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Title Page

Original Article

Inhibition of platelet mediated arterial thrombosis and platelet granule exocytosis by 3'4'-dihydroxyflavonol and quercetin

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Abstract

Flavonols are polyphenolyic compounds that produce cardio-protective effects. Antiplatelet potential of quercetin (Que) has been reported, but the antiplatelet mechanism is not fully elucidated. No studies have evaluated the antiplatelet potential of the structurally related synthetic flavonol 3', 4'-dihydroxyflavonol (DiOHF), which has been shown to have greater antioxidant capacity and improve endothelial function. Furthermore, the effect of Que or DiOHF on arterial blood flow in arterial thrombosis has not been investigated. We therefore investigated the mechanism of platelet inhibition and potential to delay arterial thrombosis by Que and DiOHF. Specifically, dense and α-granule exocytosis, GPIIb/IIIa receptor activation, fibrinogen binding and blood flow following arterial injury were investigated. Both Que and DiOHF showed a concentration dependant inhibition of collagen, adenosine diphosphate (ADP) and arachidonic acid (AA) stimulated platelet aggregation. Que and DiOHF inhibited agonist induced granule exocytosis. Greater inhibition of dense granule exocytosis occurred with DioHF, as measured by both ATP release, and fluorescent quinacrine uptake and thrombin-induced release. In contrast, while Que inhibited agonist induced P-selectin expression as measured by both platelet surface P-selectin expression and upregulation of surface GPIIb-IIIa expression, inhibition by DiOHF was not significant for either parameter. Both Que and DiOHF inhibited agonist induced GPIIb/IIIa receptor activation as demonstrated by PAC-1 and fibrinogen binding. C57BL/6 mice treated with 6 mg / kg IV Que or DiOHF maintained greater blood flow following $FeCl_3$ induced carotid artery injury when compared to the vehicle control. These data provide evidence of inhibition of platelet activation, aggregation, and granule exocytosis by Que and DiOHF. Que and DiOHF had different potency for inhibition of the dense and α-granules. We provide the first evidence that Que and DiOHF improve blood flow following arterial injury.

Flavonols, a major subgroup of the flavonoids, are polyphenolic molecules widely found in fruits and vegetables [1,2]. Flavonols exert a variety of biological activities including antioxidant, anti-inflammatory and vasorelaxant effects [3,4] which are all believed to contribute to their capacity to decrease the incidence of cardiovascular disease [5-12].

Whilst considerable attention has been paid to the antioxidant activity of flavonols as a major contributor to their cardioprotective actions, there is growing evidence of other properties that may be of importance. There have been several studies demonstrating that flavonols, particularly quercetin (Que), have anti-platelet aggregation activity that may also contribute to their beneficial effects [13-16]. Several mechanisms of action have been proposed including inhibition of cyclooxygenase or phosphodiesterases [17], antagonism of the TxA_2 receptor [18-20], as well as more recent evidence showing inhibition of kinase activity [21-24]. In particular, Que is reported to decrease Fyn and PI3 kinase activity and to inhibit tyrosine phosphorylation of Syk and PLCγ2 [21,22]. Importantly when human subjects were treated with a single dose of Que (150 or 300 mg p.o.) there was a significant inhibition of platelet aggregation accompanied by inhibition of the same kinases observed in vitro [23]. One study has demonstrated inhibition of collagen stimulated serotonin release from platelets following incubation with Que, suggesting inhibition of dense granule exocytosis [16], but the effect on α-granules has not yet been explored.

Recently we have demonstrated that the synthetic flavonol 3', 4'-dihydroxyflavonol (DiOHF) is able to reduce injury after myocardial ischaemia and reperfusion [12,25-27] and to improve endothelial function in diabetes [10]. As a structurally related molecule to Que, DiOHF may likewise inhibit platelet aggregation. However, the antiplatelet potential of DiOHF has not previously been explored.

While the functional impact of Que on the capacity of platelets to aggregate has been explored, it is not clear whether this relates to impaired activation of GPIIb/IIIa, impaired fibrinogen binding, granule exocytosis, or other mechanisms. Nor is it clear whether these reported antiplatelet activities can result in clinically relevant antithrombotic effects *in vivo* at achievable doses.

Accordingly, the aims of this study were to elucidate and compare the effects of the flavonols Que and DiOHF on human platelet GPIIb/IIIa activation, fibrinogen binding and granule exocytosis *in vitro*, and arterial blood flow in an *in vivo* model of arterial thrombosis.

