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Isorhapontigenin, a resveratrol analogue selectively inhibits ADP-stimulated platelet activation

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Abstract

Isorhapontigenin is a polyphenolic compound found in Chinese herbs and grapes. It is a methoxylated analogue of a stilbenoid, resveratrol, which is well-known for its various beneficial effects including anti-platelet activity. Isorhapontigenin possesses greater oral bioavailability than resveratrol and has also been identified to possess anti-cancer and anti-inflammatory properties. However, its effects on platelet function have not been reported previously. In this study, we report the effects of isorhapontigenin on the modulation of platelet function. Isorhapontigenin was found to selectively inhibit ADP-induced platelet aggregation with an IC_{50} of 1.85μM although it displayed marginal inhibition on platelet aggregation induced by other platelet agonists at 100μM. However, resveratrol exhibited weaker inhibition on ADP-induced platelet aggregation (IC_{50}>100μM) but inhibited collagen induced platelet aggregation at 50μM and 100μM. Isorhapontigenin also inhibited integrin αIIbβ3 mediated inside-out and outside-in signalling and dense granule secretion in ADP-induced platelet activation but interestingly, no effect was observed on α-granule secretion. Isorhapontigenin did not exert any cytotoxicity on platelets at the concentrations of up to 100μM. Furthermore, it did not affect haemostasis in mice at the IC_{50} concentration (1.85μM). In addition, the mechanistic studies demonstrated that isorhapontigenin increased cAMP levels and VASP phosphorylation at Ser157 and decreased Akt phosphorylation. This suggests that isorhapontigenin may interfere with cAMP and PI3K signalling pathways that are associated with the $P2Y_{12}$ receptor. Molecular docking studies emphasised that isorhapontigenin has greater binding affinity to $P2Y_{12}$ receptor than resveratrol. Our results demonstrate that isorhapontigenin has selective inhibitory effects on ADP-stimulated platelet activation possibly via $P2Y_{12}$ receptor.

Keywords: Isorhapontigenin; Polyphenols; Platelets; Haemostasis; Thrombosis.

1. Introduction

Platelets are small circulating blood cells that maintain haemostasis upon vascular injury, however, their unnecessary activation leads to thrombosis (George, 2000; Ruggeri, 2002). Thrombosis is the most common pathology that underpins major cardiovascular diseases such as acute coronary syndrome (ischemic heart disease) and stroke (Raskob et al., 2014). Although, anti-platelet drugs such as aspirin and clopidogrel are being used for the treatment of thrombotic complications, there is a great unmet clinical need for developing more effective and safer anti-platelet agents due to their severe side effects (Koupenova et al., 2017). Natural products, especially phytochemicals have always been an important source of new chemical entities (NCEs) in drug discovery (Cragg and Newman, 2013). Polyphenols such as stilbenoid and flavonoid classes of bioactive compounds are considered as promising molecular templates for drug design (Cuccioloni et al., 2009; Wright et al., 2013). Flavonoids such as quercetin (Hubbard et al., 2003),

chrysin (Liu et al., 2016), tangeretin (Vaiyapuri et al., 2013) and nobiletin (Vaiyapuri et al., 2015a) have been reported to modulate platelet function through distinct mechanisms. Resveratrol (Res, Fig 1Ai) [trans-3′,4′,5-trihydroxystilbene], a phytochemical that primarily found in grapes and its products such as red wine is known to exert various pharmacological activities (Berman et al., 2017; Kuršvietienė et al., 2016) including cardioprotective (Das and Das, 2010) and anti-platelet activities (Olas et al., 2002; Olas and Wachowicz, 2005). However, the therapeutic potential of resveratrol is greatly hampered by its low oral bioavailability (<1%) due to rapid metabolism in the liver and intestine (Sergides et al., 2016). Isorhapontigenin (ISO, Fig 1Aii), a new derivative of stilbene is a methoxylated analogue of resveratrol isolated from a Chinese herb, *Gnetum Cleistostachyum* (Huang et al., 2002) and is also reported to be present in grapes (Fernández-Marín et al., 2012). Isorhapontigenin has been demonstrated to exhibit anti-cancer (Fang et al., 2012), anti-oxidant (Lu et al., 2017) and anti-inflammatory (Yeo et al., 2017) effects. Notably, isorhapontigenin was found to be more orally bioavailable than resveratrol, thus possesses more favourable pharmacokinetic properties compared to resveratrol (Yeo et al., 2017). Furthermore, studies have shown that isorhapontigenin displays protective effects against cardiac hypertrophy induced by angiotensin II both *in vitro* and *in vivo* (Li et al., 2005) and it is also reported to have cardioprotective effects against isoproterenol-induced myocardial infarction in rats (Abbas, 2016). However, its effects on the modulation of platelet function have not been established previously. So, owing to the structural similarity of isorhapontigenin and resveratrol, here, we report the effects of isorhapontigenin on the modulation of platelet function in comparison to its analogue, resveratrol.

2. Materials and Methods

2.1. Human platelet preparation

Blood was obtained from healthy, aspirin-free individuals with informed consent in accordance with the methods approved by the University of Reading Research Ethics Committee (Approval number: UREC 17/17). All the experiments were performed in accordance with the relevant institutional and national guidelines and regulations and this study conform to the principles outlined in the Declaration of Helsinki. The preparation of human platelets was carried out using standard protocols as described previously (Ravishankar et al., 2018, 2017; Vaiyapuri et al., 2015b, 2013). Briefly, human blood was collected via venepuncture into vacutainers containing 3.2% (w/v) citrate and the blood samples were centrifuged at 102 g for 20 min at 20 °C to separate the platelet-rich plasma (PRP), which was used in aggregation and flow cytometry assays. Isolated platelets were prepared by mixing 50 ml of blood with 7.5 ml of ACD (acid citrate dextrose) (20 g/L glucose, 25 g/L sodium citrate and 15 g/L citric acid) and this was centrifuged at 102 g for 20 min at 20 °C. The PRP was carefully separated and mixed with 3 ml of ACD and PGI_2 (125 ng/ml) was added and then centrifuged at 1413 g for 10 min at 20 °C. The platelet pellet was washed by resuspending in modified Tyrodes-HEPES buffer (25 ml) [2.9 mM KCl, 134 mM NaCl,

0.34 mM $Na_2HPO_4.12H_2O$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 20 mM HEPES, pH 7.3] in the presence of PGI_2 (125 µg/ml) and centrifuging at 1413 g for 10 min. The obtained platelet pellet was finally resuspended in modified Tyrodes-HEPES buffer at a density of 4×10^8 cells/ml and rested for 30 min before use.

Isorhapontigenin and resveratrol (Sigma Aldrich, UK; >95% Purity) were dissolved in DMSO (100%) at 10 mg/ml stock concentration. This was further diluted to the desirable concentration for assays and the final concentration of DMSO in platelets was maintained at 0.1% (v/v), which did not affect platelet function. A DMSO vehicle control at a concentration of 0.1% (v/v) was included in all the experiments.

2.2. Platelet aggregation and dense granule secretion

Platelet aggregation assays were performed by optical aggregometry (Chrono-Log, USA) in the presence or absence of various concentrations of isorhapontigenin or resveratrol along with a vehicle control. The different concentrations of isorhapontigenin, resveratrol or a vehicle control [0.1% (v/v) DMSO] (5 µl) were added to 445 µl of PRP or isolated platelets and incubated for 5 min at 37 °C. The samples were then activated with 50 µl ADP (5 µM) (Sigma-Aldrich, UK), collagen (1 µg/ml) (Nycomed, Austria), cross-linked collagen-related peptide (0.5 µg/ml) [CRP-XL, from Professor Richard Farndale (University of Cambridge)] or U46619 (1 µM) (Tocris, UK) and the platelet aggregation was monitored for 5 min.

The dense granule secretion in platelets was determined by measuring ATP release using the luciferin-luciferase reagent by lumi-aggregometry (Chrono-Log, USA). Briefly, PRP (395 µl) was incubated with Chrono-Lume reagent (50 µl) at 37 °C for 2 min then this was further incubated for 5 min with 5 µl of various concentrations of isorhapontigenin or resveratrol and the platelets were activated with 50 µl of ADP (5 µM). The incubation with isorhapontigenin or resveratrol itself has not affected the luciferase activity (Fig S1).

2.3. Immunoblotting analysis

The platelet lysates for immunoblotting experiments were obtained by treating human isolated platelets with different concentrations of isorhapontigenin and a vehicle control [0.1% (v/v) DMSO], followed by the addition of an agonist to trigger platelet activation using optical aggregometer. After 5 min, the activation was stopped by adding lysis buffer (6X Reducing sample-treatment buffer: 12% (w/v) SDS, 30% (v/v) β-Mercaptoethanol, 30% (v/v) Glycerol and 30% (v/v) Stacking gel buffer).

SDS-PAGE and immunoblotting analyses were carried out using standard protocols as described previously (Moraes et al., 2016; Vaiyapuri et al., 2013). The phospho-specific primary antibodies for human AKT pS473, and VASP pS157 were obtained from Cell Signalling technology, UK and rabbit anti-human 14-3-3ζ antibody (Santa Cruz Biotechnology, USA) was

used to detect 14-3-3ζ protein as a loading control. The Cy5-conjugated goat anti-rabbit IgG secondary antibody (Life technologies, UK) was used for detection.

