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

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Abstract

Flavonols are polyphenolic compounds with broad-spectrum kinase inhibitory, as well as potent anti-oxidant and anti-inflammatory properties. Anti-platelet potential of quercetin (Que) and several related flavonoids have been reported; however, few studies have assessed the ability of flavonols to inhibit exocytosis of different platelet granules or to inhibit thrombus formation in vivo. 3',4'-Dihydroxyflavonol (DiOHF) is a flavonol which is structurally related to Que and has been shown to have greater anti-oxidant capacity and to improve the endothelial function in the context of diabetes and ischaemia/reperfusion injury. While the structural similarity to Que suggests DiOHF may have a potential to inhibit platelet function, no studies have assessed the anti-platelet potential of DiOHF. We therefore investigated platelet granule inhibition and potential to delay arterial thrombosis by Que and DiOHF. Both Que and DiOHF showed inhibition of collagen, adenosine diphosphate and arachidonic acid stimulated platelet aggregation, agonist-induced GPIIb/IIIa activation as demonstrated by PAC-1 and fibrinogen binding. While both flavonols inhibited agonist-induced granule exocytosis, greater inhibition of dense granule exocytosis occurred with DiOHF as measured by both ATP release and flow cytometry. In contrast, while Que inhibited agonist-induced P-selectin expression, as measured by both platelet surface P-selectin expression and upregulation of surface GPIIIa expression, inhibition by DiOHF was not significant for either parameter. C57BL/6 mice treated with 6 mgkg⁻¹ IV Que or DiOHF maintained greater blood flow following FeCl₃-induced carotid artery injury when compared to the vehicle control. We provide evidence that Que and DiOHF improve blood flow following arterial injury in part by attenuating platelet granule exocytosis.

Keywords: Granule inhibition, flavonols, platelets, arterial thrombosis

Introduction

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]. While considerable attention has been paid to the antioxidant activity of flavonols as a major contributor to their cardioprotective actions, there is a growing evidence of other properties that may be of importance. There is substantial evidence demonstrating that flavonols, particularly quercetin (Que) and related compounds, have anti-platelet aggregation activity which may contribute to their beneficial effects [13–17]. While the ability of Que and related flavonols to inhibit platelet aggregation is established, and a recent study by Jasuja et al. has demonstrated the potential for Que-3-rutinoside to inhibit laser-induced thrombosis in mouse cremaster arteries in vivo [17], the capacity of platelet inhibition by structurally related flavonols to reduce thrombus formation in vivo has not been fully investigated.

Previous studies have reported the mechanisms of platelet inhibition of Que and related flavonols include inhibition of GPIIb/IIIa and impaired fibrinogen binding secondary to inhibition of agonist-induced signalling [13, 18–21]. Several mechanisms of action have been proposed including inhibition of cyclooxygenase or phosphodiesterases [22], antagonism of the thromboxane receptor [18, 21, 23], as well as more recent evidence showing inhibition of kinase [19–21, 24] and protein disulphide isomerase [17] activity. In particular, Que is reported to decrease Fyn and PI3 kinase activity and to inhibit tyrosine phosphorylation of Syk and PLC γ 2 [19, 24]. 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 [20].

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. α -granules are the largest and most abundant secretory granules in platelets. They contain a large number of different secretory proteins involved in inflammation and wound repair, including growth factors, adhesion molecules, angiogenic factors and cytokines. Dense granules, while fewer in number, contain very high concentrations of small molecules that play important roles in platelet activation. These include adenosine diphosphate (ADP), which is necessary for amplification of platelet activation leading to aggregation. While mechanisms driving exocytosis of dense and α -granules are generally assumed to be similar, recent studies have suggested the potential for selective inhibition of different granule types [25]. Greater inhibition of dense granules, while maintaining α -granule exocytosis may be potentially beneficial, as it could inhibit the second wave of platelet activation and aggregation while maintaining the ability of platelets to adhere to the site of injury and deliver growth factors, angiogenic agents and chemokines from α -granules in situ.

