Tangeretin Regulates Platelet Function Through Inhibition of Phosphoinositide 3-Kinase and Cyclic Nucleotide Signaling

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Objective—Dietary flavonoids have long been appreciated in reducing cardiovascular disease risk factors, but their mechanisms of action are complex in nature. In this study, the effects of tangeretin, a dietary flavonoid, were explored on platelet function, signaling, and hemostasis.

Approach and Results—Tangeretin inhibited agonist-induced human platelet activation in a concentration-dependent manner. It inhibited agonist-induced integrin αIIbβ3 inside-out and outside-in signaling, intracellular calcium mobilization, and granule secretion. Tangeretin also inhibited human platelet adhesion and subsequent thrombus formation on collagen-coated surfaces under arterial flow conditions in vitro and reduced hemostasis in mice. Further characterization to explore the mechanism by which tangeretin inhibits platelet function revealed distinctive effects of platelet signaling. Tangeretin was found to inhibit phosphoinositide 3-kinase–mediated signaling and increase cGMP levels in platelets, although phosphodiesterase activity was unaffected. Consistent with increased cGMP levels, tangeretin increased the phosphorylation of vasodilator-stimulated phosphoprotein at S239.

Conclusions—This study provides support for the ability and mechanisms of action of dietary flavonoids to modulate platelet signaling and function, which may affect the risk of thrombotic disease. (Arterioscler Thromb Vasc Biol. 2013;33:2740-2749.)

Key Words: blood platelets ■ cAMP ■ cGMP ■ tangeretin ■ thrombosis

Platelets, small circulating blood cells, are activated on vessel wall damage and aggregate to form thrombi to prevent bleeding. Inappropriate activation of platelets under some pathological conditions, such as the rupture of atherosclerotic plaques, leads to thrombosis, the formation of occlusive thrombi within the circulation, which result in myocardial infarction or stroke. The pharmacological suppression of platelet function has been shown to be effective in the reduction of risk of thrombosis. Currently available drugs, however, are not effective in all patients and frequently cause side effects such as bleeding. Thus, the development of safer and more effective antithrombotic strategies is a priority. The relationship between diet and risk factors for cardiovascular diseases has long been appreciated, but the molecular basis of this is complex and poorly understood. Several epidemiological studies have suggested that regular dietary intake of citrus fruits and commonly available plant flavonoids reduce the risk of cardiovascular diseases, inflammation, and tumor progression. Several plant-derived flavonoids, such as quercetin and catechin, have been reported to reduce platelet activation by inhibiting key platelet signaling enzymes (kinases), receptor antagonism (thromboxane A2 receptors), and antioxidant activities.

Tangeretin, a flavonoid abundant in the peel of citrus fruits, has been suggested to have several beneficiary roles in human health. Indeed, the peel of lemons (Citrus limon) is incorporated in traditional medications in India and China. Tangeretin has been reported to prevent bacterial lipopolysaccharide-induced bone loss, it is implicated in increasing glucose uptake by stimulating AMP-activated protein kinase signaling and shown to modulate diet-induced hypercholesterolemia in mice. Tangeretin has also been studied as an anticancer agent and reported to possess anti-invasive properties by inhibiting cell–cell adhesion and intercellular communication in oral and ovarian cancer cells in vitro. A recent study has shown that tangeretin inhibits the platelet-derived growth factor–induced proliferation and migration of smooth muscle cells, which is of relevance to atherogenesis and restenosis after angioplasty. A previous study has reported that tangeretin inhibits ADP and collagen-induced human platelet aggregation, but the detailed analysis of this was not explored. In this report, we demonstrate the antithrombotic activities of tangeretin, which reduce platelet function and thrombus formation by modulation of platelet signaling, blocking AKT activation, and increasing cGMP levels leading to the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) on agonist-induced stimulation.
Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Tangeretin Inhibits Human Platelet Aggregation

