Materials and Methods

Platelet preparation, aggregation and immunoblotting

Human platelet preparation and aggregation assays were performed as described previously. Briefly, blood was obtained from healthy, aspirin-free, human volunteers with informed consent. Platelet-rich plasma (PRP) was prepared by centrifuging the blood for 20 minutes at 102g at room temperature for aggregation and flow cytometry assays. Washed platelets were prepared and re-suspended in modified Tyrodes-HEPES buffer (134mM NaCl, 2.9mM KCl, 0.34mM Na₂HPO₄, 12mM NaHCO₃, 20mM HEPES and 1mM MgCl₂, pH 7.3) to the final density of 4x10⁸ cells/ml for aggregation assays. Contaminating blood cells were counted by light microscopy and were mainly erythrocytes; leukocytes were rarely encountered with total cell contaminant level was <1 per 13000 platelets. Aggregation assays were performed using collagen-related peptide [(CRP-XL), a selective agonist for the platelet collagen receptor glycoprotein (GP) VI, from Prof R Farndale (University of Cambridge, UK)], thrombin (Sigma Aldrich, UK) and collagen (Horm collagen, Nycomed, Austria) in the presence or absence of various concentrations of tangeretin (Sigma Aldrich, UK), dissolved in DMSO. The final concentration of DMSO used where required as diluent was 0.01% (v/v) which does not affect platelet function (Supplementary Figure S1), and assays incorporated appropriate vehicle controls. SDS-PAGE and immunoblotting were performed using standard protocols as described previously.

Rabbit anti-human 14-3-3ζ (Santa Cruz Biotechnology, USA) was used to detect 14-3-3ζ to ensure equivalent levels of protein loading in immunoblots. The anti-phosphotyrosine antibody (4G10) was obtained from Millipore, USA and phospho-specific antibodies against various signalling proteins (Syk, PLCγ2, LAT and Lyn) were obtained from Epitomics, USA. Anti-phospho VASP antibodies were obtained from Cell signalling technology, USA. The secondary antibodies for immunoblotting; Cy5® goat anti-rabbit IgG and Cy3® goat anti-mouse IgG antibodies were obtained from Invitrogen, UK. cGMP and cAMP ELISA detection and, cyclic nucleotide phosphodiesterase assays were obtained from Enzo lifesciences, UK and performed according to manufacturer’s protocols.

Flow cytometry

Flow cytometric assays were performed as described previously. CRP-XL-stimulated fibrinogen binding and P-selectin exposure were measured in PRP or washed human platelets using FITC-labelled rabbit anti-human fibrinogen antibodies (Dako UK Ltd) and mouse anti-human CD62P antibody (BD Biosciences, UK) respectively, in the presence or absence of different concentrations of tangeretin. Platelets were incubated with tangeretin or vehicle control for 3 minutes prior to activation with CRP-XL for 20 minutes at room temperature. The cells were fixed in 0.2% (v/v) formyl saline prior to analysis by flow cytometry. Data were analysed by calculating the median fluorescence intensity.

Calcium mobilisation in platelets was measured upon agonist stimulation using flow cytometry. Three microliters of human PRP was mixed with 122µl of Fluo-4 NW dye (Invitrogen, UK) and the final volume was made up to 220µl using HEPES buffered saline and incubated at 37ºC for 27 minutes followed by 3 minutes incubation with vehicle or different concentrations of tangeretin (to the final volume of 225µl). This mix was placed in a 96 well plate and the data acquisition was initiated using Accuri C6 flow cytometry (BD Accuri flow cytometers, USA). After 10 seconds of data collection, CRP-XL (1µg/ml) was injected into the well to the final volume of 300µl and the data were continued to measure up to 5 minutes. The median fluorescence intensity and the rate of calcium release were calculated and compared the control with tangeretin inhibited samples.

Clot retraction

Human PRP (200µl) was mixed with 5µl of red blood cells and vehicle or tangeretin, and the final volume raised to 1ml with modified Tyrodes-HEPES buffer as described previously. Fibrin clot
formation was initiated by adding thrombin (1U/ml). Clot retraction around a glass capillary added prior to clot formation was observed over a period of 2 hours at room temperature. Clot weight was measured as a marker for clot retraction at different time points.

**Thrombus formation in vitro**

DiOC₆ (Sigma Aldrich, UK) labelled human citrated blood was pre-incubated with vehicle or tangeretin and perfused over a collagen coated Vena8 BioChips (Cellix Ltd, Ireland) at a shear rate of 20 dynes/cm² as reported previously.¹,² Z-stack images of thrombi were obtained for every 30 seconds for up to 10 minutes using a Nikon eclipse (TE2000-U) microscope (Nikon Instruments, UK). Fluorescence intensity and thrombus volume were calculated by analysing the data using Slidebook5 software (Intelligent Imaging Innovations, USA).

**Assessment of haemostasis**

C57BL/6 mice (7-8 weeks old) (The Jackson Laboratory, UK) were anesthetized using ketamine (80mg/kg) and xylazine (5mg/kg) administered via the intraperitoneal route 20 minutes prior to the experiment and placed on a heated mat. Tangeretin (estimated 10µM and 50µM based on the weight of mouse and respective volume of blood) or vehicle control (DMSO) was injected via femoral vein 5 minutes before 1mm of tail tip was removed using a scalpel blade and the tail tip was placed in sterile saline at 37°C. The time to cessation of bleeding was measured up to 10 minutes. Data were analysed by comparing the bleeding time obtained with vehicle or tangeretin treated mice.

**Statistical analysis**

The data obtained from aggregation, fibrinogen binding, granule secretion and clot retraction assays were analysed using student T-test. Median fluorescence intensity values obtained in fibrinogen binding and granule secretion assays were converted into percentage for comparison of controls with inhibited samples. The data obtained from in vitro thrombus formation assays were analysed using two-way Anova. The data obtained in calcium mobilisation experiments were analysed by ‘R’ statistical software. The tail bleeding assay data were analysed using the nonparametric Kruskal-Wallis global test using GraphPad Prism (version 5.04) from GraphPad Software Inc.

**References**