Methods

Human volunteers

RMIT University Human Ethics Committee approval and informed consent was obtained prior to blood collection. Subjects were healthy volunteers of both sexes, aged 18 - 60 years with no history of vascular disease, bleeding disorders or thrombosis and had not taken aspirin or any other medication that affects platelet function for at least two weeks prior to the study.

Sample preparation

Blood collection was performed using established methods for platelet function studies [28,29]. Briefly, fresh whole blood was collected by antecubital venepuncture into 3.8% (w/v) sodium citrate Vacuette tubes and used immediately for flow cytometric studies. Platelet rich plasma (PRP) for platelet aggregation studies was obtained from the fresh blood after centrifugation at 200 x g for 10 min at room temperature. Platelet poor plasma (PPP) was obtained by centrifugation of the remaining blood at 1800 x g for 15 min at room temperature. Aggregation studies were completed within 3 hours of blood collection.

Platelet aggregation

The effect of Que, DiOHF (both sourced from Indofine Chemicals Inc, NJ. USA) or vehicle on agonist induced light transmittance platelet aggregation was determined. Flavonol samples were incubated with PRP at 37°C for 5 min to achieve concentrations ranging from 0.1 to 1.0 mM in 1 % (v/v) dimethyl sulfoxide (DMSO) (n = 3). Aggregation was stimulated by 5 µg.ml^{-1} collagen, 10 µM ADP and 0.5 mM AA (all agonists were sourced from Chrono-Log Co, USA). Turbidometric platelet aggregation was calibrated against a PPP control (100% aggregation) and the maximal aggregation over a 6 minute period was recorded. ATP release was measured in the presence of luciferin-luciferase reagent (Chrono-Log Co) against a 2 nM ATP standard by luminescence at 0.1 and 1.0 mM of Que or DiOHF.

Flow cytometric immunophenotyping

The effect of Que or DiOHF on platelet activation (measured by PAC-1 binding), α-granule exocytosis (measured by P-selectin expression and changes in surface CD61 expression with alpha granule release) and fibrinogen binding was performed using established whole blood flow cytometric methods [28,30,31]. Fresh citrated whole blood from healthy volunteers (n = 6) was diluted 1:5 with HEPES saline buffer (10 mM HEPES, 0.15 M NaCl, pH 7.4) and incubated with 1 mM Que, DiOHF or vehicle at 37°C for 5 min.

For assessment of α-granule exocytosis and GPIIb/IIIa activation, samples were labelled with fluorescently conjugated monoclonal CD42b PC5 (BD-Pharmingen, USA) with either PAC-1 FITC (BD Biosciences, USA) and anti-CD62P PE (BD-Pharmingen) or PAC-1 FITC with 2 μM eptifibatide (Millennium Pharmaceuticals, Japan) and mouse IgG1 PE isotype as controls. For assessment of agonist induced changes in platelet surface GPIIb-IIIa expression, separate aliquots of vehicle, DiOHF or Que treated 1:50 whole blood in saline were labelled with CD61 FITC (BD-Pharmingen, USA). Samples were fixed with 1% (v/v) formaldehyde and analysed using a FACSCanto II flow cytometer (Becton Dickinson, USA). Platelets were identified by characteristic forward and side light scatter as well as expression of CD42b and 10,000 platelet events counted. The mean fluorescent intensities of PAC-1, CD62P and CD61 were recorded.

Other diluted whole blood aliquots were incubated with CD42a PE (BD-Pharmingen) and FITC conjugated human fibrinogen (Sigma Aldrich, USA) and stimulated with 10 μM or 20 μM TRAP at 37°C for 5 min before fixation with 1% (v/v) formaldehyde. Mean FITC fluorescent intensity was recorded.