3',4'-Dihydroxyflavonol (DiOHF) is a synthetic flavonol which is structurally related to Que, with identical substitutions on two of the three rings but lacking two hydroxyl substitutions on the third ring (Figure 1). This structure has been suggested to increase anti-oxidant potential and structure–activity relationship studies have demonstrated that DiOHF is significantly more potent than a number of natural flavones and flavonols in this regard [12, 26]. Recently, we have demonstrated

that DiOHF is able to reduce injury after myocardial ischaemia and reperfusion [12, 27–29] and to improve endothelial function in diabetes [10]. Considerable interest has been generated in the potential for therapeutic development of DiOHF. As a structurally related molecule to Que, DiOHF may likewise inhibit platelet aggregation. However, the anti-platelet potential of DiOHF has not previously been explored. Accordingly, the aims of this study were to assess the effects of structurally related flavonols Que and DiOHF on platelet activation, aggregation and granule exocytosis in vitro, and determine whether this translated to improved 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 [30, 31]. 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 xg for 10 minutes at room temperature. Platelet-poor plasma (PPP) was obtained by centrifugation of the remaining blood at 1800 xg for 15 minutes at room temperature. Aggregation studies were completed within three hours of blood collection.

Platelet aggregation

The effect of Que, DiOHF (both sourced from Indofine Chemicals Inc., Hillsborough, NJ, USA) or vehicle on agonist induced light transmittance platelet aggregation was determined. Flavonols were incubated with PRP at 37 °C for five minutes to achieve concentrations ranging from 0.1 to 1.0mM in 1% (v/v) dimethyl sulphoxide (DMSO) (n=3). Aggregation was stimulated by 5 μgml^{-1} collagen, 10 mM ADP and 0.5mM arachidonic acid (AA) (all agonists were sourced from Chrono-Log Co., Havertown, PA, USA). Turbidometric platelet aggregation was calibrated against a PPP control (100% aggregation) and the maximal aggregation over a six minutes period was recorded. ATP release was measured in the presence of luciferin–luciferase reagent (Chrono-Log Co.) against a 2nM ATP standard by luminescence at 0.1 and 1.0mM 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) and fibrinogen binding was performed using established flow cytometric methods [30, 32, 33]. Fresh citrated whole blood from healthy volunteers (n=6) was diluted 1 : 5 with HEPES saline buffer (10mM HEPES, 0.15M NaCl, pH 7.4) and incubated with 1mM Que, DiOHF or vehicle at 37 °C for five minutes. For assessment of α -granule exocytosis and GPIIb/IIIa activation, samples were labelled with fluorescently conjugated

monoclonal CD42b PC5 (BD-Pharmingen, Franklin Lakes, NJ, USA) with either PAC-1 FITC (BD-Pharmingen) and anti-CD62P PE (BD-Pharmingen) or PAC-1 FITC with 2 mM eptifibatide (Millennium Pharmaceuticals, Osaka, Japan) and mouse IgG1 PE isotype as controls.

For assessment of agonist-induced changes in platelet surface GPIIIa expression, separate aliquots of vehicle, DiOHF or Que treated 1 : 50 whole blood in saline were labelled with CD61 FITC (BDPharmingen). Samples were fixed with 1% (v/v) formaldehyde and analysed using an FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, 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, St. Louis, MO, USA) and stimulated with 10 or 20 mM TRAP at 37 °C for five minutes 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 [34]. Quinacrine is a fluorescent dye which is selectively taken up into dense granules in platelets and can be measured by fluorescent intensity of platelets by flow cytometry. The percentage decrease in fluorescence intensity following stimulation with thrombin is a measure of thrombin-induced dense granule exocytosis [30, 34]. Briefly, PRP from healthy human volunteers (n=3) was incubated with quinacrine (100 mM) (Sigma Aldrich) at 37 °C for 20 minutes in the dark, to allow quinacrine to be taken up by the dense granules. The platelets were then washed using 1ml HEPES saline buffer and incubated with 1mM Que or DiOHF at 37 °C for five minutes to remove any quinacrine not in dense granules. Thrombin (0.5Uml^{-1} , Chrono-Log Co) was added to one aliquot and incubated at 37 °C for five minutes to allow dense granule exocytosis to occur. 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 percentage difference in fluorescence intensity of thrombin treated and untreated quinacrine-labelled platelets relative to unlabelled platelets was recorded as percentage of quinacrine release.