2.4. Flow cytometry-based assays

The levels of fibrinogen binding and P-selectin exposure were measured by flow cytometry (Accuri C6, BD Biosciences, UK). The platelets (PRP) were treated with different concentrations of isorhapontigenin or resveratrol or a vehicle control for 5 min prior to activation with agonists such as ADP (5 µM), CRP-XL (0.5 µg/ml) or U46619 (1 µM) for further 20 min in the presence of FITC-conjugated anti-human fibrinogen antibodies (Dako, UK) and PECy5-conjugated CD62P antibodies (BD Biosciences, UK). The platelets were fixed using 0.2% (v/v) formyl saline and then analysed by flow cytometry by collecting 5000 events within a gated region for platelets. The median fluorescence intensity was used to assess the level of fibrinogen binding (a marker for inside-out signalling to integrin αIIbβ3) and P-selectin exposure (a marker for α-granule secretion) on the platelet surface. The level of fibrinogen binding and P-selectin exposure in the treated samples was calculated by taking the level of median fluorescence intensity obtained with the vehicle control as 100%.

2.5. cAMP assay

The cAMP levels were determined using a cAMP ELISA kit (Cambridge Bioscience, UK). Human isolated platelets were pre-incubated (for 5 min) with a vehicle [0.1% (v/v) DMSO] or different concentrations of isorhapontigenin (3.125-100 µM) followed by activation with ADP (5 µM) and the platelets treated with EGTA (1 mM) were considered as a control for maximum cAMP levels. These platelets were lysed with 0.1 M HCl and the levels of cAMP were determined according to the manufacturer's protocol.

2.6. Platelet spreading

Isolated platelets ($2x10^7$ cells/ml) treated (for 5 min) with a vehicle control [0.1% (v/v) DMSO] or isorhapontigenin (3.125 and 6.25 µM) were allowed to spread on fibrinogen- (100 µg/ml) coated cover-glasses for 45 min. The unbound platelets were removed by washing with phosphate buffered saline (PBS) and the adhered platelets were fixed using 2% (v/v) formaldehyde in PBS. Platelets were permeabilised with 0.1% (v/v) Triton-X in PBS (10 min) and then stained with Alexa Fluor 488 conjugated phalloidin for 30 min. Images were obtained using a Nikon A1-R Confocal microscope and the analysed using ImageJ (NIH, USA) to calculate the number of adhered and fully spread platelets.

2.7. Tail bleeding assay

The tail bleeding assay performed in this study was approved by the University of Reading Research Ethics Committee and the British Home Office. In brief, C57BL/6 mice [9 weeks old (6 per group; both males and females) from Envigo, UK] were anaesthetised via intraperitoneal route using ketamine (80 mg/kg) and xylazine (5 mg/kg) 20 min prior to the experiment and placed on a

heated pad at 37 °C. A vehicle control [0.1% (v/v) DMSO] or isorhapontigenin (6.25 µM and 1.85 µM- final concentrations were calculated based on the mouse weight and approximate blood volume) was injected via femoral artery and after 5 min of incubation, 3 mm of tail tip was dissected using a scalpel blade and the tail tip was placed in sterile PBS. The time taken to cessation of bleeding was measured up to 20 min when the assay was terminated.

2.8. Statistical analysis

Most of the data in this study are represented as mean ± S.E.M in a bar graph format as the data were normalised to the control for each donor. However, the data for tail bleeding assay is presented as a dot plot. All the statistical analyses in this study were carried out using GraphPad Prism 7 software (GraphPad Software Inc., USA). The statistical significance between the control and isorhapontigenin or resveratrol treated samples was determined using one-way ANOVA followed by Bonferroni *post-hoc* analysis for the normalised data obtained from aggregation and flow cytometry assays. The data obtained from tail bleeding assay were analysed using non-parametric Kruskal-Wallis test followed by Bonferroni *post-hoc* analysis. The IC_{50} values were calculated by log transforming the concentration values followed by curve fitting using non-linear regression using four parameter model [Y=Bottom+(Top-Bottom)/(1+10^((LogIC50-X)*HillSlope))] in GraphPad Prism 7 software.

The methods for the lactate dehydrogenase assay and molecular docking analysis are provided in the supplementary information.

3. Results

3.1. Isorhapontigenin inhibits platelet aggregation differently from resveratrol

Resveratrol has been reported to inhibit platelet aggregation induced by various agonists (Wang et al., 2002; Wu et al., 2007). Thus, to characterise the effects of isorhapontigenin on the modulation of platelet function in comparison to resveratrol, platelet aggregation assays were carried out using various agonists by optical aggregometry. Human PRP was incubated with a vehicle control [0.1% (v/v) DMSO] or different concentrations (3.125-100 µM) of isorhapontigenin or resveratrol for 5 min prior to activation with diverse agonists such as ADP (5 µM; acts via $P2Y_1$ and $P2Y_{12}$ receptors), collagen (1 µg/ml; acts via GPVI and α2β1 receptors), CRP-XL (0.5 µg/ml; acts via GPVI receptor) and U46619, a TxA_2 analogue (1 µM; acts via thromboxane (TP) receptor). Isorhapontigenin was found to significantly inhibit ADP-induced platelet aggregation at all the concentrations tested (~50% inhibition) (Fig 1B). However, it only marginally inhibited (<10%) both collagen (Fig 1C) and CRP-XL-induced platelet aggregation only at 100 µM (Fig 1D), and it displayed no inhibitory effect on U46619- (Fig 1E) induced platelet activation. Furthermore, the IC_{50} concentration of isorhapontigenin for ADP-induced aggregation was determined to be 1.85 ± 0.56 µM (Fig 1F). Notably, the inhibition effects of isorhapontigenin (Fig 1B and 1F) are not dose-

dependent under the current experimental settings used in this study. However, further studies will be performed to explain this lack of dose-dependent effects with isorhapontigenin in platelet aggregation.

In contrast, resveratrol inhibited ADP-induced platelet activation only at 50 and 100 µM (Fig 2A). It displayed complete inhibition of collagen-induced platelet activation at 100 µM (Fig 2B) but only moderately inhibited CRP-XL (Fig 2C) and U46619 (Fig 2D)-induced platelet activation at 100 µM. These results suggest that isorhapontigenin inhibits ADP-induced platelet activation at concentrations of less than 3 µM while, resveratrol exhibits inhibitory effects only at higher concentrations. Thus, isorhapontigenin exhibits a different inhibitory profile to resveratrol and it appears to be a selective and effective modulator of ADP-stimulated platelet aggregation.

Furthermore, the effects of isorhapontigenin and resveratrol on isolated platelets (without plasma proteins) were assessed. Isorhapontigenin showed significant inhibition of collagen-induced aggregation only at 50 and 100 µM (Fig 3A) and U46619-induced aggregation at 100 µM (Fig 3B). However, resveratrol inhibited collagen-induced aggregation at all the concentrations tested (Fig 3C) and U46619-induced aggregation at only 50 and 100 µM (Fig 3D). This again suggests that isorhapontigenin exhibits different inhibitory profile to resveratrol.

3.2. Inside-out signalling to integrin αIIbβ3 is affected by isorhapontigenin

The platelet activation initiated by diverse agonists leads to the activation of integrin αIIbβ3, a highly abundant platelet surface receptor, which plays a critical role in platelet aggregation (Shattil et al., 1998). The inside-out signalling to integrin αIIbβ3 transforms this receptor from a low-affinity, resting state to a high affinity state for fibrinogen and von Willebrand factor (vWF) binding that consequently leads to platelet aggregation (Estevez and Du, 2017). As isorhapontigenin and resveratrol inhibited platelet aggregation induced by various agonists, their effects on the inside-out signalling to integrin αIIbβ3 upon agonist-induced platelet activation were assessed. Human PRP was treated with a vehicle control [0.1% (v/v) DMSO] or different concentrations (3.125-100 µM) of isorhapontigenin or resveratrol for 5 min followed by incubation with various agonists such as ADP (5 µM), CRP-XL (0.5 µg/ml) and U46619 (1 µM) and the level of fibrinogen binding was measured by flow cytometry. Consistent with the platelet aggregation data, isorhapontigenin reduced the level of fibrinogen binding in platelets only when stimulated with ADP but at concentrations of 25 µM and above (Fig 4A). In addition, isorhapontigenin did not affect U46619 (Fig 4B) and CRP-XL (Fig 4C)- induced activation. Nevertheless, resveratrol did not affect the fibrinogen binding upon stimulation with any of the agonists (Fig 4D-F). This suggests that isorhapontigenin affects inside out signalling to integrin αIIbβ3 in platelets specifically upon stimulation with ADP. These results highlight that isorhapontigenin may be more potent than its structural analogue, resveratrol in modulating platelet function.

3.3. Isorhapontigenin selectively inhibits dense granule secretion in platelets

Platelets undergo degranulation during their activation and release several effector molecules from their granules which amplify platelet activation and thrombus formation (Flaumenhaft, 2003; White and Estensen, 1972). Platelets contain three major types of secretory granules namely, α-granules, dense granules and lysosomes. α-granules contain mainly proteins such as fibrinogen, vWF and P-selectin whereas dense granules contain small organic molecules such as ADP, ATP and serotonin (Whiteheart, 2011). To assess the effects of isorhapontigenin and resveratrol on α-granule secretion, human PRP was treated with a vehicle control [0.1% (v/v) DMSO] or different concentrations (3.125-100 µM) of isorhapontigenin or resveratrol for 5 min followed by incubation with agonists such as ADP (5 µM), CRP-XL (0.5 µg/ml) and U46619 (1 µM) for 20 min and the level of P-selectin exposure (as a marker for α-granule secretion) was measured using flow cytometry. Interestingly, isorhapontigenin did not show any inhibitory effects on the P-selectin exposure upon stimulation with any of the agonists tested (Fig 4G-I). Similarly, resveratrol also did not inhibit P-selectin exposure on platelets triggered with ADP (Fig 4J) and U46619 (Fig 4K). However, resveratrol moderately inhibited the P-selectin exposure at higher concentrations of 50 µM and 100 µM when platelets were stimulated with CRP-XL (Fig 4L).

