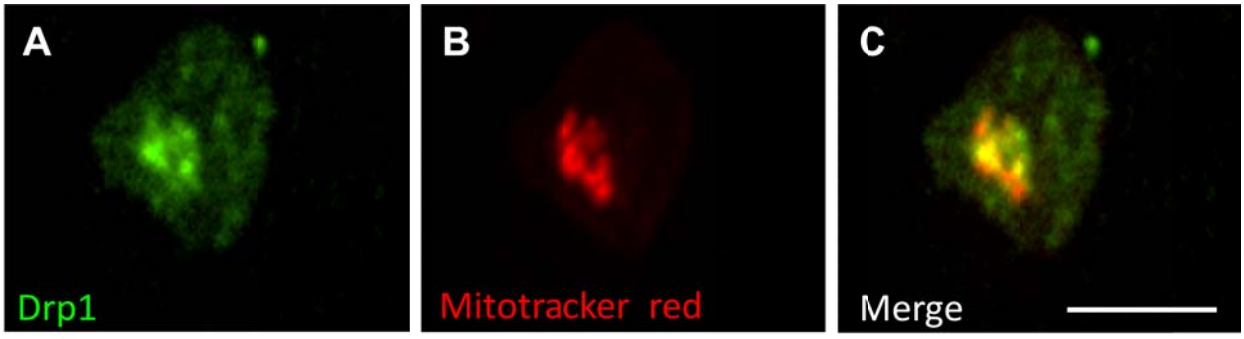

Figure IV. Colocalization of Drp1 with platelet mitochondria. Double staining confocal immunofluorescence microscopy of resting human platelets demonstrates colocalization of anti-Drp1 antibody with the mitochondrial stain, Mitotracker Red. This representative image shows staining of (A) Drp1, (B) mitochondria, and (C) the merged images. Scale bar, 5 μm. Analysis of colocalization demonstrate a Pearson's coefficient of 0.675±0.025, an M_1 value (Drp1 associated with mitochondria) of 0.428±0.038, and an M_2 value (mitochondria associated with Drp1) of 0.731±0.067. Staining with non-immune antibody showed no fluorescence, and no crossover was observed between fluorescence channels.

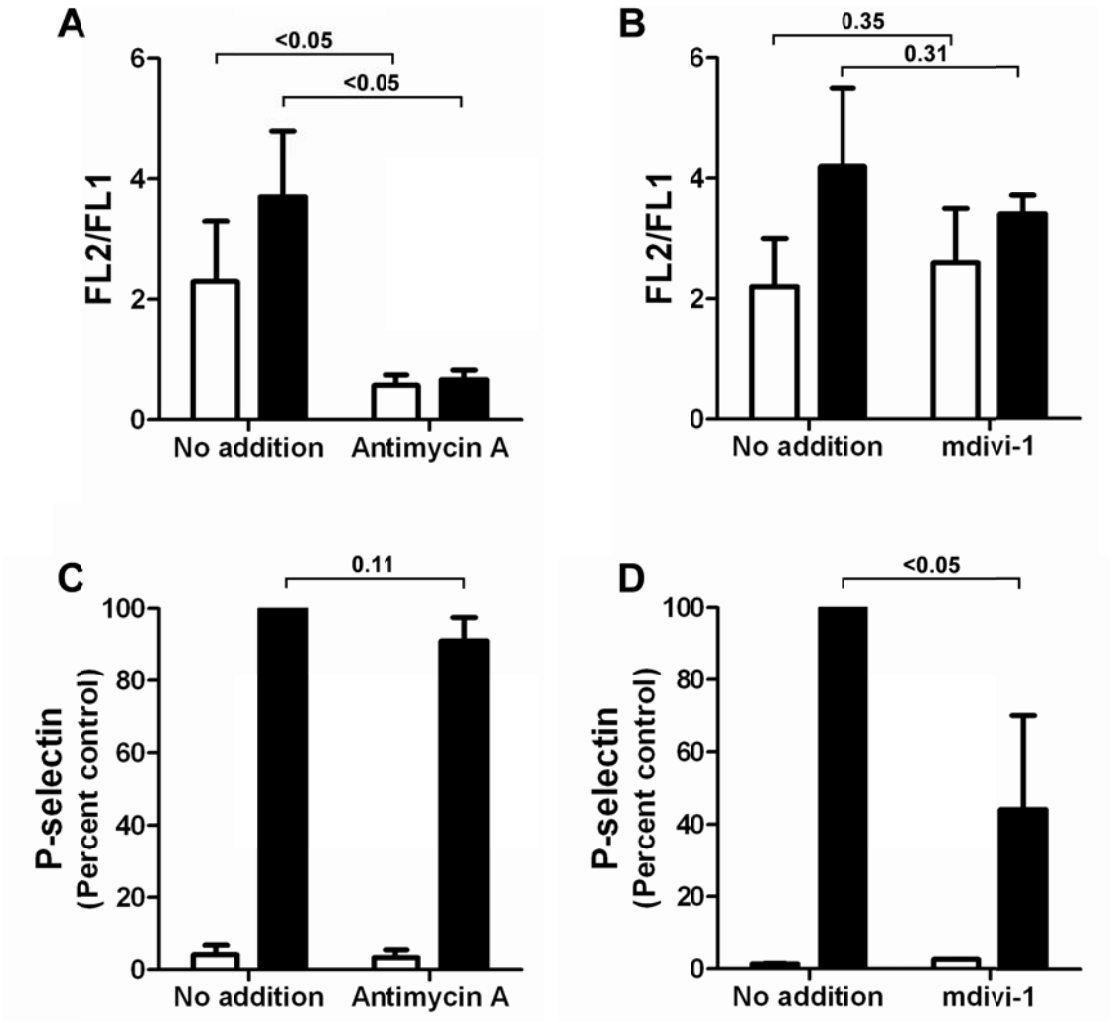

Figure V. Platelet mitochondrial membrane potential is not affected by mdivi-1. (A,B) Human platelets were incubated for 20 minutes with buffer (*No addition*) and either (A) 3 μM antimycin A or (B) 30 μM mdivi-1. Samples were then incubated in the presence or absence or 5 μM SFLLRN for 10 minutes and mitochondrial membrane potential was subsequently measured using JC-1. The ratio of red (FL2) to green (FL1) fluorescence was used to monitor mitochondrial membrane potential as previously described.[4] Data represent the ratio of FL-2/FL-1±S.E.M. of four independent samples. P-values are indicated above the graph. (C,D) Platelets were incubated for 20 minutes with buffer (*No addition*) and either (C) 3 μM antimycin A or (D) 30 μM mdivi-1. Samples were then incubated in the presence or absence or 5 μM SFLLRN for 10 minutes. P-selectin surface expression was subsequently evaluated by flow cytometry. Data represent the mean±S.E.M. of 3 samples. P-values are indicated above the graph.

Supplementary video 1. Thrombus formation following laser injury was monitored in the mouse cremaster arteriole following infusion of DMSO. The accumulation of platelets (*red*) and fibrin (*green*) is visualized during thrombus formation following laser-induced injury of a cremaster arteriole.

Supplementary video 2. Thrombus formation following laser injury was monitored in the mouse cremaster arteriole following infusion of 50 mg/kg mdivi-1. The accumulation of platelets (*red*) and fibrin (*green*) is visualized during thrombus formation following laser-induced injury of a cremaster arteriole. Following infusion of mdivi-1, fibrin formation occurs normally, but platelet accumulation is decreased.

Supplementary References