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Cucurbitacins elicit anti-platelet activity via perturbation of the actin cytoskeleton and integrin function.

Neline Kriek, Sophie Nock, Tanya Sage, Badrija Khalifa, Alexander Bye, Joanne L Mitchell, Steven Thomson, Mark G McLaughlin, Sarah Jones, Jonathan M Gibbins, Amanda Unsworth.

Affiliations below.

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Abstract:
Cucurbitacins are dietary compounds that have been shown to elicit a range of anti-tumour, anti-inflammatory and anti-atherosclerotic activities. Originally identified as STAT inhibitors, a variety of mechanisms of action have since been described, including dysregulation of the actin cytoskeleton and disruption of integrin function. Integrin outside-in signalling and cytoskeletal rearrangements are critical for the propagation of stable thrombus formation and clot retraction following platelet adhesion at the site of vessel damage. The effects of cucurbitacins on platelet function and thrombus formation are unknown.
We report for the first time anti-platelet and anti-thrombotic effects of cucurbitacins B, E and I in human platelets. Treatment of platelets with cucurbitacins resulted in attenuation of platelet aggregation, secretion and fibrinogen binding following stimulation by ADP, TRAP6, collagen and CRP-XL. Cucurbitacins were also found to potently inhibit other integrin- and cytoskeleton-mediated events, including adhesion, spreading and clot retraction. Further investigation of cytoskeletal dynamics found treatment with cucurbitacins altered cofilin phosphorylation, enhanced activation, and increased F actin polymerisation and microtubule assembly. Disruption to cytoskeletal dynamics has been previously shown to impair integrin activation, platelet spreading and clot retraction. Anti-platelet properties of cucurbitacins were found to extend to a disruption of stable thrombus formation, with an increase in thrombi instability and de-aggregation under flow.
Our research identifies novel, anti-platelet and anti-thrombotic actions of cucurbitacins that appear to be linked to dysregulation of cytoskeletal dynamics and integrin function.

Corresponding Author:
Amanda Unsworth, Manchester Metropolitan University Faculty of Science and Engineering, Department of Life Sciences,, Manchester, United Kingdom of Great Britain and Northern Ireland, a.unsworth@mmu.ac.uk

Affiliations:
Neline Kriek, University of Reading, Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, Reading, United Kingdom of Great Britain and Northern Ireland
Sophie Nock, Manchester Metropolitan University Faculty of Science and Engineering, Department of Life Sciences, Manchester, United Kingdom of Great Britain and Northern Ireland
Tanya Sage, University of Reading, Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, Reading, United

Kingdom of Great Britain and Northern Ireland
[...]
Amanda Unsworth, Manchester Metropolitan University Faculty of Science and Engineering, Department of Life Sciences,, Manchester, United Kingdom of Great Britain and Northern Ireland

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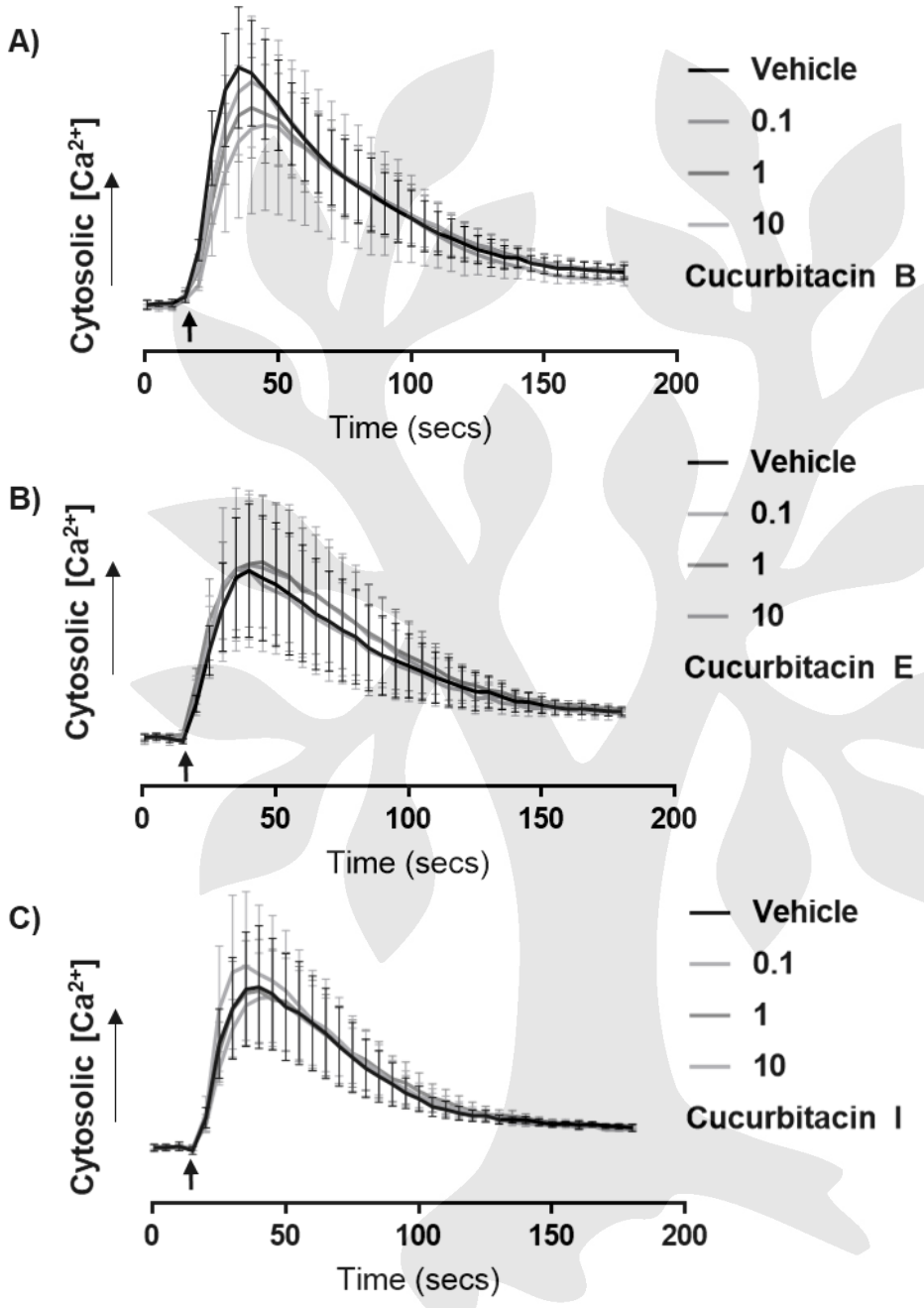
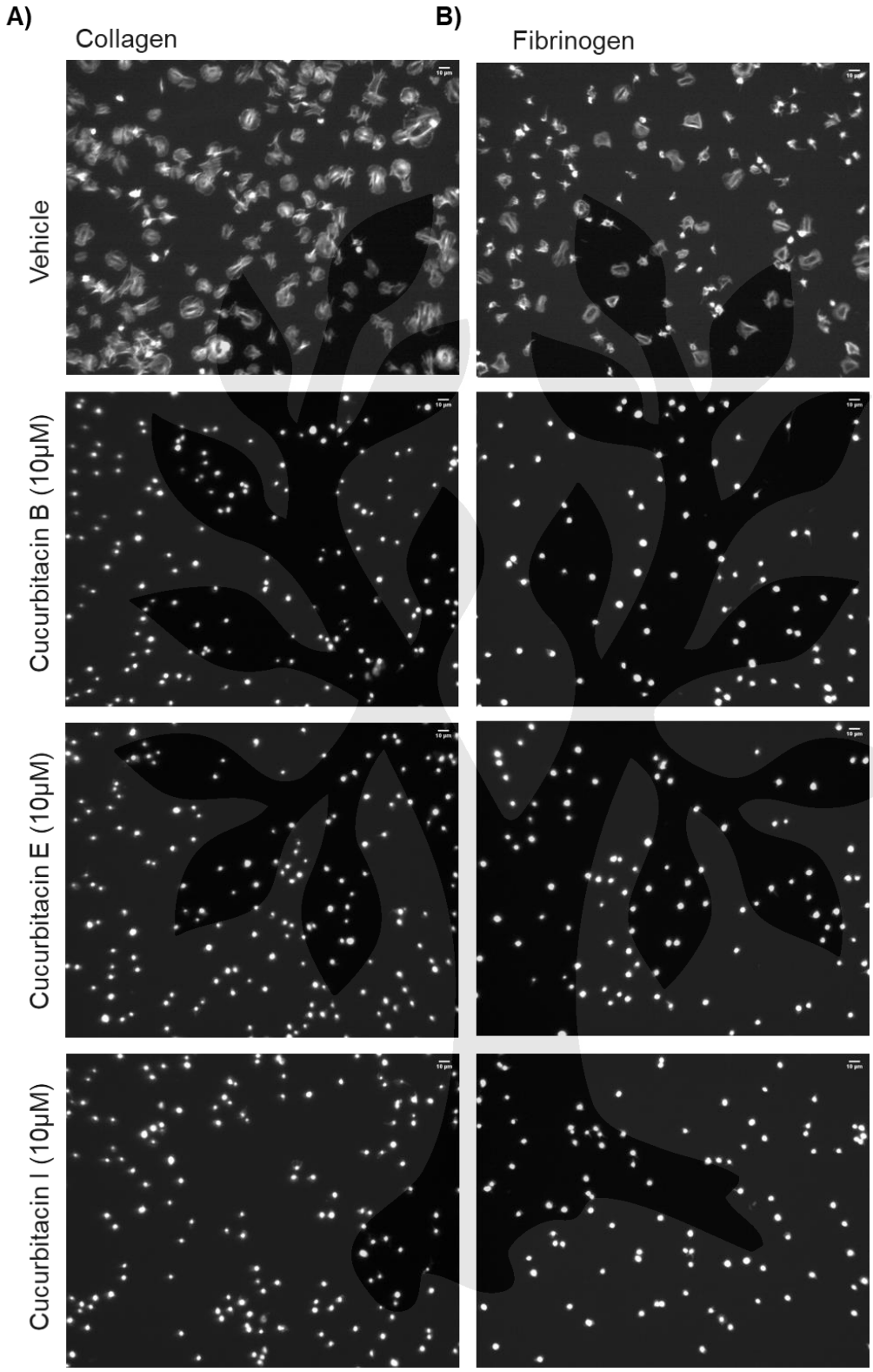
Supplemental Material