Confocal laser scanning microscopy

The ability of platelet dense granules to release their contents was visualised using laser confocal microscopy. PRP was incubated with (100 mM) 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 five minutes. Exocytosis was stimulated by incubation with thrombin (0.5Uml^{-1}) at 37 °C for five minutes. The reaction was stopped by 1 : 15 dilution in HEPES, and examined by a confocal laser microscope (Nikon A1, Nikon Corp., Tokyo, Japan) using a 60x water immersion objective (NA 1.2) and excitation with a 488 nm laser with NIS-Element advanced research software for image analysis. The proportion of platelets with fluorescent dense granules was quantified over a minimum of five fields per condition.

Viability

The effect of 1mM Que, DiOHF and the vehicle (1% DMSO) on platelet viability was determined using an established trypan blue exclusion assay [35]. Briefly, fresh human PRP (n=3) was incubated with 1mM Que, DiOHF, vehicle or 90% ethanol (EtOH, positive control) for five minutes at 37 °C

before addition of 0.4% trypan blue (Sigma Aldrich Co.) and mixed thoroughly. Viability was immediately examined under a microscope (x40 objective) using a haemocytometer for a minimum of 400 platelets. The percentage of platelets that excluded the trypan blue stain was recorded.

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 weeks old of both sexes, body weight 20.9 ± 0.47 g) were treated with a single intravenous (IV) bolus of 6 mg kg^{-1} Que (n=5), 6 mg kg^{-1} DiOHF (n=5) (Indofine Chemicals Inc.), 4.5 mg kg^{-1} eptifibatide (n=3) (Millennium Pharmaceuticals) or vehicle (0.5% DMSO plus 2.2mM polyethylene glycol in saline, n=5), using a 27-gauge needle via the tail vein. Thirty minutes 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-characterised model of platelet-mediated thrombosis [36]. In brief, C57BL/6 mice of both sexes were anaesthetised with ketamine and xylazine ($200:10 \text{ mg kg}^{-1}$ ip) (Troy Laboratories, Glendenning, NSW, Australia). 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, Devon, UK) was placed proximal to the carotid artery to measure baseline blood flow using a laser Doppler flow monitor (Moor Instruments). After baseline blood flow was established, a $2 \times 4 \text{ mm}^2$ filter paper saturated with 20% (w/v) ferric chloride (Sigma Aldrich) was applied on the adventitial surface of the vessel and removed after four minutes. Blood flow through the carotid artery was monitored for 30 minutes or until 95% vessel occlusion is reached. At the end of each experiment, and while under deep anaesthesia, the mouse was euthanised 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 mm) 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 $\pm$ standard error of mean (SEM). Comparisons between samples from the same volunteer with aliquots containing flavonol or control were performed using one-way ANOVA with repeated measures and Dunnett's test, for post hoc comparisons. Comparisons between mice randomised 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

The effect of Que and DiOHF on platelet aggregation, fibrinogen binding and GPIIb-IIIa receptor activation Incubation of PRP with 1mM Que, DiOHF or 1% DMSO vehicle did not affect platelet

trypan blue exclusion (Que=93±2%, DiOHF=94±1%, DMSO=94±2% and EtOH=18±2%), indicating no toxicity on platelets at the concentrations used.

The effect of Que and DiOHF on platelet function was investigated by measuring the ability of platelets to aggregate in response to a range of agonists using light transmission aggregometry. Flow cytometry measured the ability of fluorescently labelled fibrinogen to bind platelets, and reported binding of PAC-1, an antibody which recognises an activation-dependent conformational change which takes GPIIb-IIIa from its low affinity to high affinity state for fibrinogen.

Incubation of PRP with 1mM Que or DiOHF inhibited platelet aggregation induced by 5 µgml⁻¹ collagen, 10 mM ADP and 0.5mM AA in a concentration-dependent manner (Figure 2) 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 0.5 mM.