Furthermore, the effects of isorhapontigenin and resveratrol on dense granule secretion were assessed in human PRP. The level of ATP secretion (a marker for dense granule secretion) was measured in platelets in the presence and absence of different concentrations of isorhapontigenin or resveratrol upon activation with 5 µM ADP using a luciferin-luciferase luminescence assay. Isorhapontigenin significantly inhibited dense granule secretion at all the concentrations tested (3.125-100 µM) with 100% inhibition observed at 100 µM (Fig 5A). However, resveratrol showed significant inhibition on dense granule secretion at concentrations of 12.5 µM and above (Fig 5B). These data demonstrate that isorhapontigenin primarily affects dense granule secretion but not α-granule secretion in platelets and also, the inhibitory potency of isorhapontigenin is greater than resveratrol.

3.4. Isorhapontigenin inhibits platelet spreading

The outside-in signalling triggered by integrin αIIbβ3 in response to fibrinogen binding leads to platelet spreading (Li et al., 2010) which initiates clot retraction and wound healing. To further evaluate the effects of isorhapontigenin on integrin αIIbβ3-mediated outside-in signalling, platelet spreading on immobilised fibrinogen in the presence and absence of isorhapontigenin was carried out. As shown in Fig 6, platelets treated with isorhapontigenin (3.125 and 6.25 µM) were unable to spread in a similar manner to the untreated control. This data suggests that isorhapontigenin is able to inhibit the outside-in signalling triggered by integrin αIIbβ3.

3.5. Effect of isorhapontigenin on haemostasis in mice

Haemostasis is a normal physiological response of the body for prevention of bleeding upon vascular injury (Batty and Smith, 2010). A tail bleeding assay in mice was performed to investigate the effect of isorhapontigenin on the modulation of haemostasis under physiological conditions. A vehicle control or isorhapontigenin [final concentration of 1.85 μM (IC_{50} concentration) or 6.25 μM] was infused into anaesthetised mice through femoral arteries. After five min of incubation, 3 mm of tail tip was dissected, and the bleeding time was recorded. The mean bleeding time in the isorhapontigenin (1.85 μM)-administered group was about 384 seconds, which was not significantly different from the vehicle-treated group (Average: 347 seconds) (Fig 7A). However, a significant extension of bleeding time was observed when 6.25 μM of isorhapontigenin was used. These data illustrate that isorhapontigenin does not affect haemostasis at its IC_{50} concentration of 1.85 μM, hence, this may be safe and may not cause bleeding complications at this concentration under *in vivo* physiological settings.

3.6. Isorhapontigenin does not exert cytotoxic effects in platelets

In order to assess whether the observed effects of isorhapontigenin are due to its toxicity against platelets, LDH cytotoxicity assay, which is one of the common and sensitive assay (Kumar et al., 2018; Smith et al., 2011) was performed. This was carried out using human PRP in the presence of isorhapontigenin at the concentrations used in this study, along with a vehicle and positive controls. As shown in Fig 7B, isorhapontigenin does not possess any cytotoxic effects on platelets at all the concentrations tested in this study. This indicates that the inhibitory properties observed for isorhapontigenin are due to its pharmacological effects and not due to its adverse toxic effects in platelets.

3.7 Isorhapontigenin affects the phosphorylation of vasodilator-stimulated phosphoprotein (VASP), Akt and cAMP in platelets

Isorhapontigenin has consistently displayed selective inhibition of ADP-stimulated platelet activation. ADP acts via purinergic receptors $P2Y_1$ and $P2Y_{12}$ in platelets to initiate/promote their activation. Specifically, the interaction of ADP with $P2Y_{12}$ receptor promotes platelet aggregation (Hechler and Gachet, 2011; Wijeyeratne and Heptinstall, 2011). As isorhapontigenin inhibited fibrinogen binding and aggregation in platelets upon stimulation with ADP, we hypothesised that isorhapontigenin predominantly mediates its activity via $P2Y_{12}$ receptor. Hence, we explored the effects of isorhapontigenin on the signalling pathways relating to $P2Y_{12}$ receptor by immunoblotting analysis. Binding of ADP to the $P2Y_{12}$ receptor inhibits adenylate cyclase which reduces the levels of cyclic AMP (cAMP) allowing platelet aggregation. To assess the effect of isorhapontigenin on cAMP-mediated signalling, the level of phosphorylation of VASP; a substrate for cAMP protein kinase (Butt et al., 1994; Wentworth et al., 2006) at position S157 was analysed. As shown in Fig

7C, isorhapontigenin has increased the phosphorylation of VASP at S157 in a concentration-dependent manner. Furthermore, the effect of isorhapontigenin on Akt phosphorylation at S473 was analysed to determine the impact of isorhapontigenin on PI3K-mediated signalling. Indeed, isorhapontigenin was found to inhibit Akt phosphorylation (Fig 7D). These results imply that the inhibitory effects of isorhapontigenin may be due to its interference with the cAMP and PI3K-mediated signalling pathways that are associated with the $P2Y_{12}$ receptor in platelets (Hechler and Gachet, 2011; Kim and Kunapuli, 2011).

In order to further investigate the effect of isorhapontigenin on the cAMP signalling, which is an important mediator of platelet activation (Noe et al., 2010), the intracellular cAMP levels in the presence and the absence of isorhapontigenin was determined using a cAMP ELISA kit. The results revealed that the platelets treated with isorhapontigenin prior to the activation by the agonist (ADP-5 μM) showed an increased level of cAMP in a concentration- dependent manner when compared to the platelets treated with agonist alone (Fig 7E). This result further emphasises that isorhapontigenin may interfere with the cAMP-mediated signalling pathways.

3.8. Isorhapontigenin has greater binding affinity towards $P2Y_{12}$ receptor than resveratrol

The above results emphasise that isorhapontigenin exerts greater inhibitory effects than resveratrol on ADP-stimulated platelet activation, which is predominantly mediated via the $P2Y_{12}$ receptor. Hence, the potential molecular interactions between isorhapontigenin or resveratrol and the $P2Y_{12}$ receptor were elucidated by molecular docking analysis. For this, the molecules [isorhapontigenin, resveratrol and clopidogrel (CLO), a $P2Y_{12}$ inhibitor (Bates et al., 2011; Wallentin, 2009; Wijeyeratne and Heptinstall, 2011) for comparison] were docked sequentially into the binding site of the X-ray crystallographic structure of $P2Y_{12}$ receptor (PDB-4PXZ) (J. Zhang et al., 2014) using SYBYL-X 2.1. The docking procedure was first validated by re-docking the extracted co-crystallised ligand of the $P2Y_{12}$ receptor [2-methylthio-adenosine-5'-diphosphate (2MeSADP)] into the prepared protein to be used for docking. The RMSD between the docked conformation generated by the program and the native co-crystallized ligand conformation was found to be 0.248 Å. Since, the RMSD value was well within the 2 Å grid spacing used for docking, it indicated that the docking procedure to be used was reliable and valid. Moreover, the interactions between the docked ligand and the receptor mimicked those observed in the crystal structure of the same.

The results of ligand-directed docking into the ADP-binding site of $P2Y_{12}$ receptor are summarised in Table 1 in decreasing order of the ligand binding affinities, as determined by their docking scores. The binding modes of isorhapontigenin, resveratrol and clopidogrel with $P2Y_{12}$ receptor are depicted in Fig 8. Isorhapontigenin has shown potential interactions with the key residues, Phe-106, Gln-263, Thr-163, Cys-97 and Cys-197 of $P2Y_{12}$ receptor through hydrogen bonding (Fig 8C), whereas resveratrol has interacted only with Gln-263, Thr-163 and Cys-97 (Fig

8D) of $P2Y_{12}$ receptor through hydrogen bonds. Interestingly, the interactions of isorhapontigenin and resveratrol with the $P2Y_{12}$ receptor were found to be distinct from clopidogrel, which interacted with Arg-256 and Try-105 (Fig 8D) residues of the ligand binding pocket of $P2Y_{12}$ receptor. The docking scores suggest that isorhapontigenin (6.68) has a higher binding affinity towards $P2Y_{12}$ receptor than resveratrol (5.97) and clopidogrel (5.95). These results indicate that the superior inhibitory effects of isorhapontigenin on ADP-stimulated platelet activation in comparison to resveratrol could be due to its greater interactions with the $P2Y_{12}$ receptor.

4. Discussion

Polyphenols such as resveratrol are well acknowledged for their cardioprotective [38] and anti-platelet activities (Olas et al., 2002). The recently identified methoxylated analogue, isorhapontigenin has also been found to be bioactive. Similar to resveratrol, isorhapontigenin is a potent antioxidant and it decreases ROS (Reactive Oxygen Species) generation (Lu et al., 2017; Wang et al., 2001). Isorhapontigenin also exhibits anti-cancer activities (Fang et al., 2012; Gao et al., 2014), cardioprotective properties and attenuates cardiac hypertrophy (Li et al., 2005; Liu and Liu, 2004). Isorhapontigenin is attracting greater research interest due to its increased bioavailability (>50%) compared to resveratrol (Yeo et al., 2017). In this study, the effects of isorhapontigenin on platelet activity were investigated in comparison to resveratrol in human platelets.

This study demonstrates that isorhapontigenin selectively inhibits ADP-induced platelet activation with an IC_{50} of around 1.85 μM. However, marginal or no inhibition was observed on platelet activation when stimulated with other agonists such as collagen, CRP-XL and U46619. In contrast, resveratrol displayed inhibitory effects on all the agonists (ADP, collagen, CRP-XL and U46619)-stimulated platelet activation but only at higher concentrations (>50 μM). Notably, resveratrol completely abolished the platelet activation stimulated by collagen at 100 μM. These results are in line with the previous studies where resveratrol was found to largely inhibit platelet aggregation induced by collagen (1-2 μg/ml), U46619 (1 μM) and arachidonic acid (0.7 μM) but have a marginal inhibitory effect on ADP-induced platelet activation (5-10 μM) (Shen et al., 2007; Stef et al., 2006). Similar inhibitory profiles were observed for both isorhapontigenin and resveratrol when tested in isolated platelets upon collagen and U46619-induced activations. Furthermore, isorhapontigenin was able to inhibit inside-out signalling to integrin αIIbβ3 where the level of fibrinogen binding was significantly reduced upon stimulation with ADP although no effects were observed on CRP-XL and U46619 triggered activation. However, resveratrol did not show significant effects on the modulation of inside-out signalling to integrin αIIbβ3. This corroborates the results of a previous study, which reports that the inhibitory effect of resveratrol does not involve interaction with the integrin αIIbβ3 complex (Shen et al., 2007). The granule secretion is essential for platelet activation and thrombus formation (Sharda and Flaumenhaft, 2018).