Optical aggregometry was used to assess the effect of tangeretin on the ability of platelets to respond to different agonists, such as collagen. Washed platelets were preincubated with vehicle (containing 0.01% dimethyl sulfoxide) or different concentrations (5, 10, 15, and 20 µmol/L) of tangeretin for 3 minutes before activation with collagen (1 µg/mL) for ≤5 minutes (Figure 1A). Tangeretin was found to cause a concentration-dependent reduction in platelet aggregation (Figure 1B) at 5 minutes. Maximum inhibition (95%) was obtained with 20 µmol/L tangeretin at 5 minutes at this concentration of collagen. As collagen activates platelets by binding both glycoprotein VI (GPVI) and integrin α2β1, a GPVI-selective agonist, collagen-related peptide (CRP-XL), was used in aggregation assays to identify if the inhibition of platelet aggregation occurred through the blockade of GPVI-dependent signaling. CRP-XL (0.5 µg/mL)–stimulated platelet aggregation was also inhibited by tangeretin in a concentration-dependent manner (Figure 1C and 1D). Higher concentrations of agonists were able to partially overcome the inhibitory actions of tangeretin (Figure 1E and 1F) for CRP-XL (1 µg/mL). Other flavonoids, such as quercetin, were shown to inhibit key kinases within the GPVI signaling pathway of platelets.11,12 To determine whether the targets for the action of tangeretin are shared with other agonists, thrombin, another strong platelet agonist that activates platelets via G-protein–coupled receptors, was used. A concentration of 0.1 U/mL thrombin was chosen to produce a similar level of aggregation to 1 µg/mL collagen. Thrombin-stimulated platelet activation was also inhibited by treatment with tangeretin but only at higher concentrations (Figure 1G and 1H). For example, 200 µmol/L tangeretin inhibited thrombin (0.1 U/mL)–induced platelet aggregation by ≈80% at 5 minutes, whereas 95% inhibition was obtained with 20 µmol/L tangeretin when stimulated with collagen (1 µg/mL). Because dietary flavonoids have been shown to bind plasma proteins, the effect of tangeretin on platelet function in platelet-rich plasma (PRP) was examined to compare its effects with washed platelets. As with washed platelets, collagen (1 µg/mL)–induced aggregation of PRP was inhibited by tangeretin at different concentrations (5, 10, 25, 50, and 100 µmol/L), but the level of inhibition was reduced (Figure 1I and 1J). For example, 5 µmol/L tangeretin inhibited the aggregation of washed platelets at 5 minutes by 60%, whereas in PRP this was reduced to 20%. This suggests that tangeretin at lower micro molar concentrations (between 5 and 10 µmol/L noting the total tangeretin levels of ≈6.2 µmol/L have been measured in rodents after oral supplementation of 50 mg/kg tangeretin22) is able to cause significant levels of inhibition in PRP. These analyses together indicate that tangeretin predominantly inhibits collagen-stimulated platelet activation although it is capable of modulating platelet activation induced by other agonists at higher concentrations. These observed effects of tangeretin on platelets were similar to the effects obtained with quercetin previously.11,12

Tangeretin Inhibits Integrin αIIb3–Mediated Inside-Out Signaling in Human Platelets

Platelet aggregation is associated with the modulation of the conformation of integrin αIIb3 through inside-out signaling to
enhance its affinity for fibrinogen binding, which is necessary for platelet aggregation.\(^{23}\) Thus, fibrinogen binding (a marker for αIIbβ3 inside-out signaling) was measured using flow cytometry in the presence or absence of tangeretin. Inhibition of CRP-XL (0.5 µg/mL)–induced fibrinogen binding in washed platelets was observed at all concentrations of tangeretin tested (5, 10, 15, and 20 µmol/L; Figure 2A; median fluorescence intensity is shown in Figure IA in the online-only Data Supplement). Similarly, fibrinogen binding analyzed using PRP on stimulation with 0.5 µg/mL CRP-XL was also inhibited at different concentrations (12.5, 25, 50, and 100 µmol/L) by \(\approx 70\%\) inhibition with 50 and 100 µmol/L (Figure 2B; median fluorescence intensity is shown in Figure IIB in the online-only Data Supplement). Compared with the platelet aggregation assays and fibrinogen binding in washed platelets, higher concentrations of tangeretin were used in PRP. It is interesting to note, however, that significant levels of inhibition were observed at all concentrations of tangeretin used. These data suggest that tangeretin modulates inside-out signaling to integrin αIIbβ3 that regulates platelet aggregation.