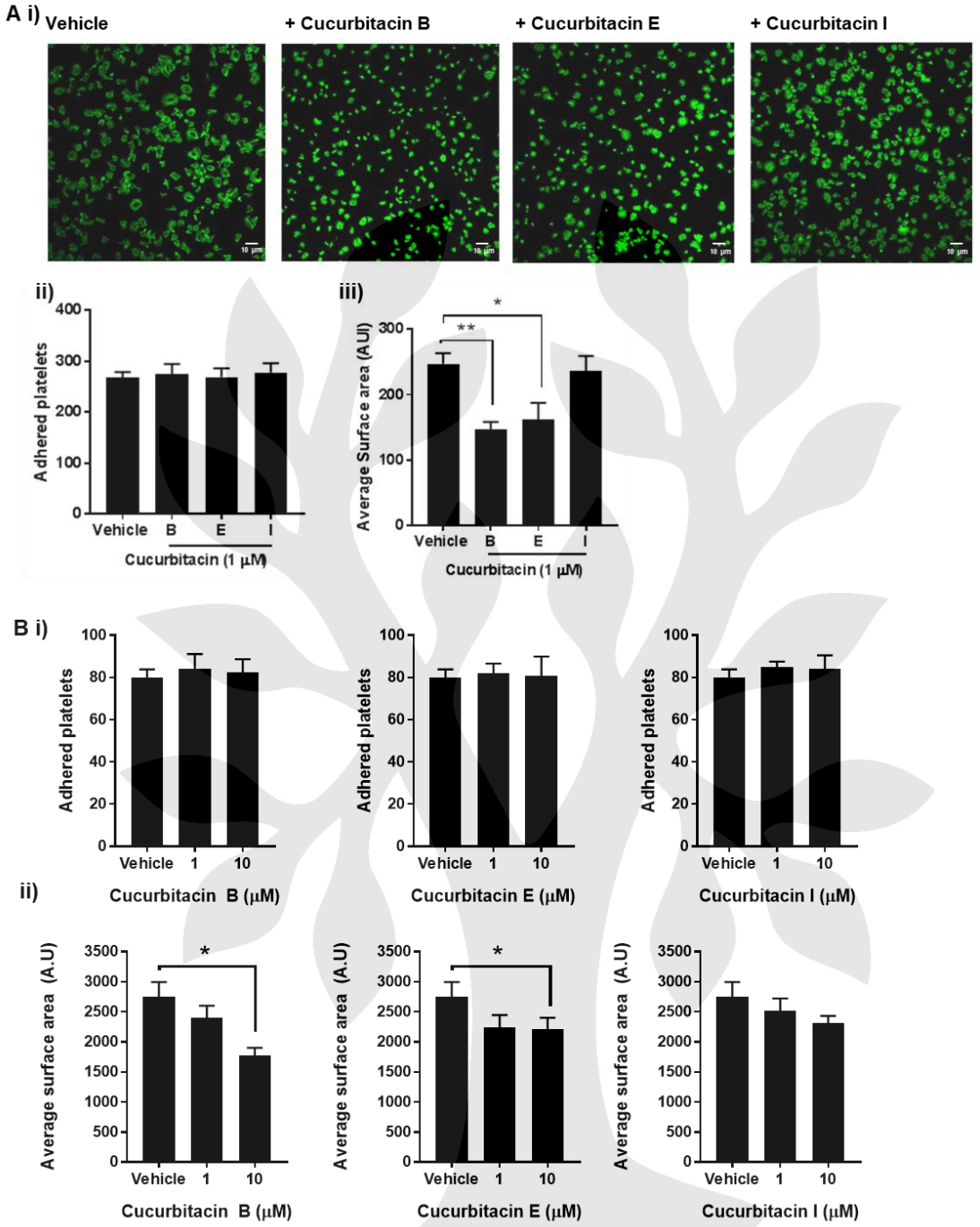
Supplemental Figure 1. Cucurbitacins B, E and I do not alter cytosolic calcium mobilisation. Mobilisation of intracellular calcium was determined in FURA-2 AM loaded washed human platelets following pre-treatment with increasing concentrations of A) Cucurbitacin B, B) Cucubitacin E or C) Cucurbitacin I (0.1-10 µM) and stimulation with TRAP-6 (1 µM). Quantified traces shown. Results are mean + S.E.M. for n≥3, * indicates $p<0.05$ in

comparison to vehicle controls, where normalised data shown, statistics were performed prior to normalisation.



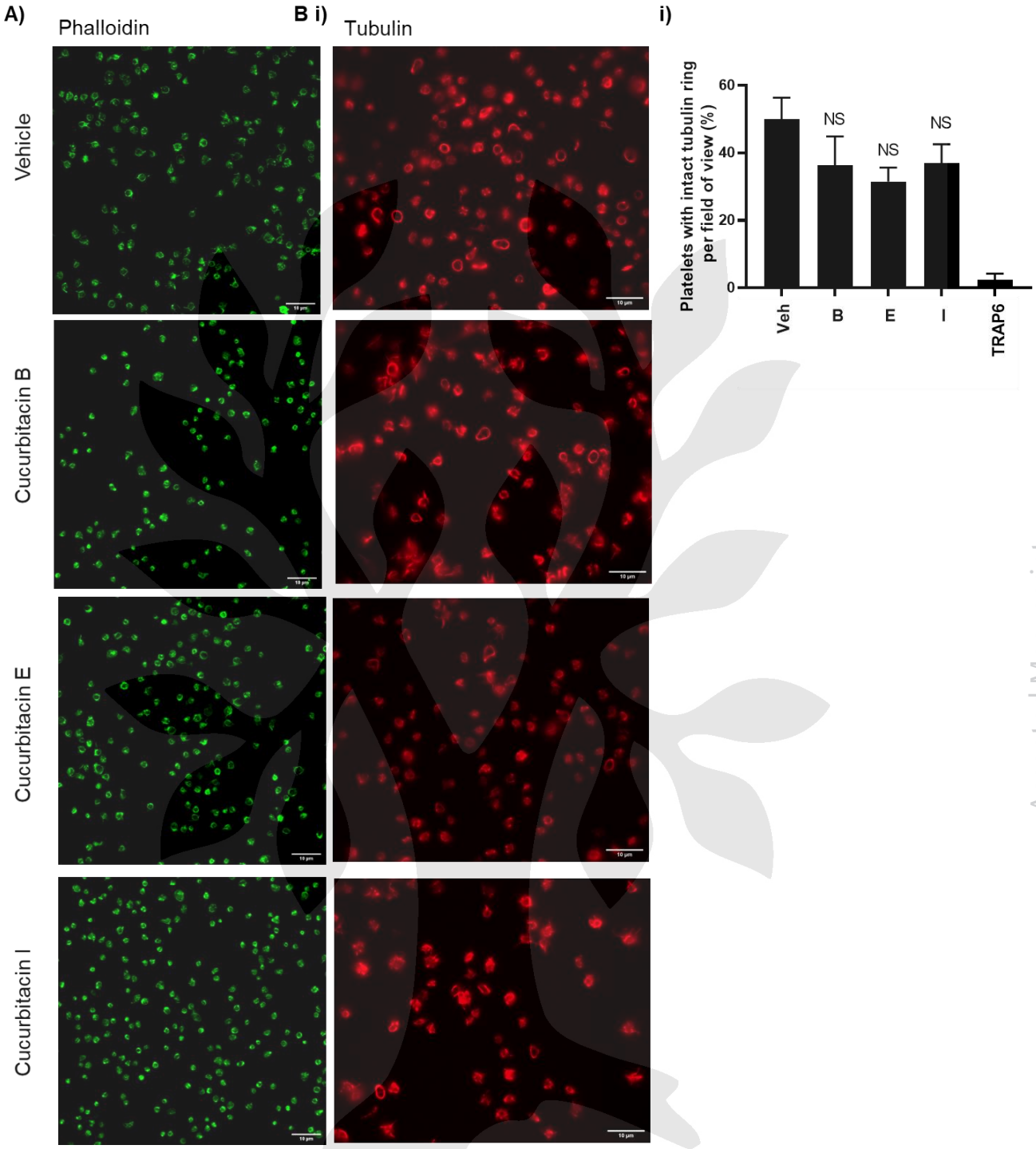
Supplemental Figure 2. Cucurbitacins inhibit platelet adhesion and spreading. Human washed platelets pretreated for 10 min with or without increasing concentrations of Cucurbitacins B, E and I (0.01-10 µM)) or vehicle control were exposed to A) collagen (100 µg/mL) and B) fibrinogen (100 µg/mL) coated coverslips and left to adhere and spread for 45 minutes. Platelets were labelled with phalloidin for visualisation. Images of adherent platelets

were captured using the 40x lens on a Celena S logos imaging system. Representative images of rhodamine-phalloidin stained platelets shown. Scale bar = 10µm