Dense granule exocytosis was quantitatively assessed by quinacrine uptake and thrombin-induced release with flow cytometry as described previously [32]. Briefly, PRP from healthy human volunteers (n = 3) was incubated with quinacrine (100 μM) (Sigma, USA) at 37°C for 20 min in the dark, to allow quinacrine to be taken up by the dense granules. The platelets were then washed using 1 ml HEPES saline buffer and incubated with 1 mM Que or DiOHF at 37°C for 5 min. Thrombin (0.5 U.ml^{-1}, Chrono-Log Co) was then added and incubated at 37°C for 5 min. The reaction was stopped by 1:25 dilution in HEPES saline buffer and

immediately read on the flow cytometer for 10,000 platelet events. The thrombin-induced decrease in quinacrine fluorescent intensity indicated dense granule release

Confocal laser scanning microscopy

The ability of platelet dense granules to release their contents was visualised using laser confocal imaging. PRP was incubated with (100 µM) quinacrine at 37°C in the dark and washed in HEPES saline buffer before incubation with Que or DiOHF (1 mM) at 37°C for 5 min. Exocytosis was stimulated by incubation with thrombin (0.5 U.ml^{-1}) at 37°C for 5 min. The reaction was stopped by 1:15 dilution in HEPES, and examined by confocal laser microscope (Nikon A1, Nikon Corp. Japan) using a 60x water immersion objective (NA 1.42) and excitation with a 488 nm laser, and NIS-Element advanced research software for image analysis. The proportion of platelets with fluorescent dense granules was quantified over a minimum of 5 fields per condition.

Animals

All experimental procedures performed in this study were approved by the Animal Experimentation Ethics Committee of RMIT University and in accordance with the guidelines of the Australian code of practice for the care and use of animals for scientific purposes.

Flavonol administration

Healthy C57BL/6 mice (13 week old of both sexes, body weight 20.9 ±0.47 g) were treated with a single intravenous (IV) bolus of Que (n = 5, 6 mg/kg), DiOHF (n = 5, 6 mg/kg) (Indofine chemicals Inc. USA), eptifibatide (n = 3, 4.5 mg/kg) (Millennium Pharmaceuticals, Japan) or vehicle (n = 5, DMSO plus polyethylene glycol and saline), using a 27 gauge needle via the tail vein. 30 min after administration of the treatment ferric chloride induced carotid artery damage was initiated.

Ferric Chloride Carotid injury model

Ferric chloride-induced arterial injury was performed using a well characterized model of platelet-mediated thrombosis [33]. In brief, C57BL/6 mice of both sexes were anaesthetised with ketamine and xylazine (200:10 mg / kg) (Troy Laboratories, Australia) by intraperitoneal (IP) injection. A midline incision was made on the right side of the neck and the carotid artery was exposed using blunt dissection. A laser Doppler flow probe (Moor Instruments, UK) was placed proximal to the carotid artery to measure baseline blood flow using a laser Doppler flow monitor (Moor Instruments, UK). After baseline blood flow was established, a 2 x 4 mm filter paper saturated with 20% (w/v) ferric chloride (Sigma Aldrich, USA) was applied on the adventitial surface of the vessel and removed after 4 min. Blood flow through the carotid artery was monitored for 30 min or until 95% vessel occlusion is reached. At the end of each experiment, and whilst under deep anaesthesia, the mouse was euthanized by cervical dislocation and the injured arterial segments were harvested for histological analysis. The harvested arterial segments were fixed in 10% (v/v) formalin and embedded in paraffin. Cross sections (4 µm) were cut from paraffin blocks and stained with haematoxylin and eosin to demonstrate no visible difference in arterial injury between groups (data not shown).

Statistical analysis

All values are expressed as mean ± standard error of mean (SEM). Comparisons between samples from the same volunteer with aliquots spiked with flavonol or control were performed using one-way ANOVA with repeated measures and Dunnett's test, for post hoc comparisons. Comparisons between mice randomized to flavonol or control were performed using one-way ANOVA and Dunnett's test. Comparisons between Que and DiOHF were performed with Bonferroni post tests. Statistical analysis was performed using PRISM Graphpad software.

Results

Platelet aggregation

Incubation of PRP with Que or DiOHF inhibited platelet aggregation induced by 5 µg.mL^{-1} collagen, 10 µM ADP and 0.5 mM AA in a concentration-dependant manner (Figure 1). Que and DiOHF caused concentration-dependant inhibition of aggregation, and achieved near complete inhibition of ADP- and collagen-induced aggregation at 1 mM. DiOHF fully inhibited AA- induced platelet aggregation at 0.2 mM, whereas Que achieved full inhibition at 0.50 mM.

Dense Granule Exocytosis

Dense granule exocytosis was measured by agonist induced ATP release and fluorescent quinacrine uptake and release.