**Tangeretin Inhibits α-Granule Secretion**

Platelets contain different granule populations: α-granules that are rich in proteins, such as fibrinogen, von Willebrand factor, and P-selectin, and dense granules that are rich in nonproteinaceous substances, such as ADP, ATP, and calcium. Activation of platelets leads to degranulation, which further enhances the platelet activation through the autocrine and paracrine actions of released factors. Various concentrations of tangeretin were used to analyze its inhibitory effects on platelet granule secretion by measuring P-selectin exposure (a marker for α-granule secretion) in washed platelets and PRP using flow cytometry. Tangeretin inhibited the exposure of P-selectin on CRP-XL (0.5 µg/mL) activation at all the concentrations used in washed platelets (Figure 2C) and PRP (Figure 2D), whereas vehicle controls caused no inhibition. Maximal inhibition of \(\approx 50\%\) was obtained with 10 to 15 µmol/L tangeretin in washed human platelets and 100 µmol/L in PRP.

**Tangeretin Inhibits Calcium Mobilization in Platelets**

Elevation of intracellular calcium levels in platelets is important in regulating platelet function, including thrombus formation, reorganization of the actin cytoskeleton necessary for shape change,\(^{24}\) degranulation, and integrin αIIbβ3 affinity modulation.\(^{25}\) In platelets, elevation of cytosolic Ca\(^{2+}\) is mediated through release from intracellular stores and influx across the plasma membrane.\(^{26}\) To assess the effects of tangeretin on elevation of calcium levels in platelets, intracellular calcium mobilization was measured in human PRP by flow cytometry. Tangeretin (50 and 100 µmol/L) inhibited the level of cytosolic calcium elevation on stimulation with CRP-XL (1 µg/mL). Maximum inhibition of 70% was achieved with 100 µmol/L tangeretin (Figure 2E and 2F). The initial acceleration rate of calcium elevation was inhibited substantially by tangeretin (Figure 2E). The level of inhibition obtained in this assay with tangeretin was lower compared with that for aggregation assays because calcium mobilization was measured in PRP under nonstirring conditions. These data suggest that tangeretin affects the elevation of intracellular calcium through inhibition of upstream signaling.

**Tangeretin Limits Thrombus Formation In Vitro**

Because platelet aggregation, granule secretion, and calcium release were reduced by tangeretin, we speculated that tangeretin would influence thrombus formation. Thrombus formation was measured in vitro under arterial flow conditions using whole fluorescently labeled human blood perfused through collagen-coated biochips in the absence or presence of 10, 20, or 100 µmol/L tangeretin. In comparison with control samples (vehicle treated; Figure 3A), tangeretin inhibited the thrombus volume (Figure 3B and 3C) and thrombus fluorescence intensity (Figure 3E) at 10 and 20 µmol/L. Tangeretin at 100 µmol/L reduced dramatically thrombus formation with
Tangeretin Inhibits Platelet Function

Vaiapuri et al

Tangeretin inhibits thrombus formation under arterial flow conditions in whole blood within a range that reported to be achievable in rat supplementation studies.22