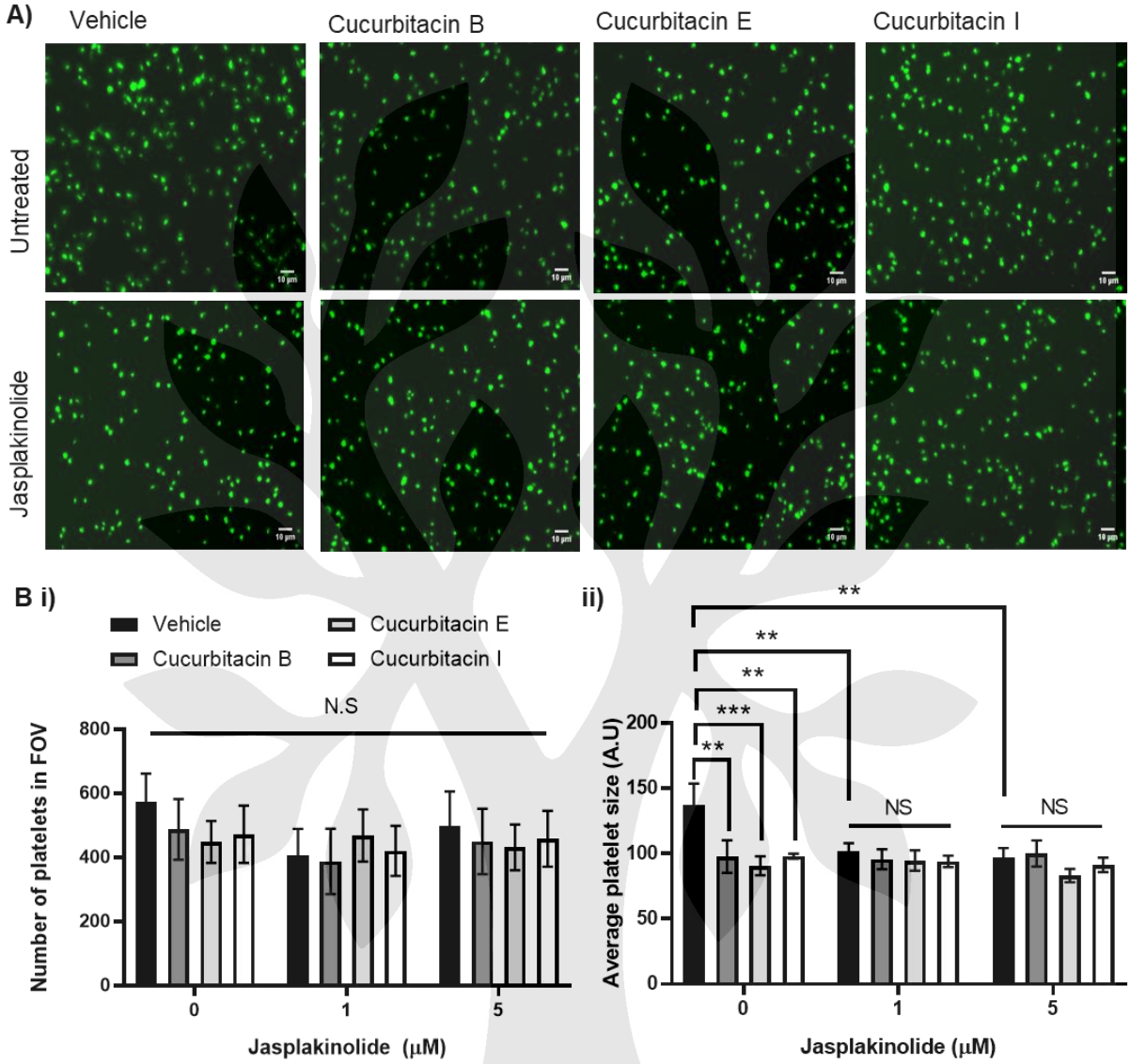
Supplemental Figure 3. Cucurbitacins B and E alter cytoskeletal rearrangements and spreading but not adhesion. Human washed platelets A) pretreated for 10 min with Cucurbitacins B, E and I (1 µM)) or vehicle control were left to adhere and spread on uncoated glass coverslips and adhesion and spreading determined after 45 min. i) representative images shown, scale bar represents 10µm, ii) number of platelets adhered (adhesion) were counted per field of view, iii) extent of spreading determined by calculating the average surface area per platelet. B) were allowed to adhere on fibrinogen (100 µg/mL) for 20 minutes before addition of cucurbitacins B, E or I (1, 10 µM) or vehicle (0.1% DMSO), platelets were left to adhere and spread for an additional 25 minutes. Platelets were labelled with Alexa 488 conjugated- phalloidin and fluorescence images of adherent platelets were captured with the 40 x objective lens of an Nikon Confocal microscope. Results are mean + S.E.M. for n≥3, *

indicates p<0.05., NS indicates p>0.05 in comparison to vehicle controls, where normalised data shown, statistics were performed prior to normalisation



Supplemental Figure 4. Cucurbitacins alter actin polymerisation but do not disrupt the microtubule marginal band. Human washed platelets were pretreated for 10 min with Cucurbitacins B, E and I (1 µM)) or vehicle control in suspension, before fixing in formalin. Samples were then added to poly-L-Lysine coverslips and stained with A) Alexa488-conjuaged phalloidin and B) Alexa647-conjugated anti-tubulin antibody for visualisation of the platelet cytoskeleton. Representative images shown and B ii) number of platelets with an intact microtubule marginal band were counted per field of view, and quantified data shown. Samples were imaged using a Leica confocal microscope. Data analysis performed using

ImageJ software. Results are mean + S.E.M. for n≥3, * indicates p<0.05., NS indicates p>0.05 in comparison to vehicle controls.



Supplemental Figure 5. Cucurbitacins do not cause further inhibition of cytoskeletal rearrangements and spreading in the presence of jasplakinolide. Human washed platelets pretreated for 10 min with Cucurbitacins B, E and I (1 μM)) or vehicle control +/- 1 and 5 μM jasplakinolide were left to adhere and spread on fibrinogen (100 μg/mL) coated coverslips and adhesion and spreading determined after 45 min. A) representative images shown, B i) number of platelets adhered (adhesion) were counted per field of view, ii) extent of spreading determined by calculating the average surface area per platelet. Platelets were labelled with DiOC6, as jasplakinolide blocks binding to phalloidin, and fluorescence images of adherent platelets were captured using the 40x lens on a Celena S logos imaging system. Data analysis performed using ImageJ software. Results are mean + S.E.M. for n≥3, * indicates p<0.05., NS indicates p>0.05 in comparison to vehicle controls, where normalised data shown, statistics were performed prior to normalisation.

Cucurbitacins Elicit Anti-Platelet Activity via Perturbation of the Actin Cytoskeleton and Integrin Function

Neline Kriek[1], Sophie H. Nock[2], T Sage[1], Badrija Khalifa[2], Alexander P. Bye[1], Joanne L. Mitchell[1], Steven Thomson[2], Mark G. McLaughlin[3], Sarah Jones[2], Jonathan M. Gibbins[1], Amanda J. Unsworth[1,2]

[1]Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, Health and Life Sciences Building, University of Reading, Whiteknights, Reading, United Kingdom

[2]Department of Life Sciences, Faculty of Science and Engineering, John Dalton Building, Manchester Metropolitan University, Manchester, United Kingdom

[3]Department of Chemistry, Lancaster University, Lancaster, United Kingdom

Address for correspondence: Amanda J Unsworth, Department of Life Sciences, Faculty of Science and Engineering, John Dalton Building, Manchester Metropolitan University, Manchester, M1 5GD, United Kingdom (e-mail: a.unsworth@mmu.ac.uk).

ABSTRACT

Cucurbitacins are dietary compounds that have been shown to elicit a range of anti-tumour, anti-inflammatory and anti-atherosclerotic activities. Originally identified as STAT inhibitors, a variety of mechanisms of action have since been described, including dysregulation of the actin cytoskeleton and disruption of integrin function. Integrin outside-in signalling and cytoskeletal rearrangements are critical for the propagation of stable thrombus formation and clot retraction following platelet adhesion at the site of vessel damage. The effects of cucurbitacins on platelet function and thrombus formation are unknown.

We report for the first time anti-platelet and anti-thrombotic effects of cucurbitacins B, E and I in human platelets. Treatment of platelets with cucurbitacins resulted in attenuation of platelet aggregation, secretion and fibrinogen binding following stimulation by ADP, TRAP6, collagen and CRP-XL. Cucurbitacins were also found to potently inhibit other integrin- and cytoskeleton-mediated events, including adhesion, spreading and clot retraction. Further

investigation of cytoskeletal dynamics found treatment with cucurbitacins altered cofilin phosphorylation, enhanced activation, and increased F actin polymerisation and microtubule assembly. Disruption to cytoskeletal dynamics has been previously shown to impair integrin activation, platelet spreading and clot retraction. Anti-platelet properties of cucurbitacins were found to extend to a disruption of stable thrombus formation, with an increase in thrombi instability and de-aggregation under flow.