Interestingly, isorhapontigenin did not affect α-granule secretion but it markedly inhibited ATP release from dense granules at all the concentrations tested. However, resveratrol moderately inhibited α-granule secretion only upon stimulation with CRP-XL and it inhibited ATP release from dense granules when triggered with ADP but at a higher concentration (>25 μM) than isorhapontigenin. A previous study reports that resveratrol inhibits dense granule secretion triggered by collagen (Shen et al., 2007). These results highlight that isorhapontigenin preferentially affects ADP-stimulated platelet activation with greater potential than resveratrol. In addition, isorhapontigenin was found to significantly reduce the platelet spreading on immobilised fibrinogen that is mediated via integrin αIIbβ3. This suggests that isorhapontigenin may modulate the bidirectional signalling of integrin αIIbβ3 (both inside-out and outside-in). Remarkably, isorhapontigenin was found to be non-toxic to platelets at the concentrations tested here and it does not affect haemostasis in mice at the IC_{50} concentration of 1.85 μM.

Polyphenolic compounds are known to inhibit enzymes and protein kinases that regulate various cellular functions (Li et al., 2012). Isorhapontigenin has been reported to inhibit NF-κB, MAPKs and PI3K/AKt signalling proteins in cardiomyocytes (Gao et al., 2014; Liu and Liu, 2004). As isorhapontigenin predominantly inhibited ADP-stimulated platelet activation, the signalling analyses of proteins related to $P2Y_{12}$ signalling pathway suggest that isorhapontigenin has increased VASP phosphorylation at serine-157, which is a marker for cAMP-mediated signalling (Wentworth et al., 2006). Phosphorylation of VASP at serine-157 is linked with reduced activation of integrin αIIbβ3 and inhibition of platelet aggregation (Horstrup et al., 1994). In addition, the phosphorylation of VASP negatively regulates actin nucleation and bundling properties and therefore reduces cytoskeletal reorganisation that is essential for platelet shape change (Aszódi et al., 1999). Therefore, increase in VASP phosphorylation by isorhapontigenin suggests that it may interfere with the cAMP signalling pathway and thereby inhibits platelet activation. Furthermore, isorhapontigenin also elevated the levels of cAMP in a concentration-dependent manner. Isorhapontigenin has also inhibited the Akt phosphorylation, which is an effector molecule of the PI3K signalling pathway (Guidetti et al., 2015) that initiates platelet activation. It is interesting to note that in a previous study, its analogue, resveratrol has been reported to be a non-inhibitor of Akt phosphorylation in platelets upon stimulation by various agonists and it had not altered the cAMP levels in activated platelets (Shen et al., 2007). Furthermore, the molecular interactions of isorhapontigenin and resveratrol with $P2Y_{12}$ receptor as predicted by the *in silico* studies suggest that isorhapontigenin has greater binding affinity towards $P2Y_{12}$ receptor than resveratrol. When compared with the interaction of clopidogrel (CLO) docked into the same site, these polyphenols were found to interact with different residues of the ligand binding pockets with relatively more hydrogen bonds. Interestingly, clopidogrel has been found to interact with Arg-256 and this was reported to be important for the activation of $P2Y_{12}$ receptor (K. Zhang et al., 2014). However, the polyphenols isorhapontigenin and resveratrol have been predicted to interact with Cys-97 that has been recognised as a covalent binding site for the active metabolites of $P2Y_{12}$ receptor drugs

(Algaier et al., 2008; Ding et al., 2009). Taken together, these results suggest that isorhapontigenin and resveratrol act differently in platelets and these differential effects could be due to the structural differences where isorhapontigenin possess an additional methoxy group. Isorhapontigenin's superior bioavailability than resveratrol coupled with its selective and potent inhibition on ADP-stimulated platelet activation suggests that isorhapontigenin may be a promising molecular template for the design and development of novel anti-platelet agents to prevent and treat thrombotic diseases.

Conflict of interest

The authors declare no competing financial and non-financial interests.

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Authors contributions

D.R and S.V designed the study, performed experiments, analysed data and wrote the paper; D.A.I.A, V.C, P.H.P, R.V, H.W, M.S and K.W performed experiments and analysed data; K.P and P.R.D provided guidance and support for the design of experiments and analysis of data.

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Figures legends

Figure 1: **Effect of isorhapontigenin on agonists-induced platelet aggregation**. The chemical structures of isorhapontigenin **(Ai)** and resveratrol **(Aii)** are shown. Human platelet-rich plasma was incubated with a vehicle control [0.1% (v/v) DMSO] or different concentrations of isorhapontigenin for 5 min prior to stimulation with agonists ADP (5 μM) **(B),** collagen (1 μg/ml) **(C)**,

CRP-XL (0.5 µg/ml) **(D)** or U46619 (1 µM) **(E)** and the level of platelet aggregation was monitored for 5 min by optical aggregometry. Representative aggregation traces of three separate experiments are shown. The percentage of aggregation for treated samples was calculated by considering the maximum aggregation obtained with the vehicle control at 5 min as 100%. The cumulative data shown represent mean ± S.E.M (n=3). *Indicates significant difference with respect to the control and #indicates significant difference with respect to the respective resveratrol concentrations presented in Fig 2, (*P<0.05, **P<0.01 and ***, ###P<0.001). **(F)** IC_{50} of isorhapontigenin on ADP-stimulated platelet aggregation: Human platelet-rich plasma was incubated with a vehicle control [0.1% (v/v) DMSO] or various concentrations of isorhapontigenin (0.19 µM–100 µM) for 5 min prior to stimulation with ADP (5 µM). The level of aggregation was monitored for 5 min. IC_{50} value was determined using GraphPad Prism. The cumulative data shown represent mean ± S.E.M (n=5); ****P<0.0001. The *P values* shown are as calculated by one-way ANOVA followed by Bonferroni *post-hoc* test; ns: non-significant.

Figure 2: **Effect of resveratrol on agonists-induced platelet aggregation**. Human platelet-rich plasma was incubated with a vehicle control [0.1% (v/v) DMSO] or different concentrations of resveratrol for 5 min prior to stimulation with agonists ADP (5 µM) **(A)**, collagen (1 µg/ml) **(B)**, CRP-XL (0.5 µg/ml) **(C)** or U46619 (1 µM) **(D)** and the level of platelet aggregation was monitored for 5 min by optical aggregometry. Representative aggregation traces of three separate experiments are shown. The percentage of aggregation for treated samples was calculated by considering the maximum aggregation obtained with the vehicle control at 5 min as 100%. The cumulative data shown represent mean ± S.E.M (n=3). *Indicates significant difference with respect to the control and #indicates significant difference with respect to the respective isorhapontigenin concentrations presented in Fig 1, (*P<0.05, **, ###P<0.01 and ***, ###P<0.001). The *P values* shown are as calculated by one-way ANOVA followed by Bonferroni *post-hoc* test.

Figure 3: **Effect of isorhapontigenin and resveratrol on agonists-induced aggregation in isolated platelets**. Human isolated platelets were incubated with a vehicle control [0.1% (v/v) DMSO] or different concentrations of isorhapontigenin (**A, B**) or resveratrol (**C, D**) for 5 min prior to stimulation with agonists collagen **(Isorhapontigenin-A; Resveratrol-C)**, or U46619 **(Isorhapontigenin -B; Resveratrol-D)** and the level of platelet aggregation was monitored for 5 min by optical aggregometry. Representative aggregation traces of three separate experiments are shown. The percentage of aggregation for treated samples was calculated by considering the maximum aggregation obtained with the vehicle control at 5 min as 100%. The cumulative data shown represent mean ± S.E.M (n=3). *Indicates significant difference with respect to the control (*P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001). The *P* values shown are as calculated by one-way ANOVA followed by Bonferroni *post-hoc* test.

Figure 4: **Effects of isorhapontigenin and resveratrol on inside-out signalling to integrin αIIbβ3 and α-granule secretion.** Human platelet-rich plasma was incubated with a vehicle control

[0.1% (v/v) DMSO] or diverse concentrations of isorhapontigenin or resveratrol for 5 min prior to the addition of ADP (5 μM), CRP-XL (0.5 μg/ml) or U46619 (1 μM) and further incubated for 20 min at room temperature. The level of fibrinogen binding (as a marker for inside-out signalling to integrin αIIbβ3) to the platelet surface and the level of P-selectin exposed (as a marker for α-granule secretion) on the platelet surface were quantified using FITC-conjugated anti-human fibrinogen antibodies and PECy5-conjugated anti-human P-selectin antibodies, respectively by flow cytometry. The bar graph shows the effects on inside-out signalling by isorhapontigenin upon stimulation with agonists such as ADP **(A)**, U46619 **(B)** and CRP-XL **(C)** and by resveratrol upon stimulation with agonists on ADP **(D)**, U46619 **(E)** and CRP-XL **(F)**. The bar graph shows the effects on α-granule secretion by isorhapontigenin upon activation of platelets with agonists such as ADP **(G)**, U46619 **(H)** and CRP-XL **(I)** and by resveratrol on agonists ADP **(J)**, U46619 **(K)** and CRP-XL **(L)-**induced platelet activation. The extent of inhibition in treated samples was calculated by considering the level of fluorescence obtained with the vehicle control as 100%. Cumulative data represent mean ± S.E.M (n=3). The *P values* shown (*P<0.05) are as calculated by one-way ANOVA followed by Bonferroni *post-hoc* test.