ATP Release: 1 mM of Que or DiOHF achieved complete, or near complete, inhibition of ATP release from dense granules caused by collagen (Que $91 \pm 4\%$ and DiOHF $93 \pm 2\%$) and AA (Que, DiOHF, 100% inhibition at 1 mM,) (Figure 2).

Quinacrine release: Dense granule exocytosis, as measured by the thrombin-induced decrease in quinacrine fluorescence, was significantly inhibited by concentrations of either Que or DiOHF greater than 0.5 mM (Figure 3). Inhibition of quinacrine release by DiOHF was significantly greater than Que when used at the same concentration and identical experimental conditions. Failure of flavonol treated platelets to release quinacrine labelled dense granules was visually confirmed by confocal laser microscopy. Thrombin caused visible shape change associated with activation for all treatments, but retained visible dense granules in platelets treated with Que and DiOHF (Figure 4). The proportion of platelets with fluorescent granules by confocal microscopy following stimulation with 0.5 U.ml^{-1} thrombin is shown in Figure 5.

α-granule Exocytosis

α-granule exocytosis was measured by platelet surface P-selectin expression and agonist induced changes in GPIIIa expression.

1 mM Que significantly inhibited ADP (58% inhibition, $p < 0.05$), AA (36% inhibition, $p < 0.05$), TRAP (14% inhibition, $p < 0.05$), and adrenaline + collagen (54% inhibition, $p < 0.05$) induced α-granule exocytosis as measured by P-selectin mean fluorescence intensity (Figure 6). Inhibition was observed with DiOHF also, but this failed to achieve statistical significance; ADP (25% inhibition, $p = ns$) AA (18% inhibition, $p = ns$), TRAP (3% inhibition, $p = ns$) adrenaline + collagen (31% inhibition, $p = ns$) (Figure 6).

Figure 7 shows the agonist induced increase in platelet surface CD61 expression from intracelleular α-granule stores. 0.5 mM AA induced a significant increase in CD61 MFI in the presence of vehicle (40% increase, $p < 0.05$) and 1 mM DiOHF (45% increase, $p < 0.05$) but not in the presence of 1 mM Que (19% increase, $p = ns$) (Figure 7A). Similarly, 20 μM TRAP induced a significant increase in CD61 MFI in the presence of vehicle (30% increase, $p < 0.05$) and 1 mM DiOHF (27% increase, $p < 0.05$) but not in the presence of 1 mM Que (14% decrease, $p = ns$).

GPIIb/IIIa Receptor Activation and Fibrinogen Binding

1 mM Que, and to a lesser extent DiOHF, significantly inhibited ADP (DiOHF = 56%, Que = 71% inhibition, both $p < 0.05$), AA (DiOHF = ns, Que = 45% inhibition, $p < 0.05$ for Que only), TRAP (DiOHF = 43%, Que = 59% inhibition, both $p < 0.05$), and adrenaline + collagen (DiOHF = 59%, Que = 78% inhibition, both $p < 0.05$) induced GPIIb/IIIa activation as measured by PAC-1 binding (Figure 8).

Correspondingly, 1 mM Que achieved greater inhibition of 10 and 20 μM of TRAP- induced fibrinogen binding to platelets than 1 mM DiOHF (Que inhibited $60 \pm 2\%$ $p < 0.05$, DiOHF ns $35 \pm 7\%$, $p = ns$) (Figure 9).

Blood Flow Following Arterial Injury

Treatment with 6 mg/kg of Que or DiOHF better maintained blood flow when administered IV 30 min prior to initiation of platelet mediated thrombosis in the carotid artery compared to the vehicle control (Figure 10A). Vehicle treated mice had a 95% vessel occlusion at 15 min while the flavonol and eptifibatide treated mice had less than 20% vessel occlusion (Figure 10B).

Discussion

We have shown that both the naturally occurring flavonol Que, as well as a synthetic, structurally related flavonol, DiOHF, better maintain blood flow in a well characterized model of platelet mediated arterial thrombosis. We provide further insight into the mechanism by which flavonols can inhibit platelet activation and aggregation, as well as different inhibition of dense and α-granule exocytosis in response to a range of agonists for Que and DioHF.