Tangeretin Reduces Clot Retraction

Subsequent to fibrinogen binding, the integrin αIIbβ3 transduces signals into the cell triggering platelet spreading, and in the latter phase of thrombus formation, clot retraction.23 The effects of tangeretin on outside-in integrin signaling through αIIbβ3 were assessed through the measurement of clot retraction in vitro. Plasma clots were initiated by adding thrombin to platelet-rich plasma in the absence or presence of tangeretin (12.5, 25, 50, and 100 µmol/L), and the rate of clot retraction was monitored for 2 hours by measuring the remaining clot weight. Clot retraction was reduced 4-fold in the presence of tangeretin (50 and 100 µmol/L) at 2 hours compared with vehicle-treated samples (Figure 3F and 3G). These data suggest that outside-in signaling through αIIbβ3, which controls the coordinated process of clot retraction, is modulated by tangeretin.

Tangeretin Extends Bleeding Time in Mice

To analyze the effects of tangeretin on hemostasis, tail bleeding assays were performed on mice in the presence of vehicle (dimethyl sulfoxide [0.01%]) or tangeretin (estimated 10 and 50 µmol/L based on the mouse weight and respective volume of blood). Vehicle-treated mice bled for a mean time of 200 seconds (between 195 and 228 seconds). As shown in Figure 3H, after administration of tangeretin, bleeding was extended modestly at a concentration of 10 µmol/L (mean time of 252 seconds [between 210 and 294 seconds]) and to a greater extent at 50 µmol/L tangeretin (mean time of 300 seconds [between 237 and 635 seconds]), displaying the level of variability that is normally encountered in the use of this assay. These data suggest that tangeretin moderately inhibits hemostasis.

Tangeretin Blocks AKT Activation

Because collagen and CRP-XL–stimulated platelet aggregation were more predominantly inhibited by tangeretin, the phosphorylation levels of various proteins involved in the GPVI pathway, which is stimulated on binding these agonists, were analyzed. Washed platelets were stimulated under non-aggregation conditions (in the presence of 1 mM EGTA, 10 µmol/L indomethacin, and 2 U/mL apyrase) with CRP-XL (1 µg/mL) in the presence of tangeretin (10, 15, 20, 50, 100, 150, and 200 µmol/L) or vehicle control and lysates were prepared to analyze the phosphorylation of different proteins. Phospho-tyrosine and phospho-specific antibodies against Syk (pY323), linker for activation of T cells (pY200), phospholipase Cγ2 (PLCγ2) (pY759), and AKT (pS473) proteins were used to assess phosphorylation status by immunoblot analysis. The total (Figure 4A) and individual proteins, such as Syk and linker for activation of T cells (Figure 4B) tyrosine phosphorylation levels, were unaffected after treatment with tangeretin. But phosphorylation of PLCγ2 was diminished at all the concentrations of tangeretin (particularly at 100, 150, and 200 µmol/L) and extends tail bleeding in mice. DiOC_6(Tangeretin inhibits thrombus formation in vitro, outside-in signaling and extends tail bleeding in mice. DiOC_6-

**Figure 3.** Tangeretin inhibits thrombus formation in vitro, outside-in signaling and extends tail bleeding in mice. DiOC_6–labeled human blood was preincubated with vehicle (A) or tangeretin (10, 20, 50, and 100 µmol/L, D) and perfused over a collagen-coated Vena8 BioChips. Images were obtained every 30 seconds for ≤10 minutes and representative images (at 10 minutes) are shown. The fluorescence (Fl) intensity of thrombi measured at 2 hours. The P values shown are as calculated by Student’s t test (*P≤0.05 and **P≤0.01). Effect of tangeretin (12.5, 25, 50, and 100 µmol/L) on clot retraction was analyzed in vitro (F and G). F. A representative image of clot retraction at 90 minutes in the presence and absence of different concentrations of tangeretin. Data represent mean±SD (n=4) of clot weights measured at 2 hours. The P values calculated by Student’s t test (**P≤0.01 and ***P≤0.01). The effect of tangeretin (estimated 10 and 50 µmol/L) on hemostasis of mice was analyzed by measuring the bleeding time after tail tip excision. The bleeding time obtained with vehicle-treated mice was compared with the treated thrombi levels (Figure 3F). These data suggest that tangeretin moderately inhibits hemostasis.