Figure 5: **Effect of isorhapontigenin and resveratrol on dense granule secretion**. Dense granule secretion was determined based on the amount of ATP released from platelets by lumi-aggregometry. Human PRP was mixed with 50 μl of luciferin-luciferase reagent for 2 min, followed by incubation with various concentrations of isorhapontigenin or resveratrol for a further 5 min. Platelets were activated with ADP (5 μM) and the level of ATP release was monitored for 5 min. Inhibitory effects of isorhapontigenin **(A)** and resveratrol **(B)** on dense granule secretion are shown. The cumulative data represent mean ± S.E.M (n=6). *Indicates significant difference with respect to controls and $^{\#}$indicates significant difference with respect to the respective resveratrol concentrations. The *P values* shown (*, $^{\#}$P<0.05, **P<0.01, ***P<0.01 and ***P<0.001) are as calculated by one-way ANOVA followed by Bonferroni *post-hoc* test.

Figure 6: **Effect of isorhapontigenin on platelet spreading**. Human isolated platelets were treated with vehicle control [0.1% (v/v) DMSO] or isorhapontigenin (3.125 and 6.25 μM) for 5 min and were allowed to spread on fibrinogen- (100 μg/ml) coated cover-glasses (45 min). The platelets stained with Alexa Fluor 488 conjugated phalloidin were imaged using confocal microscopy. **(A)** Representative images of platelet spreading (n=3). **(B)** The graph showing the number of adhered and fully spread platelets. *Indicates significant difference with respect to control. The *P values* shown (***P<0.01 and ****P<0.001) are as calculated by one-way ANOVA followed by Bonferroni *post-hoc* test.

Figure 7: **Effects of isorhapontigenin on haemostasis, cytotoxicity, phosphorylation of VASP and Akt, and on cAMP levels**. **(A)** Effect of isorhapontigenin on haemostasis in mice: Mice were anaesthetised and a vehicle control [0.1% (v/v) DMSO] or isorhapontigenin (1.85 μM or 6.25 μM) was injected via femoral artery. After 5 min of incubation, 3 mm of tail tip was dissected using a

scalpel blade and the tail tip was placed in sterile PBS. The time for cessation of bleeding was measured up to 20 min. Data represent ± S.E.M (n=6 mice in each group). The *p* value shown (***P<0.01) was calculated by non-parametric Kruskal-Wallis test using Graphpad Prism; ns: non-significant**. (B)** The cytotoxicity of isorhapontigenin on human platelets: Human PRP was treated with a positive control, a vehicle control [0.1% (v/v) DMSO] or various concentrations of isorhapontigenin for 30 min and the amount of LDH released (a marker for cytotoxicity) was measured at 490 nm and 650 nm using spectrofluorimetry. The level of LDH release for isorhapontigenin-treated samples was calculated by considering the maximum LDH release obtained with the positive control as 100%. Data represent mean ± S.E.M (n=3). The statistical significance was calculated by one-way ANOVA. Impact of isorhapontigenin on the phosphorylation of VASP **(C)** and Akt **(D)**: Human isolated platelets were treated with a vehicle control (R) or various concentrations of isorhapontigenin for 5 min before the addition of 5 µM ADP and further incubation for 5 min in an aggregometer at 37 °C. The level of VASP phosphorylation at residue S157 and Akt phosphorylation at residue S473 was detected using platelet lysates by immunoblot analysis. The level of 14-3-3ζ was detected as a loading control. The provided blots are representative of three separate experiments. The original uncropped full-length images of these blots are provided in SI (Fig S2). Effect of isorhapontigenin on cAMP levels **(E):** The total cellular cAMP levels in platelets pre-treated with vehicle [0.1% (v/v) DMSO] or different concentrations of isorhapontigenin (3.125-100 µM) and stimulated with ADP (10 µM) were determined using cAMP ELISA kit. Control corresponds to platelets treated with EGTA (1 mM), a platelet inhibitor (to achieve maximum cAMP levels). Cumulative data represent mean ± S.E.M (n=3). $^{\#}$Indicates significant difference with respect to control and *indicates significant difference with respect to '0' concentration of isorhapontigenin. The *P values* shown (*P<0.05, ***P<0.01 and $^{\#,}$***P<0.001) are as calculated by one-way ANOVA followed by Bonferroni *post-hoc* test.

Figure 8: **Molecular interactions of isorhapontigenin and resveratrol with $P2Y_{12}$ receptor**. Full view of the $P2Y_{12}$ receptor showing the binding mode of isorhapontigenin as predicted by *in silico* docking analysis **(A)**. Superimposition of isorhapontigenin**,** resveratrol and clopidogrel interacting with the ligand binding pocket of $P2Y_{12}$ receptor **(B)**. Docking modes of isorhapontigenin **(C)**, resveratrol **(D)** and clopidogrel **(E)** with $P2Y_{12}$ receptor as predicted by molecular docking of isorhapontigenin and resveratrol into the ADP binding site of the X-ray crystallographic structure of $P2Y_{12}$ receptor (PDB:4PXZ) using SYBYL-X 2.1 software. The 2D interactions of isorhapontigenin **(F)**, resveratrol **(G)** and clopidogrel **(H)** with the key amino acid residues of $P2Y_{12}$ receptor as generated by LigPlot^{+} (Laskowski and Swindells, 2011).

Figure 1

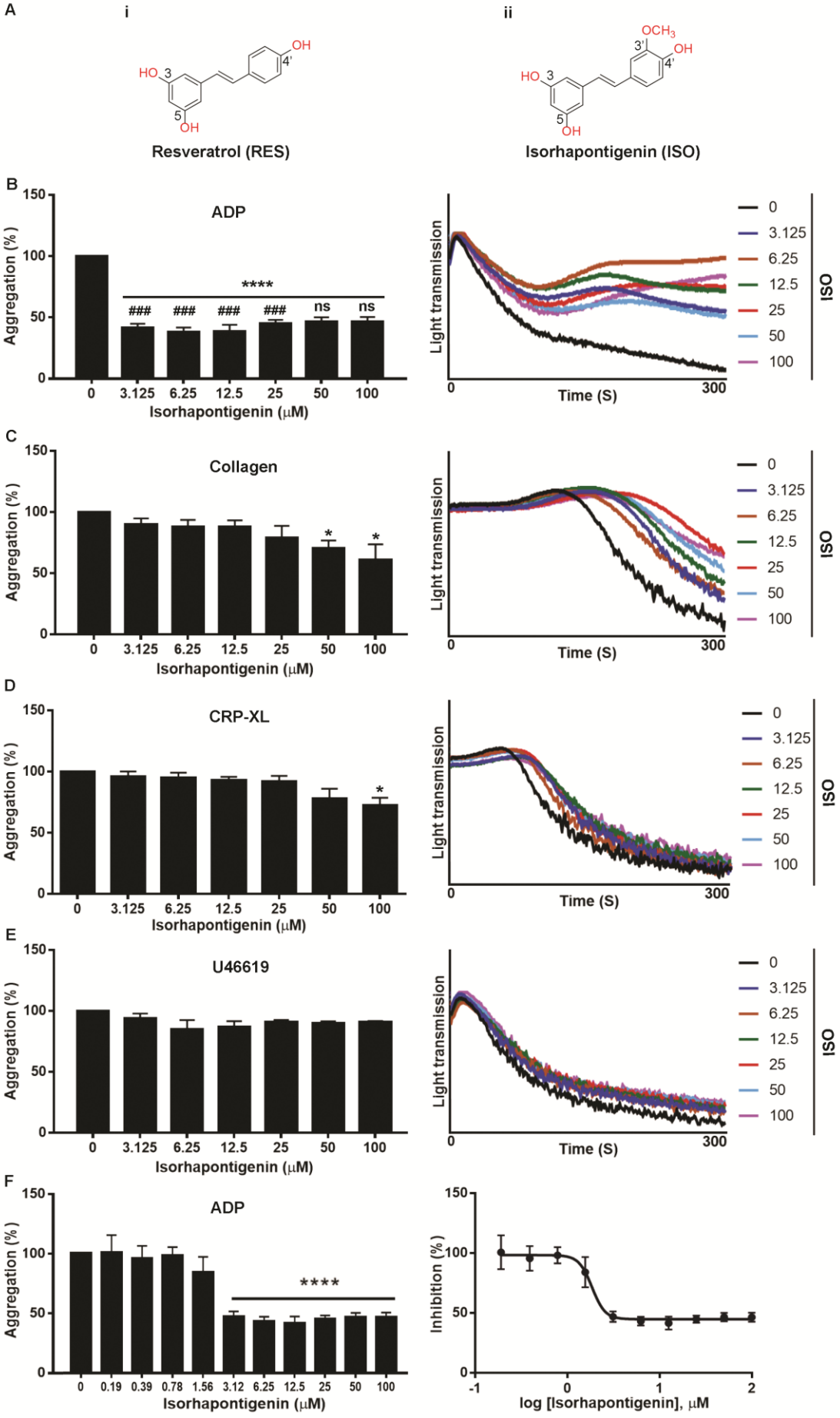
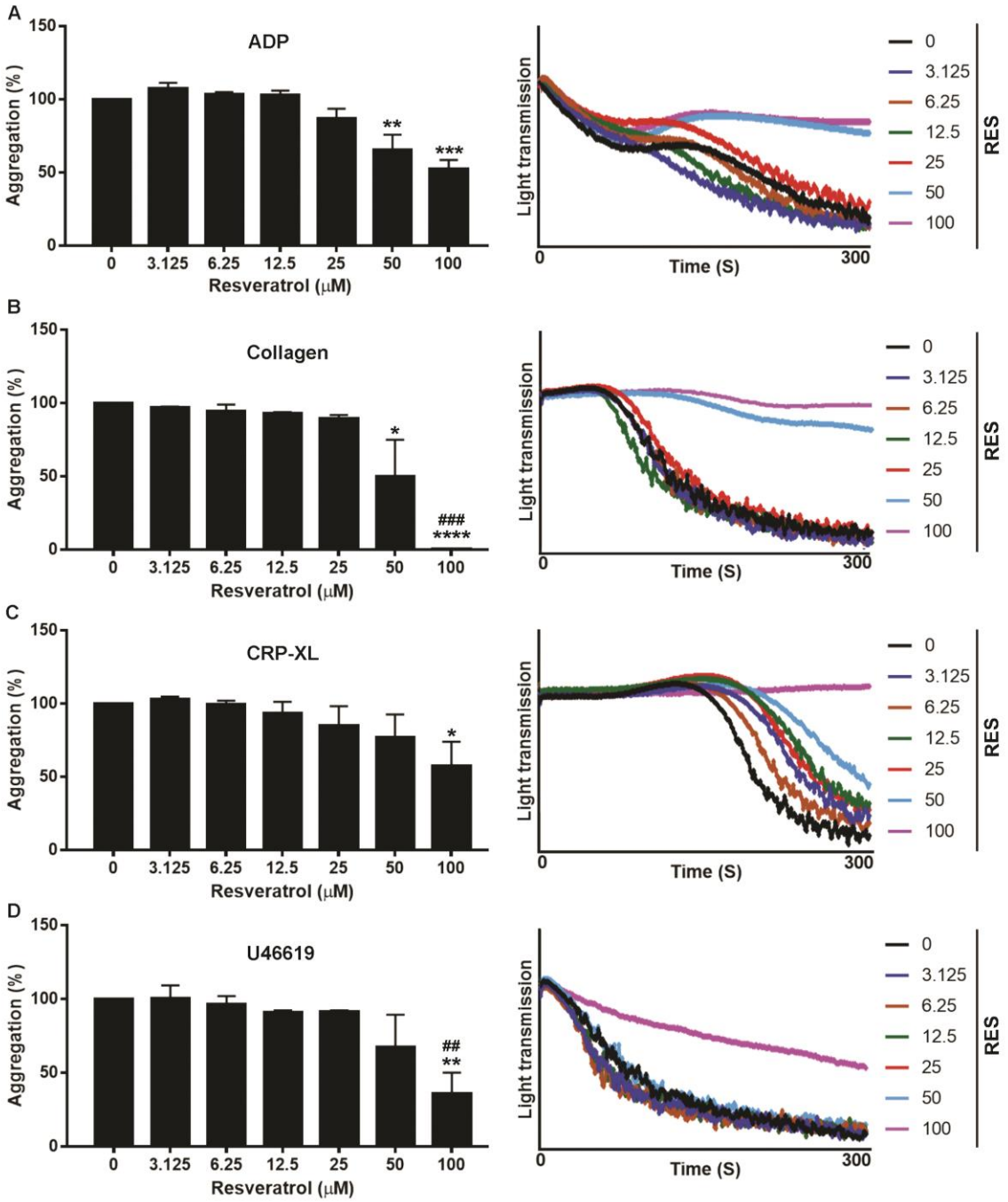