Both Que and DiOHF inhibited dense granule exocytosis at concentrations corresponding to those inhibiting agonist induced platelet aggregation. Consistent with inhibition of aggregation, both 1 mM Que and DiOHF inhibited GPIIb/IIIa receptor activation, as demonstrated by PAC-1 binding. Que significantly inhibited α-granule exocytosis with a range of agonists, as demonstrated by CD62P expression and prevention of an agonist induced increase in CD61 expression. While some inhibition of P-selectin expression was observed with DiOHF, this failed to achieve statistical significance, and was not supported by any inhibition of agonist induced release of α-granule GPIIb. Thus the potency of α-granule inhibition may be less in DiOHF than in Que. Furthermore, Que significantly inhibited fluorescently labelled fibrinogen binding, whereas inhibition with DiOHF was less and did not achieve statistical significance. However, DiOHF showed significantly greater inhibition of dense granule exocytosis across a range of agonists as measured by ATP release and by thrombin induced fluorescent quinacrine uptake and release. This enhanced inhibition of dense granule exocytosis with DiOHF relative to Que may offset the relatively lower inhibition of α-granule exocytosis and fibrinogen binding, and ADP or collagen induced platelet aggregation and delay of *in vivo* thrombus formation occurred equally as well with DiOHF as with Que. Inhibition of arachidonic acid induced aggregation was enhanced with DiOHF relative to quercetin. Further investigation of the structure function relationship responsible for the different mechanisms of inhibition of platelet activation, aggregation and granule exocytosis with DiOHF and Que is justified.

An unexpected finding of this study was differences in the potency of inhibition of α-versus dense granule exocytosis by the two structurally related flavonols. Platelet α-granule secretion occurs more readily than dense granule secretion, however the mechanisms leading

to membrane fusion and exocytosis of the two granule types have generally been assumed to be similar [34,35]. Studies have shown that aspirin at certain concentrations is capable of inhibiting ADP induced serotonin release (a dense granule component) whilst P-selectin expression is unaffected [35], suggesting potential for selective inhibition of exocytosis the different granule types. The results obtained in the current study suggest enhanced inhibition of dense granule exocytosis with DiOHF, while greater inhibition of α-granule exocytosis was seen with Que. This supports the concept that release of dense and α-granules may be independently regulated, and therefore potentially independently inhibitible. This represents a potentially interesting therapeutic strategy.

Platelet granule-cell membrane fusion necessary for exocytosis is governed, in part, by the matching of a vesicle SNARE (v-SNARE) with SNAP or syntaxin proteins in the plasma membrane [36]. In platelets, syntaxin 2 and 4 function to mediate α- granule release, but dense granules lack syntaxin 4. This dual usage of syntaxin 2 and 4 in α-granules may potentially explain how differential release of dense and α-granules could occur. Different inhibition of syntaxin function by Que and DiOHF has the potential to explain the differences in relative potency of inhibition of dense and α-granule exocytosis observed in this study, but has not been examined. Further studies are warranted to elucidate the potential role of syntaxin in the mechanism of different inhibition of α-and dense granule exocytosis by structurally related flavonols.

Recent studies have suggested that α-granules are heterogeneous in composition [34,37]. While all α-granules contain P-selectin, subtypes have been identified with differential expression of pro- and anti-angiogenic factors [37] and vWF [38]. While our results demonstrate that overall α-granule exocytosis, as measured by P-selectin expression, inhibited by Que, it remains possible that subtypes of α-granules may be uninhibited, and further studies are warranted to elucidate this.

Platelet exocytosis is a critical component of platelet function and thrombus growth, as it allows both the site specific release of pre-formed thrombo-inflammatory mediators, as well as alterations of the platelet surface membrane adhesion molecule and receptor expression [39]. The ability to modulate the inhibition of dense granule exocytosis relative to α-granule

exocytosis by structural modification of flavonols represents a potential novel therapeutic target for antiplatelet therapy. Such an approach would inhibit release of ADP and serotonin, which are critical molecules involved in the positive feedback loop of platelet activation and thrombus propagation, whilst providing less inhibition of the capacity of platelets to activate, adhere to the site of injury, and deliver important immune and growth factor molecules from α-granules.

While a potential for different inhibition of dense and α-granule exocytosis by two structurally related flavonols is shown in this study, it is clear that this is in addition to antiplatelet effects of flavonols that have been previously described. Both flavonols effectively inhibited agonist induced aggregation in an independent manner, and delayed thrombus formation in an *in vivo* model of platelet mediated arterial thrombosis.