Que of 1mM achieved inhibition of TRAP-induced fibrinogen binding to platelets (60±2% decrease from vehicle, p<0.05) while inhibition by 1mM DiOHF was smaller and not statistically significant (35±7% decrease from vehicle, p=ns; Figure 3). Correspondingly, 1mM Que, and to a lesser extent DiOHF, significantly inhibited GPIIb/IIIa activation induced by 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) as measured by PAC-1 binding (Figure 4).

As expected, these findings show dose-dependent inhibition of platelet aggregation across the full range of agonists by both DiOHF and Que. Consistent with inhibition of aggregation, both 1mM Que and DiOHF inhibited GPIIb/IIIa receptor activation, as demonstrated by PAC-1 binding. However, while Que significantly inhibited fluorescently labelled fibrinogen binding, inhibition with DiOHF was less and did not achieve statistical significance.

The effect of Que and DiOHF on granule exocytosis

Dense granule exocytosis was measured by agonist-induced ATP release by chemiluminescent aggregometry and release of fluorescent quinacrine by both flow cytometry and confocal microscopy. α-Granule exocytosis was measured by agonist induced expression of P-selectin and increase in GPIIb expression on the platelet surface by flow cytometry.

ATP release. 1mM Que or DiOHF achieved complete, or near complete, inhibition of ATP release from dense granules caused by collagen (Que 91±4% and DiOHF 93±2% inhibition) and AA (Que, DiOHF, 100% inhibition) (Figure 5).

Quinacrine release. Dense granule exocytosis, as measured by the thrombin-induced decrease in quinacrine fluorescence, was significantly inhibited by 0.5mM Que vs. vehicle (53.8±0.5% vs. 79.7±0.9% decrease, p<0.05) and 0.5mM DiOHF (17.0±6.3% vs. 79.7±0.9% decrease, p<0.05) and continued with dose dependent inhibition to 1mM Que (26.5±1.3% decrease, p<0.05) and DiOHF (5.9±1.7% decrease, p<0.05) (Figure 6). Inhibition of quinacrine release by DiOHF was significantly greater than Que at 0.5mM (17.0±6.3% vs. 53.8±0.5% release, respectively, p<0.05), 0.75mM (11.9±5.1% vs. 46.9±0.5% release, respectively, p<0.05) and 1mM (5.9±1.7% vs. 26.5±1.3% release, respectively, p<0.05) (Figure 6). Failure of flavonol treated platelets to release quinacrine labelled dense granules with thrombin stimulation 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 1mM Que and DiOHF (Figure 7). Following stimulation 0.5Uml^{-1} of thrombin $17.8\pm9.3\%$ of platelets per field treated with vehicle had fluorescent granules, while $87.4\pm3.8\%$ of 1mMQue ($p<0.05$ vs. vehicle) and $86.3\pm6.0\%$ of 1mM DiOHF ($p<0.05$ vs.vehicle) treated platelets per field had fluorescent granules (Figure 8).

P-selectin expression. 1mM 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 9). Inhibition was observed with DiOHF also, but this failed to achieve statistical significance; ADP (25% inhibition, $p=\text{ns}$) AA (18% inhibition, $p=\text{ns}$), TRAP (3% inhibition, $p=\text{ns}$) and adrenaline + collagen (31% inhibition, $p=\text{ns}$) (Figure 9). _

α -Granule GPIIb expression. Figure 10 shows the agonist induced increase in platelet surface CD61 expression from intracellular α -granule stores. 0.5mM AA induced a significant increase in CD61 MFI (Mean fluorescence intensity) in the presence of vehicle (40% increase, $p<0.05$) and 1mM DiOHF (45% increase, $p<0.05$) but not in the presence of 1mM Que (19% increase, $p=\text{ns}$) (Figure 10A). Similarly, 20 mM TRAP induced a significant increase in CD61 MFI in the presence of vehicle (30% increase, $p<0.05$) and 1mM DiOHF (27% increase, $p<0.05$) but not in the presence of 1mM Que (14% decrease, $p=\text{ns}$) (Figure 10B).

Therefore, both Que and DiOHF inhibited dense granule exocytosis at concentrations corresponding to those which inhibited agonist-induced platelet aggregation. 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. 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.