GraphPad Prism.
µmol/L) used (Figure 4C). The serine phosphorylation of AKT (pS473) was also affected in a concentration-dependent manner by tangeretin (Figure 4D). Compared with the aggregation assays, the levels of inhibition obtained in the phosphorylation of proteins were small at lower concentrations of tangeretin. This is mainly because of the preparation of platelet lysates under conditions that disfavor aggregation and limitations of immunoblotting sensitivity. These data, however, suggest that although tangeretin may not influence the immediate effectors (Syk and linker for activation of T cells) within the GPVI pathway, it can modulate later signaling events controlled by PLC and AKT, which are shared with activation mechanisms stimulated by other agonists such as thrombin. The inhibitory actions of tangeretin on PLCγ2 and AKT phosphorylation indicate its potential impact on phosphoinositide 3-kinase (PI3K)–mediated platelet signaling.

### Tangeretin Elevates Platelet cGMP Levels But Does Not Inhibit Phosphodiesterase Activity

Under normal physiological conditions, endothelium-derived NO and prostaglandin, PGI₂, inhibit platelet function by elevating cGMP and cAMP, respectively. Because plant flavonoids have shown to act on cyclic nucleotide signaling in other cell types previously,²⁷ the effects of tangeretin on the levels of cGMP and cAMP on agonist stimulation in platelets (at the density of 4×10⁸) were analyzed in the presence of vehicle control or different concentrations (10, 25, 50, and 100 µmol/L) of tangeretin. The levels of cGMP (Figure 5A) and cAMP (Figure 5B) were reduced slightly on stimulation with CRP-XL (0.5 µg/mL), and the addition of tangeretin elevated cGMP levels significantly but not cAMP levels at all the concentrations used. Tangeretin alone did not interfere with the performance of the immunobassay (Figure III in the online-only Data Supplement). These data suggest that tangeretin may inhibit platelet function through the elevation of cGMP and not through cAMP. To explore whether the elevation of cGMP levels by tangeretin is dependent on the activation by CRP-XL, the level of cGMP was measured in resting platelets incubated with vehicle control or tangeretin (50 and 100 µmol/L) for 3 minutes. Platelet cGMP levels were stimulated in platelets treated with tangeretin alone (Figure 5C), indicating that cGMP elevation is not dependent on agonist stimulation or platelet activation.

Dietary flavonoids have been shown previously to inhibit phosphodiesterases,⁸ which hydrolyze cAMP and cGMP to terminate cyclic nucleotide signaling. Because tangeretin elevates cGMP levels, the effect of tangeretin on platelet phosphodiesterase activity was measured using cGMP and cAMP as substrates. Different concentrations (10, 25, 50, and 100 µmol/L) of tangeretin used in these assays did not show any inhibitory effects of phosphodiesterase activity on the hydrolysis of either cGMP (Figure 5D) or cAMP (Figure 5E). The phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, inhibited phosphodiesterase activity by 30% at 10 µmol/L and ≈50% at 40 µmol/L concentration. This result suggests that tangeretin may elevate the cGMP levels through increased production and not through increased hydrolysis of this cyclic nucleotide by phosphodiesterases.