Our research identifies novel, anti-platelet and anti-thrombotic actions of cucurbitacins that appear to be linked to dysregulation of cytoskeletal dynamics and integrin function.

Keywords

Thrombosis

Platelet

Cytoskeleton

Cucurbitacins

signalling

INTRODUCTION

Natural compound libraries produced from plant products have identified a variety of promising compounds with a range of pharmacological properties for further development as therapeutic agents [1]. The dietary flavonoids quercetin, tangeritin and nobilietin [2-4], derived from citrus fruits and dietary polyphenols, [5] have all been shown to elicit anti-platelet properties via a variety of mechanisms [3, 4] including the regulation of cGMP and cAMP signalling pathways, reactive oxygen species generation and kinase linked signalling pathways [6].

Cucurbitacins are a group of tetracyclic triterpernoid compounds that are found in members of the Cucurbitacae plant family which includes pumpkins, squash, cucumber and melons [7].

There are over 40 members of the cucurbitacin family, the most widely studied being cucurbitacins B, E and I, which have been shown to elicit a range of pharmacological properties including anti-cancer, anti-diabetic, anti-inflammatory, anti-oxidant and anti-atherosclerotic activities [1, 7, 8]. A variety of targets and mechanisms of action have been described including regulation of JAK/STAT [9, 10], cofilin [11, 12], cyclins [13], cyclooxygenase 2 [14] and NFkB activity and function [15]. Cucurbitacins have also been identified to cause dysregulation of actin cytoskeleton dynamics [11, 12, 16] and disruption of integrin function [17, 18].

Platelets perform a critical protective role in haemostasis preventing blood loss upon injury. Platelet activation and thrombosis, however, can also be triggered by arterial damage following rupture or erosion of an atherosclerotic plaque resulting in myocardial infarction or stroke. Drugs which disrupt platelet function therefore represent the cornerstone of antithrombotic treatment in cardiovascular disease patients.

Integrin outside-in signalling and cytoskeletal rearrangements are critical events in platelet activation and are required for the formation of stable thrombi [19 20 21]. The initial activation of platelets is followed by rearrangement of the platelet cytoskeleton to form filopodia and lamellipodia that leads to platelet shape change and spreading [22]. Integrin adhesions support platelet aggregation via fibrinogen binding to integrin αIIbβ3, and clot retraction is then driven through integrin outside-in signalling and cytoskeleton contraction that subsequently stabilises and supports the growing thrombus [20, 23].

We have identified anti-platelet and anti-thrombotic activity of cucurbitacins B, E and I. We show that these inhibitory effects are associated with dysregulation of platelet cytoskeletal dynamics and the subsequent inhibition of platelet integrin αIIbβ3-mediated aggregation and thrombus formation thus identifying the future potential for the development of cucurbitacin derived compounds as novel anti-platelet and anti-thrombotic agents.

MATERIALS AND METHODS

Reagents

Cucurbitacins B, E and I were purchased from Cambridge Bioscience, (Cambridge UK). ADP, TRAP-6, fibrinogen, fibronectin and laminin were purchased from Sigma Aldrich (Poole, UK). Horm collagen was purchased from Nycomed, Austria, and Labmedics, CRP-XL was purchased from Prof. R Farndale (University of Cambridge, UK).

Primary phospho-antibodies for (P) cofilin Ser3 (#3311), (P) LIMK T508/505 (#3841), (P) Myosin light chain S19 (#3671), (P) VASP S157 (#3111), were purchased from New England Biosciences (Cell Signalling Hitchin, UK). β-Actin (C4) antibody was purchased from Santa Cruz (# sc47778), tubulin antibodies (#ATN02) from Cytoskeleton Inc. (Denver, USA), and β-tubulin (# PAS-16863) from Invitrogen and PF4 (#ab129183) antibody from Abcam (Cambridge, UK). Fluorophore conjugated secondary antibodies, FURA-2AM calcium indicator dye and Alexa-488 conjugated phalloidin were purchased Life Technologies (Paisely, UK).

Platelet preparation

For human experiments, blood was obtained from consenting aspirin-free healthy volunteers following procedures approved by the Manchester Metropolitan University and University of Reading Research Ethics Committees. 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 xg for 20 minutes at room temperature. ADP sensitive washed platelets were prepared from PRP (containing 1:8 v/v ACD) by centrifugation at 350 xg 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.

Platelet aggregation

Aggregation of human washed platelets was measured by optical aggregometry (Helena Biosciences, Gateshead, UK) as described previously [24], following pretreatment of platelets with cucurbitacins or vehicle (0.1% DMSO) for 10 minutes prior to agonist stimulation.

Agonists used to stimulate platelet aggregation include 1 µg/mL collagen, 1 µg/mL CRP-XL, 1 µM TRAP-6 and 10 µM ADP.

Granule secretion and fibrinogen binding by flow cytometry

Flow cytometry was used to examine alpha and dense-granule secretion and affinity up regulation of the integrin αIIbβ3 by detecting levels of P-selectin exposure, CD63 exposure and fibrinogen binding respectively. Following incubation with the cucurbitacins or vehicle for 10 minutes and stimulation with different platelet agonists (ADP, CRP-XL and TRAP-6), platelets were incubated at room temperature for 20 minutes with either a PE/Cy5 anti-human CD62P (P-selectin), PE anti-human CD63, or fluorescein isothiocyanate-labelled (FITC) anti-fibrinogen antibody. Reactions were stopped after 20 minutes, and platelets fixed by addition of 0.2% (v/v) paraformaldehyde. Data for 5000 events were collected using either a BD Accuri C6 flow cytometer and analysed using the CFlow Sampler software or MACsQuant Analyser 16 flow cytometer and analysed using FlowLogic software as described previously [25].

PF4 release

Washed platelets were preincubated for 10 minutes with 10 µM CucB, E or I or vehicle and stimulated with a concentration of TRAP-6 that elicited full aggregation for the vehicle but showed reduced aggregation with the cucurbitacins. After 3 minutes aggregation, the reaction was stopped by addition of PGI_2 (500ng/ml), platelets were pelleted and PF4 in the supernatants detected by Western blot analysis.

Microvesicle formation and Annexin V binding

Flow cytometry was used to investigate platelet microvesicle formation and phosphatidylserine exposure. Washed platelets were resuspended in 1x Annexin V binding buffer (BD biosciences) and treated with the cucurbitacins or vehicle for 10 minutes prior to stimulation with 10 µM A23817 for 10 minutes at room temperature. PE-conjugated anti-human CD41a antibody was used to identify platelets and platelet derived microvesicles. FITC- conjugated Annexin V (Ann) was used to detect surface PS exposure on platelet

derived microvesicles and platelets. Microvesicles were gated from platelets based on FSC profile as described previously [26]. Platelet Annexin V binding was determined from data collected for 10,000 events in the platelet gate, and number of platelet derived microvesicles (Low FSC, CD41+/Ann+ events) was determined per 1uL, using a MACsQuant Analyser 16 flow cytometer and analysed using FlowLogic software.

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 Tyrodes-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 FlexStation (Molecular Devices, Winnersh, UK). $[Ca^{2+}]_i$ was estimated using the ratio of the 340 and 380 nm excited signals and the method of Grynkiewicz *et al* was utilised [27-29].

Platelet Adhesion and Spreading

Washed platelets at 2×10^7 cell/ml were exposed to collagen- (100 µg/ml), fibrinogen (100 µg/ml), fibronectin (200 µg/ml) or laminin (50 µg/ml) -coated 96-well assay plates and allowed to adhere for 45 minutes at 37°C following treatment with or without cucurbitacins, Non-adherent platelets were removed by washing in PBS before fixing with 10% formyl saline for 10 minutes. The effect on cucurbitacin treatment on platelet spreading was also determined post adhesion, with spreading assays performed on platelets allowed to adhere to fibrinogen coated surface for 20 minutes prior to addition of cucurbitacins B, E or I. Non-adherent platelets were removed, and adhered platelets treated with cucurbitacins B, E or I and continue to spread for an additional 25 minutes before fixing as described above. The wells were then washed and labelled with DiOC6, to visualise the membrane, or FITC conjugated phalloidin, to visualise the actin cytoskeleton. Fluorescence images of adherent platelets were captured with the 20 x objective lens of an ImageXpress Pico high content imaging system and manually counted using CellReporterXpress software (Molecular

Devices, Winnersh, UK) or imaged on Celena S logos imaging system and manually counted using ImageJ software. All platelets per field of view were counted, with a minimum of 100 platelets per field of view required for the data set to be included.

Thrombus formation under flow *in vitro*

Thrombus formation on and platelet interaction with immobilized type I collagen was performed as described previously [25] using the VenaFlux Platform and Vena8Fluor+ Biochips (Cellix) [30]. Channels were coated overnight at 4°C with collagen, then blocked with 1% BSA/PBS for 1 h and replaced with PBS. 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 10 minutes (human) at 37°C with an arterial shear rate of 45 dynes/cm^2 /1000s^{-1}. Human thrombus formation was visualised using a 20 x magnification lens on a Nikon A1-R confocal microscope with images taken over a 10 minute time period. Images were analysed using Fiji ImageJ software to determine surface area coverage and fluorescence intensity over time as described previously [31-34], and the thrombus instability index (Δ Sd/ ΔT (%)) was calculated. (Δ Sd/ ΔT (%) is the change in surface distribution relative to change in time and is described in detail by Pugh et al [31 32]. A low ΔSd/ ΔT (%) value represents stable thrombi where there is little change in fluorescent intensity over time. In contrast, non-stable thrombi or rolling platelets display higher ΔSd/ ΔT (%) values, as platelets detach.