The effect of Que and DiOHF on blood flow following arterial injury

In order to assess the effect of Que and DiOHF on platelet mediated thrombosis in vivo, blood flow through the carotid artery of C57BL/6 mice was measured following FeCl_3 injury (Figure 11A). Vehicle treated mice had near complete vessel occlusion within the first 15 minutes following ferric chloride induced carotid artery damage with both the single IV dose ($1.7\pm1.7\%$ flow, Figure 11B). As expected, the platelet GPIIb/IIIa receptor antagonist eptifibatide, administered at 4.5 mg kg^{-1} as a positive control maintained blood flow at near pre-injury levels ($96.7\pm3.3\%$ flow, Figure 11B, $p<0.05$ vs. vehicle). Blood flow at 15 minutes was maintained at near pre-injury levels for mice treated with 6 mgkg^{-1} of Que ($83.1\pm17.0\%$ flow, $p<0.05$ vs. vehicle, Figure 11B) or with 6mgkg^{-1} DiOHF ($100\pm0\%$ flow, $p<0.05$ vs. vehicle, Figure 11B).

Discussion

We have shown that Que and its synthetic, structurally related flavonol, DiOHF, improve blood flow in a well-characterised model of platelet-mediated arterial thrombosis. This corresponds to well-established inhibition of platelet aggregation and GPIIb-IIIa activation. These findings corroborate

those of a recent study showing the anti-thrombotic effect of a related dietary flavonol, Que-3-rutinoside in laser-induced injury to mouse cremaster arteries [17]. In this study, we show differences in inhibition of dense and α -granule exocytosis in response to a range of agonists between Que and DiOHF. Enhanced inhibition of dense granule exocytosis with DiOHF relative to Que may offset the relatively lower inhibition of α -granule exocytosis and fibrinogen binding. 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 -granule vs. 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 [25, 37]. Studies have shown that aspirin at certain concentrations is capable of inhibiting ADP-induced serotonin release (a dense granule component) while P-selectin expression is unaffected [25], suggesting selective inhibition of exocytosis the different granule types. The results obtained in this 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 modulated representing an interesting therapeutic strategy.

Platelet granule–cell membrane fusion necessary for exocytosis is governed, in part, by the matching of a vesicle SNARE with SNAP or syntaxin proteins in the plasma membrane [38]. 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 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 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 [37, 39]. While all α -granules contain P-selectin, subtypes have been identified with differential expression of pro- and anti-angiogenic factors [39] and vWF [40]. 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 [41]. 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 anti-platelet 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, while 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. [42], 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, 19, 20, 24] including Fyn and PI3 kinase activity and the tyrosine phosphorylation of Syk and PLC γ 2 [16, 43, 44] 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 [22] or binding to the thromboxane receptor [23, 45] as has been previously demonstrated.

A significant finding in this study is that doses of these flavonols which were pharmacologically achievable in mice 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 [36]. 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 or laser-induced injury model is warranted to verify this finding, although care must be taken that anti-inflammatory properties of these agents do not mask anti-platelet effects.

Limitations

In this study, high concentrations of Que and DiOHF were used in in vitro to inhibit different parameters of platelet function. However, significant improvement in carotid blood flow in a model of platelet-mediated thrombosis was observed at doses likely to have resulted in much lower plasma concentrations, indicating that incomplete inhibition of platelet function is sufficient for improvement in arterial thrombosis in vivo. The concentrations used in the in vivo are consistent with concentration previously shown to improve endothelial function in diabetes [46]. It is possible that only partial platelet inhibition is necessary to obtain clinical benefits, consistent with clinical dosage strategies intended to provide partial inhibition in established anti-platelet therapy. 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 were necessary to maintain solubility. However, similar concentrations have been previously used without affecting parameters of platelet function [44], 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 this study and no evidence of platelet toxicity was observed in aggregation tracings or flow cytometric dot plots.