The concentrations of DiOHF and Que that were found to significantly inhibit collagen and ADP induced platelet aggregation were higher than previously reported by Sheu et al. [13] and Yin et al. [40], but are consistent with Raghavendra et al. [15]. Because ADP, collagen and AA induced aggregation were all inhibited, these flavonols may inhibit platelet function by multiple mechanisms or a common pathway that is shared by these agonists. The ability of flavonols to inhibit kinase activity [16,21-23] including Fyn and PI3 kinase activity and the tyrosine phosphorylation of Syk and PLCγ2 [16,41,42] may contribute to the inhibition of platelet activation, aggregation and granule exocytosis observed in this study. However, more potent inhibition of AA induced platelet aggregation suggest an additional mechanism may be through inhibition of cyclooxygenases [17] or binding to the thromboxane receptor [18,43] as has been previously demonstrated.

A significant and novel finding in this study is that doses of these flavonols achievable *in vivo* were able to significantly improve blood flow following arterial injury in an *in vivo* model of platelet mediated thrombosis. In our well characterised model of platelet mediated arterial thrombosis ferric chloride initiates thrombus formation via iron mediated endothelial damage and platelet activation [33]. The significant improvements in blood flow following injury reported in this study suggest the potential for flavonols to be developed as a clinically relevant approach to inhibit platelet aggregation. However, ferric chloride induces oxidative

injury, and flavonols have widely characterised antioxidant activity. While no visible difference in the magnitude of arterial injury was observed by histological examination of carotid artery (data not shown), it remains possible that administration of flavonols improved blood flow by affecting the nature of the ferric chloride induced oxidative injury, rather than by platelet mediated mechanisms. Further investigation using a crush injury model is warranted to verify this.

Limitations

In this study high concentrations of Que and DiOHF were used in order to demonstrate a novel mechanism of action of inhibition of different parameters of platelet function. However, significant improvement in carotid blood flow in a model of platelet-mediated thrombosis was observed at much lower concentrations, indicating that incomplete inhibition of platelet function is necessary for improvement in arterial thrombosis *in vivo*. This is consistent with clinical benefits of partial platelet inhibition seen with established antiplatelet agents. Further studies are warranted to investigate whether structural modification might increase anti-platelet potency to achieve this novel mechanism of selective dense granule exocytosis more clinically achievable concentrations.

In order to achieve these high concentrations of flavonols in blood and plasma concentrations of up to 1% DMSO was necessary to maintain solubility. However, similar concentrations have been previously used without affecting parameters of platelet function [42], and did not affect platelet function in response to the high concentrations of agonists used in this study (data not shown). Furthermore, appropriate 1% DMSO vehicle controls were used in the current study and no evidence of platelet toxicity was observed in aggregation tracings or flow cytometric dot plots.

Conclusion

These data provide the first evidence of inhibition of platelet activation, aggregation and granule secretion by DiOHF and report antithrombotic properties of both quercetin and DioHF in an *in vivo* model of platelet mediated arterial thrombosis. We demonstrated differing potency of inhibition of dense and α-granule exocytosis by these two structurally

related flavonols. This study outlines important antiplatelet mechanisms of these flavonols that could lead to the design of selective inhibitors of platelet secretion and new antiplatelet therapy.

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Declaration of Interest Statement

The authors report no conflict of interest.

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Figure 1. Inhibition of platelet aggregation in the presence of Que (squares) and DiOHF (circles). Increasing concentrations of Que or DiOHF dissolved in DMSO were incubated with fresh PRP (n=3) at 37°C for 5 min. Maximal turbidimetric platelet aggregation over 6 minutes was recorded. Platelet aggregation was induced by (A) 5 µg.ml^{-1} collagen, (B) 10 µM ADP and (C) 0.5 mM AA.

Figure 2. Effect of Que or DiOHF on ATP release. ATP release from platelets treated with vehicle, 1 mM Que or 1 mM DiOHF at 37°C over 5 minutes was measured against a 2 nM ATP standard by chemiluminescence of luciferin-luciferase stimulated by (A) 5 µg.ml^{-1} collagen, (B) 10 µM ADP and (C) 0.5 mM AA. Mean ± SEM. One way ANOVA with Bonferroni post test (n=3). * $p < 0.05$ vs vehicle, ** $p < 0.05$ between DiOHF and Que.