Flow cytometry Actin Polymerisation Assay

Human washed platelets were resuspended at 4 x10^8/mL and treated with cucurbitacins B, E, or I (0.1, 1, 10 µM) or vehicle (0.1 % DMSO) for 10 minutes prior to stimulation with or without 10 µM TRAP-6 for 3 minutes at room temperature before fixing with an equal volume of 2% formyl saline. Samples were centrifuged at 1000 xg for 10min at 4˚C. Supernatant was discarded and pellet resuspended in PBS, before a repeat centrifugation step. The pellet was resuspended in BD Phosflow Perm Buffer III and incubated on ice for 30 mins. Platelets were then washed three times as described above using PBS and a centrifugation step of

1000 xg. The pellet was resuspended in PBS containing 1:500 dilution of Alexa488-conjugated phalloidin and incubated for 1 hour in the dark. Phalloidin staining (marker of polymerised actin) was then determined by flow cytometry using a BD Accuri C6 plus and data analysed using the CFlow Sampler software as described previously [25].

F/G actin ratio Actin Polymerisation assays

Human washed platelets were resuspended at 8 x10^8/mL and treated with cucurbitacins B, E or I (10 µM) or vehicle (0.1 % DMSO) for 10 minutes prior to stimulation with or without 10 µM TRAP-6 for 3 minutes at room temperature. Following stimulation, an equal volume of (2x) actin stabilisation buffer (0.2M PIPES, pH 6.9, 60% glycerol, 10% DMSO, 2mM MgSO4, 2mM EGTA, 2% Triton X-100, 1mM PMSF, 2mM NaVO4, 10ug/ml leupeptin, 10ug/ml aprotinin, 1ug/ml pepstatin A, 2mM ATP) was added to the samples at 4°C. Samples were centrifuged at 16,000xg for 75 minutes at 4°C and the supernatant (containing monomeric G actin) removed. The pellet (containing polymerised F actin) was then resuspended in an equivalent volume of actin depolymerisation buffer (0.1M PIPES, pH 6.9, 1mM MgSO4, 10mM CaCl2, 5 µM cytochalasin D) containing cytochalasin D (5 µM). Both G actin and F actin samples were then lysed in Laemmli sample buffer and boiled. Samples were separated by SDS PAGE electrophoresis and transferred to PVDF membranes by Western blotting.

Cytoskeleton imaging

Washed platelets were resuspended to 4x10^7 cells/mL and incubated with cucurbitacins or vehicle control for 10 minutes at room temperature. Reactions were stopped with an equal volume of 10% formalin and samples were pelleted onto slides as described in the work by Kahn et al [35]. Samples were permeabilised with 0.1% Triton for 5 minutes before washing 3x with PBS. Wells were blocked with 1% BSA/PBS for 1 hour at room temperature, before samples were stained with beta Tubulin Polyclonal Antibody (Invitrogen) with a dilution of 1:200 in BSA/PBS for 1 hour at room temperature. Samples were washed before staining with Goat anti rabbit AlexaFluor 647 antibody (Invitrogen) and AlexaFluor 488 phallodin

(1:750) for 1 hour at room temperature. Slides were washed, mounted and imaged on a Zeiss Imager M2 100x lens.

Immunoblotting

Immunoblotting was performed using standard techniques as described previously [32,33]. 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 total protein were used to normalize the phosphorylation data using Image Quant software and ImageJ.

Statistics

All experiments were performed using blood from a minimum of three separate donors. 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 or Tukey correction (multiple). Where data were 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.

RESULTS

Cucurbitacins attenuate platelet aggregation and granule secretion.

Platelet aggregation mediated through activation of integrin αIIbβ3 and the formation of inter-platelet fibrinogen bridges is a key step in the process of thrombus formation. It has been previously described that treatment with cucurbitacins disrupts integrin function in cancer cells through inactivation of the integrin α subunit [17, 18]. Light transmission aggregometry was used to determine the effect of treatment of human washed platelets with increasing concentrations of cucurbitacins on platelet aggregation. We found that treatment with cucurbitacin B, E or I caused a dose dependent attenuation of platelet aggregation to a

range of platelet agonists, including Collagen, CRP-XL, ADP, and PAR-1 activating peptide TRAP6 (Figure 1). With approximately 30-50% inhibition of aggregation observed following treatment with the highest concentration tested of each cucurbitacin (10 μM). Consistent with an inhibition of platelet aggregation, integrin αIIbβ3 activation, assessed by fibrinogen binding, was reduced following treatment of washed platelets with cucurbitacin B, E or I (10 μM) prior to stimulation with TRAP6 or ADP. Interestingly no inhibition of fibrinogen binding was observed following stimulation with 1 μM CRP-XL (Figure 2) despite the observed inhibition of aggregation responses at the same concentration. This may be due to assay differences and/or the sensitivity of the CRP-XL used.

In contrast to integrin αIIbβ3 activation, P-selectin exposure a marker of alpha granule secretion, another key process in platelet activation and thrombus formation, was found to be unaffected by treatment with cucurbitacin B, E or I (Figure 2). We therefore determined whether this was also the case for secretion of dense granules. Interestingly in contrast to P-selectin exposure, we did see an inhibition of TRAP-6 induced CD63 exposure (a component of dense granules) following treatment with cucurbitacins B, E and I (10μM) (Figure 2D) indicating dense granule secretion is affected. It has been previously described that P-selectin exposure is not always representative of alpha granule secretion [36]. We therefore analysed secretion of soluble mediator and alpha granule component platelet factor 4 (PF4) following stimulation by TRAP6. In contrast to P-selectin exposure a reduction in PF4 release was observed following cucurbitacin treatment, under the same conditions where cucurbitacins cause inhibition of platelet aggregation. Indicating that both alpha and dense granule secretion appear to be affected following treatment with cucurbitacins.

We next determined whether cucurbitacins caused inhibition of platelet functional responses as a result of alteration of a global regulatory mechanism such as activation of central signalling components. Interestingly treatment of platelets with cucurbitacin B, E or I at concentrations that attenuate platelet aggregation, integrin function and granule secretion does not alter cytosolic calcium mobilisation following stimulation with TRAP6 (1 μM) a key

regulator of downstream platelet signalling pathways and platelet activation (Supplemental Figure 1). Indicating cucurbitacins inhibit platelet function via regulation of processes that occur downstream of calcium signalling.

Cucurbitacin treatment inhibits platelet adhesion and spreading

Platelet adhesion and activation on extracellular matrix components is mediated by integrin activity. Following platelet adhesion, activation and subsequent fibrinogen binding, clustering of integrin αIIbβ3 occurs and outside-in signalling is initiated leading to platelet cytoskeleton rearrangements, platelet shape change and spreading. The effect of cucurbitacins on platelet adhesion and spreading was therefore determined on a range of platelet adhesive surfaces.

As shown in figure 3 (and supplemental Figure 2), treatment of platelets with increasing concentrations of the different cucurbitacins caused a decrease in the ability of the platelets to adhere (cell count) and spread (surface area) on fibrinogen, collagen, fibronectin and laminin coated surfaces (inhibition of ~ 20-50%). IC50 values (< 1 μM) showed cucurbitacins B, E and I to inhibit platelet adhesion and spreading on the different adhesive surfaces more potently than the concentrations required to achieve 50% inhibition of aggregation or integrin activation (10 μM). Attenuation of platelet adhesion and spreading was not due to reduction in surface expression levels of integrin αIIbβ3 and other platelet surface receptors, as surface expression levels of αIIb, β3, α2, β1 and GPVI were unaltered following treatment with cucurbitacins (Figure 3C).

The observed impairment of platelet adhesion and spreading on multiple adhesive surfaces indicates global disruption of adhesive integrin receptor signalling or cytoskeletal rearrangements. In further support of this, analysis of platelet spreading on glass coverslips identified no significant alteration in platelet adhesion following treatment with either cucurbitacin B, E or I (1 μM), but did cause a significant reduction in platelet surface area (cucurbitacin B and E), indicating an inhibition of the platelets to undergo normal shape change and spreading (Supplemental Figure 3A). In addition, when platelets were left to

adhere to a fibrinogen-coated surface for 20 minutes prior to treatment with cucurbitacins, no alteration in platelet adhesion was observed, indicating no alteration in the ability of platelets to maintain adhesion and outside-in signalling. However, some reduction in spreading (cell size – surface area) was observed following treatment with cucurbitacin B and E (10 µM) indicating some disruption to cytoskeletal dynamics following treatment with the cucurbitacins (Supplemental Figure 3B).