A
i
ii
Resveratrol (RES)
Isorhapontigenin (ISO)
B
ADP
Aggregation (%)
Isorhapontigenin (μM)
Light transmission
Time (S)
ISO
C
Collagen
D
CRP-XL
E
U46619
F
ADP
Inhibition (%)
log [Isorhapontigenin], μM

Figure 2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ADP
Aggregation (%)
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100
50
0
**
***
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Resveratrol (μM)
Light transmission
0
Time (S)
300
0
3.125
6.25
12.5
25
50
100
RES
B
Collagen
Aggregation (%)
*
###
****
0 3.125 6.25 12.5 25 50 100
Resveratrol (μM)
Light transmission
Time (S)
RES
C
CRP-XL
Aggregation (%)
*
0 3.125 6.25 12.5 25 50 100
Resveratrol (μM)
Light transmission
Time (S)
RES
D
U46619
Aggregation (%)
##
**
0 3.125 6.25 12.5 25 50 100
Resveratrol (μM)
Light transmission
Time (S)
RES

Figure 3

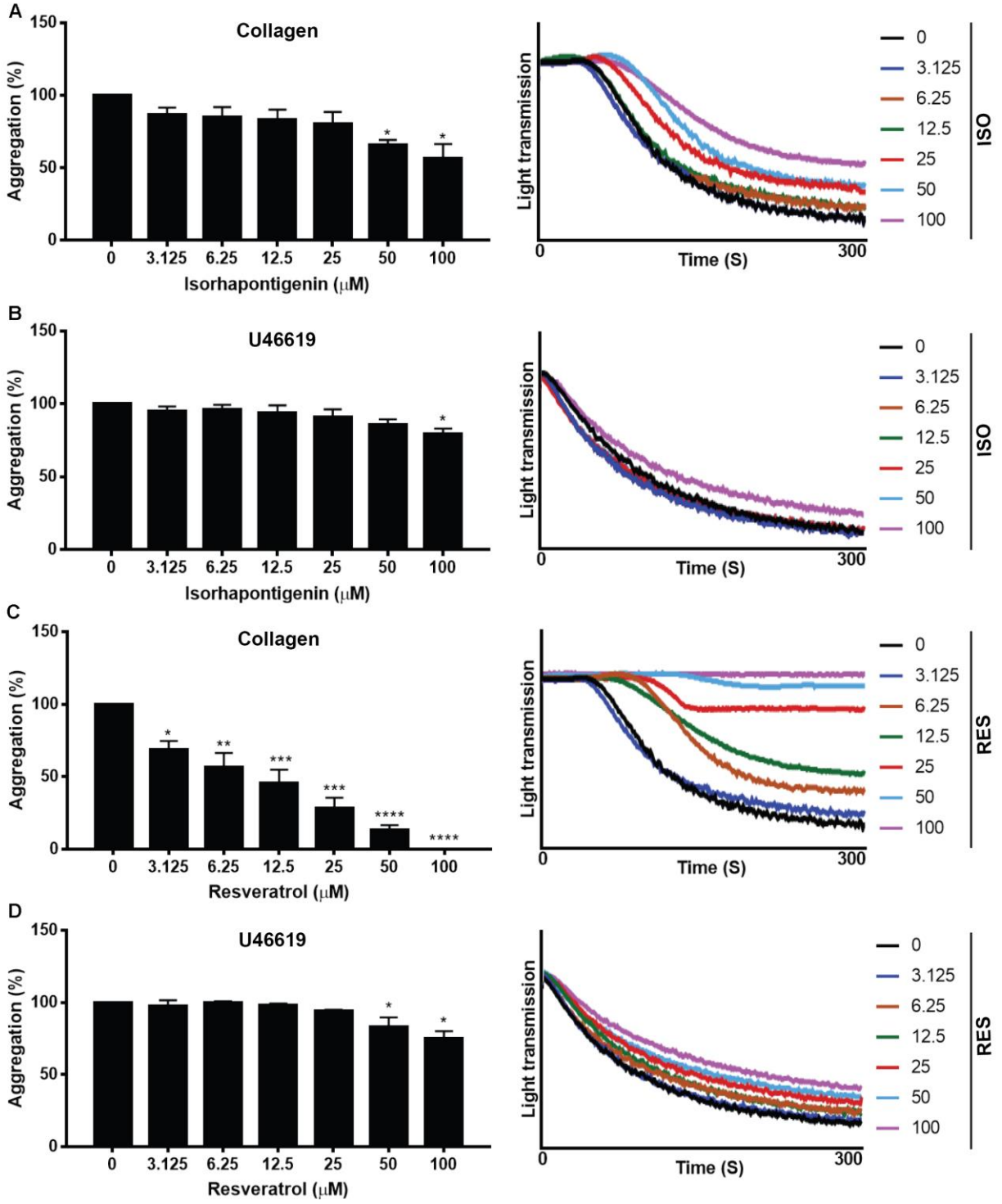

A
Collagen
Aggregation (%)
150
100
50
0
0
3.125
6.25
12.5
25
50
100
Isorhapontigenin (μM)
Light transmission
Time (S)
0
300
0
3.125
6.25
12.5
25
50
100
ISO
B
U46619
Aggregation (%)
Isorhapontigenin (μM)
Light transmission
Time (S)
ISO
C
Collagen
Aggregation (%)
Resveratrol (μM)
Light transmission
Time (S)
RES
D
U46619
Aggregation (%)
Resveratrol (μM)
Light transmission
Time (S)
RES