Conclusion

DiOHF is a synthetic flavonol which is structurally related to Que. DiOHF is of considerable interest for clinical development in cardiovascular disease for its potent anti-oxidant and anti-inflammatory properties. These data provide the first evidence of inhibition of platelet activation, aggregation, granule secretion and improvements in arterial blood flow following injury by DiOHF. We observed differing potency of inhibition of dense and α -granule exocytosis by Que and DiOHF flavonols. DiOHF represents a potentially interesting anti-platelet therapy, obtaining similar inhibition of platelet aggregation and thrombus formation with less inhibition of α -granule exocytosis. This study outlines important antiplatelet mechanisms of these flavonols that represent additional therapeutic properties of flavonols and could inform the design of selective inhibitors of platelet secretion and new anti-platelet therapy.

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Figure 1. Flavonol structure: (A) natural flavonol Que and (B) synthetic flavonol 3', 4' dihydroxyflavonol.

Figure 2. 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 five minutes. Maximal turbidimetric platelet aggregation over six minutes was recorded. Platelet aggregation was induced by: (A) 5 μgml^{-1} collagen, (B) 10 mM ADP and (C) 0.5mM AA.

Figure 3. Effect of 1mM 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 five minutes. 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 mM and (B) 20 mM TRAP. Notes: Mean $\pm$ SEM. Paired t-test (n=6). *p<0.05 vs. vehicle.

Figure 4. Effect of 1mM Que or DiOHF on PAC-1 binding by flow cytometry. Whole blood aliquots were incubated with vehicle, 1mM Que or DiOHF at 37 °C for five minutes. 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 mM ADP, (B) 0.5mM AA, (C) 20 mM TRAP or (D) 25 μgml^{-1} collagen + 250 mM adrenalin. Notes: One-way ANOVA with Dunnett's post test (n=6). *p<0.05 vs. vehicle. Mean $\pm$ SEM.

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

Figure 6. Inhibition of 0.5 U ml^{-1} thrombin-induced dense granule exocytosis by 1mM Que or DiOHF by flow cytometry. Fresh PRP was incubated with quinacrine in the presence of vehicle, 1mM Que or DiOHF in the dark at 37 °C for 20 minutes. Platelets were identified by characteristic forward and side light scatter. The thrombin-induced decrease in fluorescence indicating dense granule exocytosis was recorded. Notes: Mean $\pm$ SEM. One-way ANOVA with Bonferroni post test ($n = 3$). * $p < 0.05$ vs. vehicle, ** $p < 0.05$ between DiOHF and Que.

Figure 7. Inhibition of dense granule exocytosis was visually confirmed by confocal microscopy. Quinacrine-labelled platelets were incubated with vehicle, 1mM Que or DiOHF in the dark at 37 °C for 20 minutes. Representative images of quinacrine-labelled platelets with: (A) Que only, (B) Que + 0.5Uml⁻¹ thrombin, (C) DiOHF only, (D) DiOHF + 0.5Uml⁻¹ thrombin, (E) Vehicle only and (F) Vehicle + 0.5Uml⁻¹ thrombin.

Figure 8. 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 six fields per condition.

Notes: Mean $\pm$ SEM. One-way ANOVA with Dunnett's post test.

* $p < 0.05$ vs. vehicle.

Figure 9. Effect of 1mM Que or DiOHF on platelet surface P-selectin (CD62P) expression by flow cytometry. Whole blood aliquots were incubated with vehicle, 1mM Que or DiOHF at 37 °C for five minutes. 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 mM ADP, (B) 0.5mM AA, (C) 20 mM TRAP or (D) 5 μgml^{-1} collagen + 250 mM adrenalin. Mean $\pm$ SEM.

Notes: One-way ANOVA with Dunnett's post test (n=6). *p<0.05 vs. vehicle.

Figure 10. Effect of 1mM Que or DiOHF on platelet surface GPIIb (CD61) expression by flow cytometry. Whole blood aliquots were incubated with vehicle, 1mM Que or DioHF at 37 °C for five minutes. Platelets were identified by characteristic forward and side light scatter and expression of the platelet-specific CD61. MFI of CD61 relative to circulating (no agonist) levels for 0.5mM AA (A) and 20 mM TRAP (B).
Notes: Mean $\pm$ SEM. One-way ANOVA with Dunnett's post test (n=3). *p<0.05 vs. no agonist.

Figure 11. 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 four minutes 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).