Cucurbitacins inhibit stable thrombus formation

Having observed an inhibitory effect of cucurbitacins on platelet adhesion and aggregation, the effect of the cucurbitacins on thrombus formation was assessed in whole blood under arterial shear conditions in collagen coated microfluidic chambers. Human whole blood was pre-incubated with vehicle control or cucurbitacin B, E or I (10 µM) for 10 minutes and perfused over collagen-coated (100 µg/mL) Vena8 biochips at arterial shear rate (45 dyn/cm^3 for 10 mins). Interestingly, whilst initial adhesion to collagen appeared to be unaffected, cucurbitacin treated platelets exhibited a reduced ability to form stable platelet aggregates and stable thrombi (Figure 4A). In the presence of vehicle, platelets accumulated on collagen fibres while continuously contracting into dense stable thrombi. In contrast, treatment with cucurbitacins prevented retraction of platelet aggregates, which consequently remained unstable, appeared loose, and were prone to disaggregation with several micro-thrombi observed detaching from the site of adhesion. In support of this, surface area coverage was significantly increased in cucurbitacin B, E or I treated samples compared to vehicle treated control (Figure 4B) indicating their inability to contract and form dense thrombi. Fluorescence intensity, used to analyse thrombus formation, was unaffected following treatment with cucurbitacins B, E or I (Figure 4C). This anomaly could be attributed to the increased surface area coverage but lack of stable dense thrombi compared to vehicle controls. To quantify thrombus stability, the thrombus instability index ΔSd/ ΔT (%) [31, 34] was used (Figure 4D). A significant increase in ΔSd/ ΔT (%) following treatment with cucurbitacin B, E or I was observed compared to vehicle treated controls. These observations indicate

that treatment with cucurbitacins prevents platelets from being able to form stable thrombi under flow conditions.

Cucurbitacins attenuate Clot retraction

The significant reduction in the ability of platelets to form stable thrombi following incubation with cucurbitacins indicates inhibited clot retraction. The effect of cucurbitacins on clot retraction was therefore explored (Figure 5). Pre-incubation of platelets with cucurbitacins B, E or I resulted in an increase in clot weight and therefore an inhibition of clot retraction after 90 minutes following stimulation with thrombin, compared to vehicle treated controls (0.1% v/v DMSO). Lack of stable thrombus formation and inhibition of clot retraction is typical of attenuated outside-in signalling via integrin αIIbβ3 or disruption of cytoskeletal rearrangements preventing platelets from changing shape and 'contracting' within a thrombus.

Cucurbitacins alter platelet cytoskeleton dynamics

During platelet activation, integrin activity is tightly associated with cytoskeletal dynamics and the platelet cytoskeleton has been shown to regulate platelet dense and alpha granule secretion [36-38]. The anti-cancer cytotoxic properties of some of the cucurbitacins have been shown to be associated with disruption of cytoskeletal dynamics which ultimately leads to an inhibition of cell division and/or cell migration [11, 12, 16, 39]. To determine whether cucurbitacins altered cytoskeleton remodelling in platelets, we monitored actin polymerisation in platelets stimulated with or without 10 µM TRAP6 following treatment with and without cucurbitacin B, E and I. We identified that treatment with cucurbitacins resulted in an increase in the platelet pool of polymerised actin (F actin) (Figure 6A) and an increase in the F/G actin ratio in both resting and stimulated platelets (TRAP6) compared to vehicle treated controls (Figure 6B and C) indicating that cucurbitacins alter actin turnover. A decrease in tubulin:microtubule ratio was also observed in both resting and stimulated platelets (TRAP6) following cucurbitacin treatment (6D, and 6E) suggesting that dysregulation of platelet cytoskeleton dynamics following treatment with cucurbitacins occurs independently of platelet activation.

Visualisation of the actin and microtubule cytoskeleton using microscopy identified no changes in microtubule coiling in cucurbitacin treated platelets compared to vehicle treated controls (supplemental Figure 4).

The process of platelet cytoskeleton rearrangements is dependent on proteins that bind to actin and tubulin to facilitate their polymerisation. Phosphorylation of myosin light chains (MLC) on Ser19 enables interaction of Myosin II with actin filaments which is required for platelet shape change. In further support of an increase in actin polymerisation and alteration of cytoskeleton dynamics we observed an increase in myosin light chain phosphorylation following treatment of platelets with cucurbitacins compared to vehicle treated controls, similar to that observed following stimulation of platelets with TRAP6 (Figure 6D).

It has been previously demonstrated that actin polymerisation agent jasplakinolide impairs platelet function via the inhibition of integrin mediated responses [40]. Jasplakinolide is not associated with dysregulation of microtubule dynamics. To ascertain whether the observed cucurbitacin mediated effects were due to disruption of the actin cytoskeleton, platelet adhesion and spreading assays were performed in the presence of jasplakinolide (1 and 5 μM) (Supplemental Figure 5). Treatment with jasplakinolide caused an inhibition of platelet spreading on fibrinogen, with ~30% reduction in average platelet size, which was not further inhibited by addition of cucurbitacins. This suggests that cucurbitacins act either via a similar mechanism to jasplakinolide, disrupting actin polymerisation and cytoskeleton dynamics or that the mechanism is masked in the presence of the actin stabilising agent.

Cucurbitacins do not alter procoagulant platelet or microvesicle formation

The actin cytoskeleton plays a key role in platelet structural integrity. Reorganisation of the cytoskeleton could have the potential to favour membrane budding and microvesicle release [41]. To determine whether treatment with the cucurbitacins altered platelet procoagulant activity and the ability of platelets to form microvesicles, the effect of cucurbitacin treatment on phosphatidylserine exposure and platelet derived-microvesicle formation was determined in platelets in the presence and absence of the calcium ionophore A23187 (10 μM). As

shown in Figure 7, treatment with cucurbitacins alone did not induce an increase in Annexin V binding in the absence of platelet agonist stimulation, and we observed no alteration of Annexin V binding (% positive cells and MFI) in A23187 stimulated platelets following treatment with cucurbitacins compared to vehicle treated controls. It has been previously demonstrated that actin polymerisation agent jasplakinolide increases platelet microvesicle formation due to its disruption of cytoskeletal dynamics [41]. Interestingly we observed no cucurbitacin mediated increase in microvesicle formation in unstimulated platelets, and no alteration in agonist mediated platelet microvesicle formation following stimulation with calcium ionophore A23187. Taken together these observations indicate that despite their ability to alter platelet cytoskeleton dynamics, cucurbitacin B, E and I unlike other actin destabilising agents, do not alter platelet procoagulant activity and microvesicle formation .

Cucurbitacins upregulate cofilin activity

Protein kinase A is a known mediator of Rho/ROCK signalling and a key mediator of cytoskeletal rearrangements via regulating phosphorylation of MLC. However, we show that cucurbitacins do not increase or alter PKA activity as determined by monitoring VASP S157 phosphorylation (the PKA phosphorylation site) which is unaffected by treatment with cucurbitacins (Figure 8A). Previously published papers have identified activation of the Rho/ROCK pathway independently of PKA following treatment with cucurbitacin I [42]. ROCK kinase phosphorylates and deactivates MYPT at Thr850 which leads to increased phosphorylation of MLC on S19 [12, 43]. However, under the same conditions that cause an increase in MLC S19 phosphorylation we observed no increase in MYPT phosphorylation compared to vehicle treated controls (Figure 8B). Taken together these data indicate cucurbitacin induced regulation of platelet cytoskeletal changes occurs independently of PKA and RhoA/ROCK activity.

Platelet shape change requires the input of a variety of actin filament-related proteins that regulate actin cytoskeleton dynamics. Cofilin is an ‘actin dynamising factor’ which binds to actin filaments stabilising F actin. Due to its multiple activities, cofilin can favour both actin

depolymerisation and polymerisation. To see whether treatment with cucurbitacins altered cofilin activation, phosphorylation of cofilin at Ser3 was determined in unstimulated platelets following treatment with cucurbitacins B, E or I. As described by Falet et al (2005) we observed phosphorylation of cofilin at Ser3 in vehicle treated unstimulated platelets. We demonstrated that treatment with either cucurbitacin B, E or I caused a reduction in cofilin ser3 phosphorylation compared to vehicle treated controls, and similar to that observed following stimulation of platelets with TRAP6 peptide (10uM) (Figure 8C). Dephosphorylation of cofilin at Ser3 has been shown to accelerate actin filament turnover, further supporting our observations that cucurbitacins disrupt actin cytoskeleton dynamics in platelets.

Cofilin is phosphorylated and inactivated by Lim kinase family LIMK-1 and 2 [43]. Treatment with cucurbitacins and subsequent reduced phosphorylation of cofilin indicates inhibition of LIMK-1 and/or activation of cofilin phosphatase slingshot (SSH1L). To determine whether alteration of platelet actin cytoskeleton dynamics is due to regulation of LIMK activity, phosphorylation of LIMK at Thr 508/505, a marker of activation, was determined. As shown in Figure 7D no significant increase or decrease in LIMK Thr 508/505 phosphorylation was observed following treatment with cucurbitacins B, E and I compared to vehicle-treated control in resting platelets. No increase in LIMK phosphorylation is consistent with cofilin activation, but a lack of a decrease in LIMK phosphorylation indicates cucurbitacin induced cofilin activation is not due to upstream inhibition of LIMK activity, and possibly points to an increase in SSH1L activity.

In summary, cucurbitacins B, E and I increase cofilin dephosphorylation and activation leading to dysregulation of cytoskeleton dynamics, inhibition of platelet shape change and integrin mediated events, resulting in inhibition of stable thrombus formation.

DISCUSSION

Compounds that target integrin function or integrin signalling pathways, preventing fibrinogen binding, platelet aggregation and thrombus formation are promising anti-platelet agents [44]. However, despite their early promise as potent inhibitors of platelet aggregation, oral platelet integrin αIIbβ3 inhibitors show no benefit and/or increased mortality rate in clinical trials [45, 46]. New approaches that indirectly target integrin function could therefore offer a safer, more efficacious approach to integrin αIIbβ3 targeted anti-platelet therapy. Novel therapies targeting regulation of platelet shape change and cytoskeleton rearrangements are also of increasing interest [21].