Figure 4

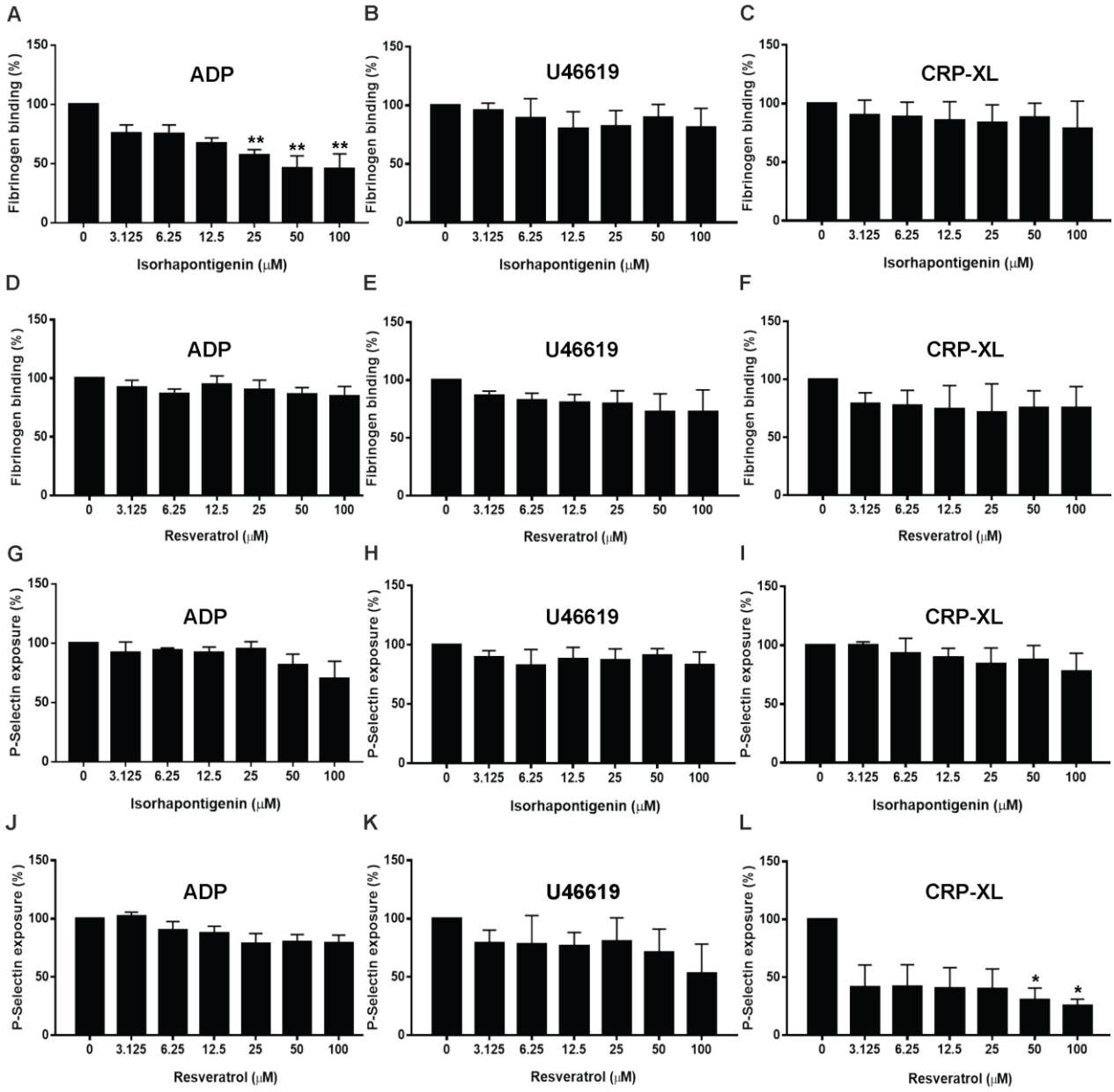

A
ADP
Fibrinogen binding (%)
Isorhapontigenin (μM)
B
U46619
Fibrinogen binding (%)
Isorhapontigenin (μM)
C
CRP-XL
Fibrinogen binding (%)
Isorhapontigenin (μM)
D
ADP
Fibrinogen binding (%)
Resveratrol (μM)
E
U46619
Fibrinogen binding (%)
Resveratrol (μM)
F
CRP-XL
Fibrinogen binding (%)
Resveratrol (μM)
G
ADP
P-Selectin exposure (%)
Isorhapontigenin (μM)
H
U46619
P-Selectin exposure (%)
Isorhapontigenin (μM)
I
CRP-XL
P-Selectin exposure (%)
Isorhapontigenin (μM)
J
ADP
P-Selectin exposure (%)
Resveratrol (μM)
K
U46619
P-Selectin exposure (%)
Resveratrol (μM)
L
CRP-XL
P-Selectin exposure (%)
Resveratrol (μM)
0 3.125 6.25 12.5 25 50 100