Figure 3. Inhibition of 0.5 U.mL^{-1} thrombin induced dense granule exocytosis by 1 mM of Que or DiOHF by flow cytometry. Fresh PRP was incubated with quinacrine in the presence of vehicle, 1 mM Que or 1 mM DiOHF in the dark at 37°C for 20 min. Platelets were identified by characteristic forward and side light scatter. The thrombin induced decrease in fluorescence indicating dense granule exocytosis was recorded. Mean ± SEM. One way ANOVA with Bonferroni post test (n=3) * $p < 0.05$ vs vehicle, ** $p < 0.05$ between DiOHF and Que.

Figure 4. Inhibition of dense granule exocytosis was visually confirmed by confocal microscopy. Quinacrine labelled platelets were incubated with vehicle, 1 mM Que or 1 mM DiOHF in the dark at 37°C for 20 min. Representative images of quinacrine labelled platelets with (A) Que only, (B) Que + 0.5 U.ml^{-1} thrombin, (C) DiOHF only, (D) DiOHF + 0.5 U.ml^{-1} thrombin, (E) Vehicle only, (F) Vehicle + 0.5 U.ml^{-1} thrombin.

Figure 5. Inhibition of dense granule exocytosis was quantified by confocal microscopy. The percentage of platelets per field with fluorescent dense granules was quantified over a minimum of 6 fields per condition. * $P < 0.05$ vs vehicle. Mean ± SEM. One way ANOVA with Dunnett's post test.

Figure 6. Effect of 1 mM Que or DiOHF on platelet surface P-selectin (CD62P) expression by flow cytometry. Whole blood aliquots were incubated with vehicle, 1 mM Que or 1 mM DiOHF at 37°C for 5 min. Platelets were identified by characteristic forward and side light scatter and expression of the platelet-specific CD42b. Platelet surface P-selectin expression was determined by CD62P fluorescence induced by (A) 25 μM ADP, (B) 0.5 mM AA, (C) 20 μM TRAP or (D) 5 μg.ml^{-1} collagen. Mean ± SEM. * $P < 0.05$ vs vehicle. One way ANOVA with Dunnett's post test (n = 6).

Figure 7. Effect of 1 mM Que or DiOHF on platelet surface GPIIIb (CD61) expression by flow cytometry. Whole blood aliquots were incubated with vehicle, 1 mM Que or 1 mM DioHF at 37°C for 5 min. Platelets were identified by characteristic forward and side light scatter and expression of the platelet-specific CD61. Mean fluorescence intensity (MFI) of CD61 relative to circulating (No Agonist) levels for 0.5 mM AA (A) and 20 μM TRAP (B). Mean ± SEM. * $P < 0.05$ vs No Agonist. One way ANOVA with Dunnett's post test (n = 3).

Figure 8. Effect of 1 mM Que or DiOHF on PAC-1 binding by flow cytometry. Whole blood aliquots were incubated with vehicle, 1 mM Que or 1 mM DiOHF at 37°C for 5 min. Platelets were identified by characteristic forward and side light scatter and expression of the platelet-specific CD42b. PAC-1 binding was determined by increase in fluorescence upon stimulation by (A) 25 μM ADP, (B) 0.5 mM AA, (C) 20 μM TRAP or (D) 5 μg.mL^{-1} collagen.* $P < 0.05$ vs vehicle. Mean ± SEM. One way ANOVA with Dunnett's post test (n=6)

Figure 9. Effect of 1 mM Que or DiOHF on FITC conjugated fibrinogen platelet binding by flow cytometry. Que or DiOHF treated platelets were incubated with FITC conjugated fibrinogen at 37°C for 5 min. Platelets were identified by characteristic forward and side light scatter and expression of the platelet-specific CD42a. Platelet surface fibrinogen binding was determined by fluorescent detection of FITC labelled fibrinogen on the platelets. Fibrinogen binding was induced by (A) 10 μM and (B) 20 μM TRAP. * $P < 0.05$ vs vehicle. Mean ± SEM. Paired t-test (n=6).

Figure 10. Effect of flavonols on blood flow through ferric chloride induced injury to the carotid artery. Ferric chloride (20%) was applied on the surface of the carotid artery for 4 min. blood flow through the carotid artery was recorded using a Doppler flow laser (A) and blood flow at 15 minutes following injury was recorded (B). (n = 5 for each group).