Cucurbitacins, tetracyclic triterpernoid compounds, have been shown to elicit a range of beneficial properties including anti-cancer, anti-diabetic, anti-inflammatory, anti-oxidant and anti-atherosclerotic activities through a variety of mechanisms including regulation of cofilin, dysregulation of actin cytoskeleton dynamics [11, 12, 16] and disruption of integrin function [17, 18].

We observed anti-platelet activity of cucurbitacins B, E and I which results in a significant attenuation in the ability of platelets to form stable thrombi. Most notably, whilst initial platelet adhesion to collagen (and fibrinogen) under flow appeared to be unaffected by treatment with cucurbitacins, the morphology of the thrombi was altered, with frequent embolization observed and clot retraction perturbed. Analysis of thrombus stability using the thrombus instability index ΔSd/ΔT(%) [31] identified increased ΔSd/ΔT (%) values following cucurbitacin treatment, indicating a reduction in stable thrombi formation. These observations are likely resultant from the actions of cucurbitacins on the cytoskeleton, including alteration of actin dynamics and microtubule organisation.

Following treatment with cucurbitacins we observed an increase in actin polymerisation, F actin/ G actin ratio, a decrease in tubulin:microtubule ratio, and increase in myosin light chain phosphorylation. This occurred independently of platelet agonist activation, indicating that cytoskeletal dynamics and actin and microtubule turnover are arrested at the stage of polymerisation. Consistent with this, platelet adhesion and spreading on a range of surfaces, a process dependent on cytoskeletal remodelling and polymerisation, was disrupted by

cucurbitacins. Furthermore, clot retraction, which is driven by the cytoskeletal tensile strength and dynamics was also inhibited by cucurbitacins B, E and I. Interestingly we did observe some differences in the concentrations of cucurbitacins required to cause inhibition of functional responses. Treatment with higher concentrations of cucurbitacins were needed to inhibit platelet aggregation compared to platelet spreading and cytoskeleton rearrangements. We hypothesise that this is due to the differences in the nature of the assays and their reliance on cytoskeleton rearrangements.

The mode of action of cucurbitacins observed in this study is different to that observed following treatment with latrunculin A or cytochlasin D, inhibitors of actin polymerisation. Instead our observations align with those made previously using Jasplakinolide, a stabiliser of actin filaments [40, 47]. This is supported by experiments that show cucurbitacins are unable to further inhibit Jasplakinolide induced inhibition of platelet spreading (Supplemental Figure 3). Jasplakinolide induces actin polymerisation by promoting actin nucleation and preventing depolymerisation of actin stress fibres. Bennet et al (1999) and Bury et al (2016) describe how treatment with jasplakinolide leads to impairment of platelet function, specifically as a result of impaired $\alpha_{IIb}\beta_3$ activation, reduced aggregation, altered spreading on fibrinogen and clot retraction, observations that are similar to what we observed following treatment with cucurbitacins. Like our observations with cucurbitacins B, E and I, Bury et al also identify that actin polymerisation (following treatment with jasplakinolide) does not alter P-selectin exposure [40]. However further investigation did identify cucurbitacin mediated inhibition of PF4 release, under conditions where cucurbitacins inhibit platelet aggregation, indicating cucurbitacin mediated disruption of the actin dynamics reduces the release of soluble α-granule contents. In contrast to our findings with the cucurbitacins, Bennet al demonstrate jasplakinolide does not alter dense granule secretion, whilst we observed decreased CD63 exposure following treatment with cucurbitacins. This could be reflective of differences in the mechanism by which the two groups of compounds elicit actin polymerisation, although it should also be noted that Bennet et al, measured serotonin release to 2 U/mL thrombin

which is a very high concentration of the strong activatory agonist, and therefore is likely difficult to inhibit.

We also observed no alteration of platelet derived microvesicle formation following treatment with cucurbitacins. This is a particularly promising observation as other actin disrupting agents are associated with increased microvesicle formation [41]. Platelet derived procoagulant microvesicles have been shown to enhance coagulation and thrombosis, which could potentially outweigh any anti-platelet activity of other actin disrupting agents. Specific therapeutic targeting of the platelet cytoskeleton and enhancing thrombus destabilisation opens new avenues for the development of novel anti-platelet therapies. The lack of effect on microvesicle formation and platelet procoagulant activity indicates that the cucurbitacins are a suitable scaffold for developing novel anti-platelet agents.

Several publications in other cell types have identified that cucurbitacins cause an increase and stabilisation of polymerised actin, resulting in a condensed actin network [11, 12, 16, 42]. Possible mechanisms of action include regulation of actin binding or actin remodelling proteins, or via direct interaction with and stabilisation of the actin cytoskeleton, however, the precise mechanism is still not fully understood.

Previous publications have identified cucurbitacin mediated activation of the Rho/ROCK pathways [12, 42]. This pathway in platelets regulates cytoskeleton dynamics via deactivation of MYPT by phosphorylation (at Thr850), increased phosphorylation of the myosin light chain at S19 and increased actin polymerisation [48]. However, we observed no increase in MYPT Thr850 phosphorylation following treatment with the cucurbitacins indicating an alternative mechanism of action.

Cofilin activity and subsequent actin depolymerisation has been proposed to be altered following treatment with cucurbitacins in other cell types both via direct and indirect mechanisms [11, 12]. Cofilin acts as an ‘actin dynamising factor’ which binds to actin filaments stabilising F-actin, where depending on the cellular content of actin filaments, cofilin can

favour actin depolymerisation or polymerisation. We identified reduced phosphorylation at Ser3 and activation of cofilin following treatment with cucurbitacins in platelets. Both Falet et al [49] and Pandey et al [43] identified that cofilin dephosphorylation is accompanied by an increase in F-actin content and increased association of cofilin with F-actin. Taken together these findings indicate that cucurbitacins alter actin polymerisation and cytoskeleton dynamics via regulation of cofilin dephosphorylation and activity.

Cofilin is phosphorylated and inactivated by LIMK-1 and 2 [43] downstream of the RhoA/PKA/VASP signalling pathway and activated by dephosphorylation via cofilin phosphatase slingshot (SSH1L). Cucurbitacins have been shown to induce stimulation of the small GTPases observed in other cell types [40, 50]. However, in support of lack of activation of the RhoA/PKA/VASP–LIMK-1 pathway in platelets, we observed no alteration in VASP S157 phosphorylation, or phosphorylation of LIMK at Thr 508/505, following treatment with cucurbitacins B, E or I in resting platelets, indicating no activation of PKA or LIMK activity. This is further supported by our observations that treatment with cucurbitacins B, E or I does not alter platelet cytosolic calcium mobilisation or granule secretion, processes known to be negatively regulated by increases in PKA activity.

Similar to our observations, a study investigating the anti-cancer properties of cucurbitacins using HeLa cells identified that cucurbitacin I induced the formation of actin and myosin II coaggregates that led to an alteration in cytoskeletal dynamics [40]. Interestingly this study identified reduced cofilin phosphorylation which the authors attributed to an inhibition of LIMK via direct binding of cucurbitacin I to the kinase. In contrast, our observations of a lack of increase in both PKA and LIMK activity is consistent with cofilin activation, but indicates that cucurbitacin mediated cofilin activation is likely associated with either an increase in SSH1L phosphatase activity which leads to cofilin dephosphorylation, or activation of an alternative regulatory pathway that results in a negative regulation of cofilin phosphorylation and subsequent activation (summarised in Figure 8).

Our research identifies novel, anti-platelet and anti-thrombotic actions of cucurbitacins that appear to be linked to cofilin mediated dysregulation of cytoskeletal dynamics, and subsequent inhibition of integrin activity. Therapeutic targeting of the platelet cytoskeleton and thrombus destabilisation is an attractive novel approach for anti-platelet therapy.

At this stage it should be noted that these compounds are associated with toxicity issues at therapeutic doses, and therefore in their current form have limited pharmacological use. This is not surprising nor is it a major cause of concern, noting that natural products have provided chemical leads for the development of many drugs for a range of indications [51]. Due to the molecular complexity of the cucurbitacins scaffold, intelligent compound design and pharmacophore modelling will allow us to simplify the scaffold into more "drug-like" motifs [52], incorporating covalent warheads [53], shape complementarity [54] and functional group mapping [55] to develop novel more 'drug-like' compounds capable of eliciting a similar biological response.

Cucurbitacin-derived compounds [1] therefore represent an exciting scaffold for a novel anti-platelet targeting strategy that can control and inhibit platelet function by preferentially targeting the formation of stable platelet aggregates and thrombi.

What is known on this topic?

- Natural compound libraries produced from plant products have identified a variety of promising compounds for further development as therapeutic agents
- Cucurbitacins are dietary compounds that have been shown to elicit a range of anti-tumour, anti-inflammatory and anti-atherosclerotic activities.
- The effects of cucurbitacins on platelet function and thrombus formation are unknown.

What does this paper add?

- This research identifies novel, anti-platelet and anti-thrombotic actions of cucurbitacins
- Cucurbitacins disrupt platelet cytoskeletal dynamics, increasing actin polymerisation, microtubule formation, myosin light chain activation and cofilin activity.
- Cucurbitacins potently inhibit integrin- and cytoskeleton-mediated events, including adhesion, spreading, clot retraction and stable thrombus formation.