Figure 5

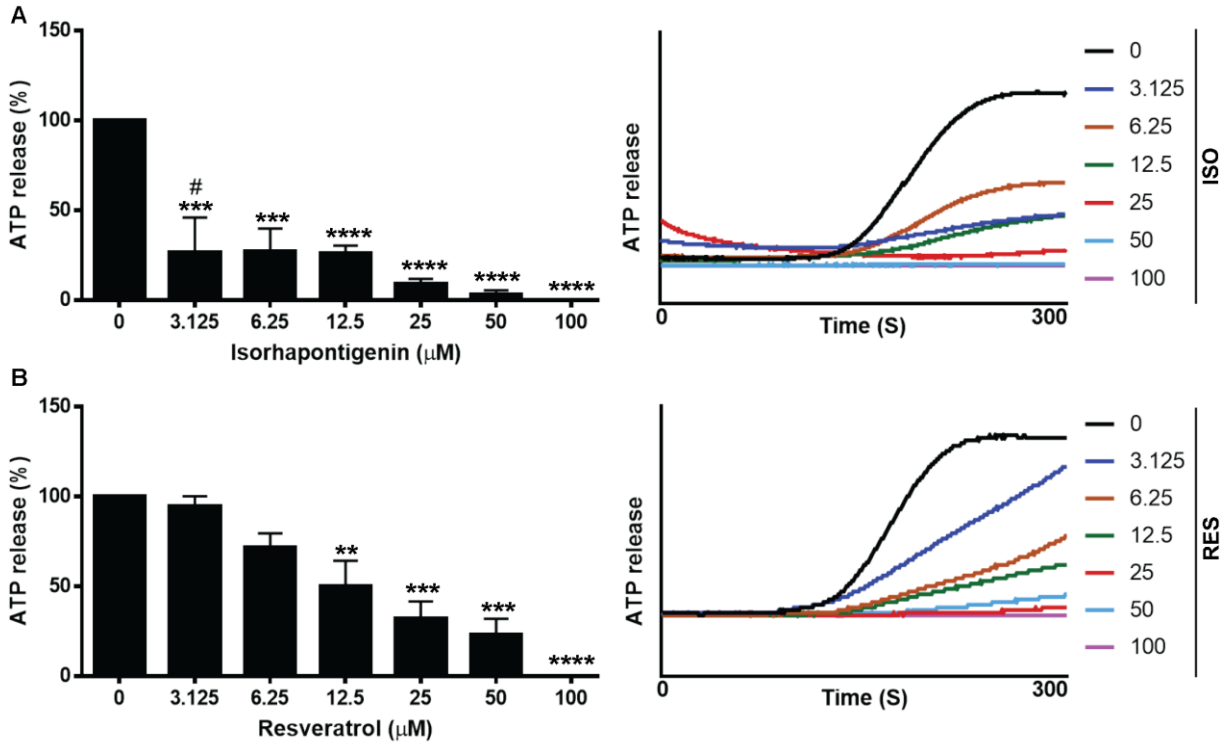


Figure 6

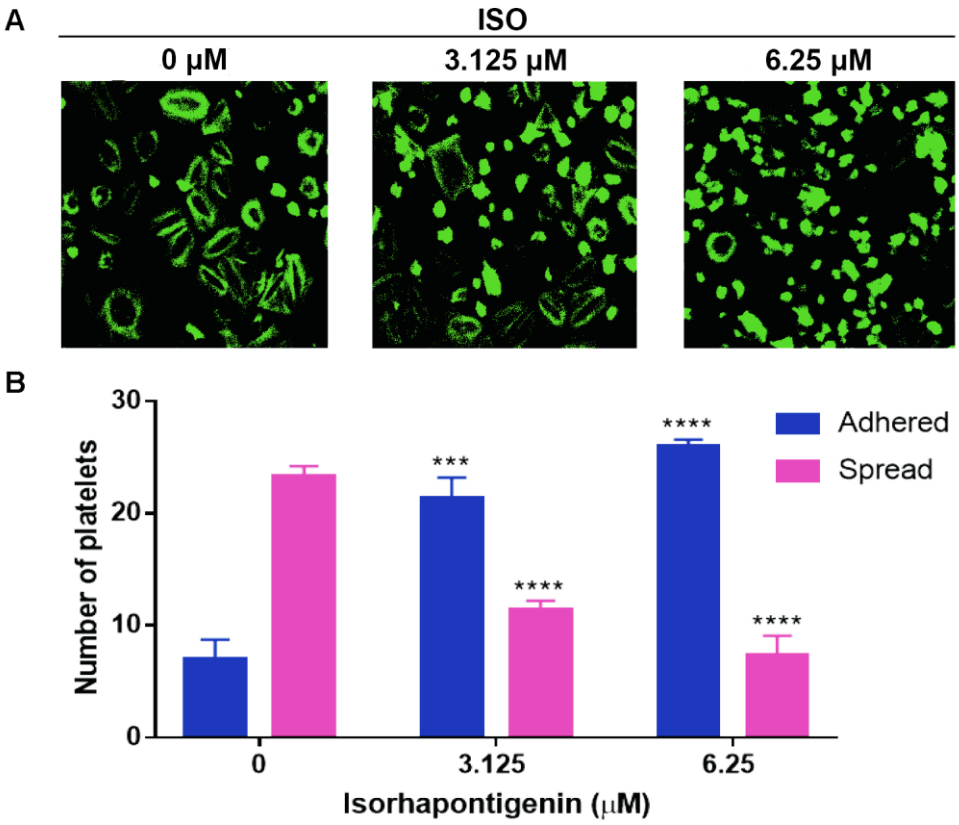


Figure 7

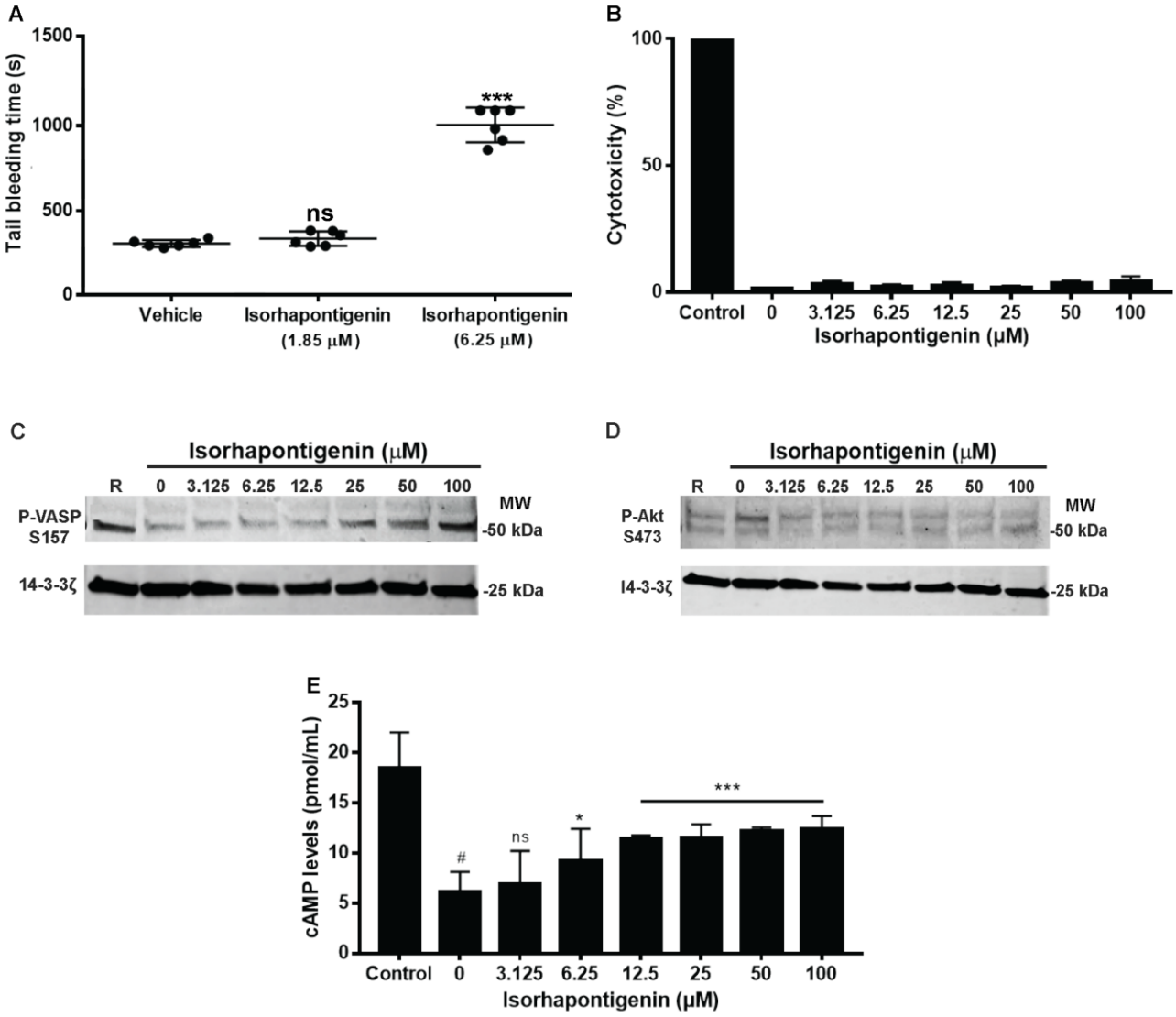
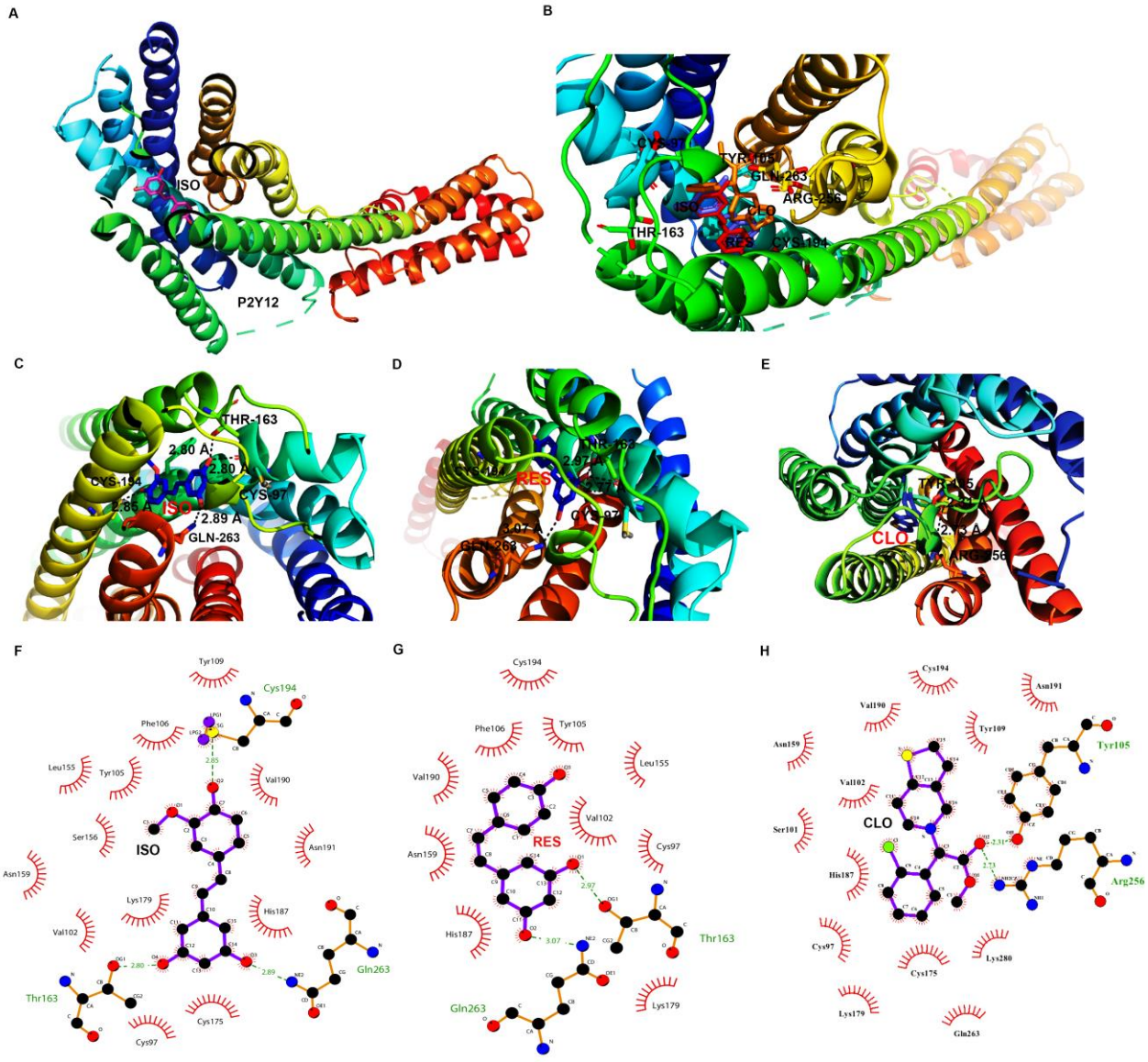

A
Tail bleeding time (s)
1500
1000
500
0
***
ns
Vehicle
Isorhapontigenin (1.85 μM)
Isorhapontigenin (6.25 μM)
B
Cytotoxicity (%)
100
50
0
Control
0
3.125
6.25
12.5
25
50
100
Isorhapontigenin (μM)
C
Isorhapontigenin (μM)
R
0
3.125
6.25
12.5
25
50
100
P-VASP S157
MW
-50 kDa
14-3-3ζ
-25 kDa
D
Isorhapontigenin (μM)
R
0
3.125
6.25
12.5
25
50
100
P-Akt S473
MW
-50 kDa
14-3-3ζ
-25 kDa
E
cAMP levels (pmol/mL)
25
20
15
10
5
0
#
ns
*
***
Control
0
3.125
6.25
12.5
25
50
100
Isorhapontigenin (μM)

Figure 8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ISO
P2Y12
B
TYR-105
GLN-263
ARG-256
ISO
CLO
THR-163
RES
CYS-194
C
THR-163
2.80 A
2.80 A
CYS-194
CYS-97
2.86 A
ISO
2.89 A
GLN-263
D
RES
E
CLO
F
Tyr109
Cys194
Phe106
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Tyr105
Val190
Ser156
ISO
Asn191
Asn159
Lys179
His187
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Thr163
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Cys97
G
Cys194
Phe106
Tyr105
Val190
Leu155
Val102
RES
Cys97
Asn159
His187
Thr163
Gln263
Lys179
H
Cys194
Asn191
Val190
Tyr109
Tyr105
Asn159
Val102
CLO
Ser101
His187
Arg256
Cys97
Lys280
Cys175
Lys179
Gln263

Table 1 Summary of the polar contacts and van der Waals interaction between isorhapontigenin, resveratrol and clopidogrel and $P2Y_{12}$ receptor

Molecule	Docking score	Hydrogen bond interactions			Number of van der Waal's interaction
		Interacting ligand atom	Interacting receptor residues	Average distance (Å)	
Isorhapontigenin	6.68	4' (O)	Cys-194 (S)	2.85	149
		5 (O)	Gln-263 (N)	2.89	
		3 (O)	Thr-163 (OG1)	2.80	
		3 (O)	Cys-97 (O)	2.80	
Resveratrol	5.97	3 (O)	Thr-163 (OG1)	2.97	112
		3 (O)	Cys-97 (O)	2.77	
		5 (O)	Gln-263 (NE2)	3.07	
Clopidogrel	5.95	2 (O)	Arg-256 (N)	2.73	117
		2 (O)	Tyr-105 (O)	2.31	