AUTHOR CONTRIBUTIONS

Contribution: N.K, S.N , T.S, B.K, A.P.B, J.LM, S.T, performed experiments and analysed results, M.G.M, S.J and J.M.G designed the research and wrote the paper, A.J.U designed the research, performed experiments, analysed results and wrote the paper.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Figure 1. Cucurbitacin B, E and I inhibit platelet aggregation. Washed human platelets were pre-treated with increasing concentrations of cucurbitacin i) B, ii) E or iii) I (0.1-10 μM) prior to stimulation with either A) Collagen (1 μg/mL), B) CRP-XL (1 μg/mL), C) ADP (10 μM), D) TRAP6 (1 μM), and aggregation monitored using optical light transmission aggregometry, representative traces and quantified data shown. Results are mean + S.E.M. for n≥3, * indicates $p<0.05$ in comparison to vehicle controls, where normalised data shown, statistics were performed prior to normalisation.

Figure 2. Cucurbitacin B, E and I inhibit integrin αIIbβ3 activation but not alpha granule secretion. Washed human platelets were pre-treated with increasing concentrations of cucurbitacin B, E or I (0.1-10 μM) prior to stimulation with either A) CRP-XL (1 μg/mL), B) TRAP6 (1 μM) or C) ADP (10 μM) and alpha granule secretion determined by monitoring P-selectin exposure (grey) and integrin activation measured as fibrinogen binding (black) determined using flow cytometry, quantified data shown. D) Washed human platelets were pre-treated with cucurbitacin B, E or I (10 μM) prior to stimulation with TRAP6 (1 μM) and i) dense granule secretion determined by monitoring CD63 surface expression

via flow cytometry and ii) release of soluble alpha granule contents determined by measuring PF4 levels of the platelet releasate by western blotting, quantified data shown. Results are mean + S.E.M. for n≥3, * indicates $p<0.05$ in comparison to vehicle controls, where normalised data shown, statistics were performed prior to normalisation.

Figure. 3. Cucurbitacins inhibit platelet adhesion and spreading. Human washed platelets pretreated for 10 min with or without increasing concentrations of cucurbitacins B, E and I (0.01-10 μM)) or vehicle control were exposed to i) collagen (100 μg/mL), ii) fibrinogen (100 μg/mL), iii) fibronectin (200 μg/mL) or iv) laminin (50 μg/mL) -coated coverslips and A) adhesion and B) spreading determined after 45 min. Platelets were labelled with DiOC6 and fluorescence images of adherent platelets were captured with the 20 x objective lens of an ImageXpress Pico high content imaging system and manually counted using CellReporterXpress software with. A) Number of platelets adhered (adhesion) were counted per field of view for each condition and the number of cells adhered expressed as a percentage of the vehicle treated control. (B) Extent of spreading determined by calculating the average surface area per platelet and expressed as a percentage of vehicle treated control. All platelets per field of view counted, with a minimum of 100 platelets per field of view required for the data set to be included. C) Receptor surface expression levels of αIIb, β3, GPVI, α2 and β1 were determined by flow cytometry using fluorophore conjugated antibodies and by measuring median fluorescence intensity following treatment with or without 10 μM cucurbitacin B, E or I. Data expressed as percentage of vehicle control Results are mean + S.E.M. for n≥3, * indicates $p<0.05$., NS indicates $p>0.05$ in comparison to vehicle controls, where normalised data shown, statistics were performed prior to normalisation.

Figure 4. Cucurbitacins inhibit stable thrombus formation on collagen in vitro. DiOC6 loaded human whole blood was pretreated with vehicle or 10 μM cucurbitacin B, E or I for 10 minutes before perfusion through collagen coated (100 μg/mL) Vena8Biochips at an arterial shear rate of 45 dynes/cm^2/1000s^{-1}. Thrombus formation was determined for 10 minutes.

The channels were imaged on a Nikon A1-R confocal microscope to visualise thrombus formation over time A) representative images at 10 minutes shown. Image sequences were then analysed using image J to determine B) surface area coverage, C) fluorescence intensity and D) thrombus instability index (dSd/dT (%)) compared to vehicle. D) Representative images taken at end of each video. Results are expressed as mean ± S.E.M for n≥4. * indicates $p<0.05$ in comparison to vehicle controls, where normalised data shown, statistics were performed prior to normalisation. Data was analysed using two-way Anova.

Figure 5. Cucurbitacins inhibit clot retraction. Human washed platelets pretreated for 10 min with or without increasing concentrations of cucurbitacin B, E or I (0.1-10 μM) or vehicle control were added to aggregometer tubes in the presence of 2 mg/mL fibrinogen and 2 mM $CaCl_2$. Clot retraction was initiated by adding 1 U/mL thrombin and left to proceed for 90 minutes at room temperature. Clot retraction was determined by weighing the clot and data expressed as percentage of vehicle treated control. Results are mean + S.E.M. for n≥3, * indicates $p\leq0.05$ in comparison to vehicle control, where normalised data shown, statistics were performed prior to normalisation.

Figure 6. Cucurbitacins alter cytoskeletal dynamics. Human washed platelets were A) pretreated for 10 min with increasing concentrations of cucurbitacin B, E or I (0.1-10 μM) or vehicle before fixing, permeabilising and staining with AlexaFluor 488-conjugated phalloidin. Phalloidin staining was measured by flow cytometry, quantified data shown, B-E) pretreated for 10 min with or without cucurbitacin B, E or I (10 μM) C) and then stimulated with C and E) TRAP6 (1 μM) for 3 min and reactions stopped by lysis in Triton-X lysis buffer. The Triton insoluble pellet (polymerised F actin) was then separated from the soluble fraction (monomeric G actin) by centrifugation. B and C) The F and G actin ratio was then calculated under each treatment condition. Cytochalasin D (an inhibitor of actin polymerisation was included as a control. (i) Representative blot shown. (ii) F/G actin ratio in each sample were quantified and expressed as a fold change over resting control. D and E) under the same

conditions the tubulin:microtubule ratio was then calculated for each sample, (i) Representative blot shown. (ii) tubulin:microtubule ratio in each sample were quantified and expressed as a fold change compared to resting control. F) Human washed platelets were pretreated for 10 min with or without cucurbitacin B, E or I (1 μM) and reactions stopped by lysis in SDS Laemmli sample buffer and myosin light chain phosphorylation at Ser19 determined using a phospho-specific antibody (that recognises the phosphorylated myosin light chain). TRAP6 (1 μM) stimulated platelets was included as a positive control for activation, 14-3-3 was included as a loading control. (i) Representative blot shown. (ii) quantified data expressed as a percentage of untreated control. Results represent mean + SEM for n ≥ 3. *Indicates $P < 0.05$ in comparison with vehicle control, where normalised data shown, statistics were performed prior to normalisation.

Figure 7. Cucurbitacins do not initiate procoagulant platelet or microvesicle formation. Human washed platelets pretreated for 10 min with or without cucurbitacin B, E or I (1 μM) for 10 minutes prior to stimulation with 10 μM A23817 for 10 minutes at room temperature and A and B) phosphatidylserine exposure determined by measuring A) and B i) quantified data for Annexin V % positive platelets and B ii) quantified data for median fluorescence intensity using FITC- conjugated Annexin V shown. C) microvesicle formation following treatment with or without cucurbitacins in the ii) absence and ii) presence of calcium ionophore (10 μM A23817) was determined by counting the number of CD41+/Annexin V+ events per μL using flow cytometry and staining with an PE-conjugated anti-human CD41a antibody and FITC- conjugated Annexin V. i) representative histogram plots demonstrating the gating strategy based on FSC and CD41+ events, ii) and iii) quantified data shown. When samples were treated with cucurbitacins alone in the absence of ionophore stimulation (A and Ci), 10 μM A23817 was included as a positive control. Data was collected using a MACsQuant Analyser 16 flow cytometer and analysed using FlowLogic software. Results are mean + S.E.M. for n≥3, *Indicates $P < 0.05$, **indicates $P<0.01$, ***$P<0.001$ in comparison with vehicle control

Figure 8. Cucurbitacins regulate cofilin activity. Human washed platelets were pretreated for 10 min with or without Cucurbitacin B, E or I (10 µM) or for 3 minutes with TRAP6 (1 µM) (positive control) and reactions stopped by lysis in SDS Laemmli sample buffer and A) PKA activity was determined by blotting these samples for VASP S157 phosphorylation, B) Myosin light chain phosphatase activity determined by blotting samples for MYPT T853 phosphorylation C) Cofilin activity was determined by blotting these samples for Ser3 phosphorylation and D) LIMK1/2 activity was determined by blotting these samples for LIMK1/2 Thr 505/508 phosphorylation using phospho-specific antibodies. Actin, cofilin and GAPDH were used to confirm equal loading respectively. i) representative blots and ii) quantified data shown. Levels of total phosphorylation were quantified and expressed as a percentage of the vehicle control. Results are mean + S.E.M. for n≥3, *Indicates $P < 0.05$, **indicates $P<0.01$ in comparison with vehicle control, where normalised data shown, statistics were performed prior to normalisation.

Figure 9. Cucurbitacins perturb actin cytoskeleton rearrangements and integrin function in platelets. Cucurbitacins inhibit stable thrombus formation via disruption of platelet cytoskeleton dynamics. Cucurbitacins increase the platelet F/G actin ratio, actin polymerisation and microtubule formation, alongside up-regulation of cofilin activity and myosin light chain phosphorylation via a mechanism which appears to be independent of regulation of PKA, ROCK or LIMK.