To further confirm the inhibitory effects of tangeretin on platelet function through the elevation of cGMP levels, the effect of tangeretin on platelet aggregation was assessed in the presence of soluble guanylate cyclase inhibitor, 1H-[1,2,4] oxadiazolo [3,4-a] quinoxalin-1-one (ODQ) and cGMP-dependent protein kinase G (PKG) inhibitor (Rp-8-Bromo-β-phenyl-1,2-ethenoguanosine 3′,5′-cyclic monophosphorothioate sodium salt [Rp-8-Br-PET-cGMP]). Although ODQ (20 µmol/L) delayed slightly the acceleration of aggregation, it did not significantly affect the final extent of platelet aggregation, but its preincubation at this concentration before treatment with tangeretin (20 µmol/L) restored platelet aggregation by ≈60% (Figure 5F and 5G). Similarly, the PKG inhibitor (30 µmol/L) did not affect the level of platelet aggregation but its preincubation restored aggregation levels to ≈70% when incubated with tangeretin (Figure 5H and 5I). The prior treatment of platelets with ODQ (20 µmol/L) also significantly reduced the tangeretin-mediated elevation of cGMP levels in resting (Figure 5J) and CRP-XL (0.5 µg/mL)–stimulated platelets (Figure 5K). These data suggest that the
Figure 5. Tangeretin elevates cGMP levels and does not inhibit phosphodiesterase activity. The levels of cGMP (A) and cAMP (B) were measured in platelets on stimulation with collagen-related peptide (CRP-XL) (0.5 µg/mL) using ELISA kits in the presence and absence of various concentrations of tangeretin. Similarly, the level of cGMP was measured in resting platelets incubated with different concentrations of tangeretin for 3 minutes (C). The concentration of cGMP and cAMP was calculated based on standard curves. The effects of various concentrations of tangeretin on phosphodiesterase activity were measured using assay kits on the hydrolysis of cGMP (D) and cAMP (E). IBMX represents the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine at 10 and 40 µmol/L concentration. D and E. The level of phosphodiesterase activity obtained in the absence of inhibitor or tangeretin was taken as 100%. Human platelet aggregation was measured in the presence or absence of 20 µmol/L 1H-(1,2,4) oxadiazolo [3,4-a] quinoxalin-1-one (ODQ), a soluble guanylate cyclase inhibitor (F and G), or 30 µmol/L protein kinase G (PKG) inhibitor (Rp-8-Br-PET-cGMPS) (H and I), after stimulation with CRP-XL (0.5 µg/mL) for 5 minutes. Similarly, after incubation with 20 µmol/L ODQ (F and G) or 30 µmol/L PKG inhibitor (H and I), platelets were treated with 20 µmol/L tangeretin or vehicle control before stimulation with CRP-XL (0.5 µg/mL) for 5 minutes. The level of aggregation obtained with CRP-XL at 5 minutes was taken as 100%. As shown for A, the level of cGMP was measured in resting (J) and CRP-XL (0.5 µg/mL; K) stimulated platelets after the treatment with 20 µmol/L ODQ before the addition of tangeretin. The level of cGMP released was calculated using standard curves. Cumulative data represent means±SD (n=3). P values shown are calculated using Student t test (**P≤0.01, ***P≤0.001, and ****P≤0.0001). C-CRP-XL (0.5 µg/mL), T-tangeretin (20 µmol/L), O-ODQ (20 µmol/L), and P-30 µmol/L PKG inhibitor.

Tangeretin Inhibits Platelet Function

VASCAs are substrates for cAMP- and cGMP-dependent protein kinases, which mediate cAMP- and cGMP-dependent inhibitory signaling in platelets, respectively. On elevation of cAMP, the cAMP-dependent protein kinase A phosphorylates VASP at position S157, whereas increased level of cGMP results in phosphorylation of S239 in VASP by cGMP-dependent protein kinase G (PKG), although some debate exists within the literature as to whether this site (S239) may also be phosphorylated by a cAMP-dependent mechanism in platelets. To further analyze whether tangeretin is involved in cAMP- or cGMP-mediated inhibitory signaling, the phosphorylation of VASP at these 2 sites were assessed using phospho-specific antibodies on stimulation with agonist, CRP-XL (1 µg/mL). Immunoblot analysis showed that tangeretin increased the phosphorylation of VASP at S239 (Figure 6A and 6B) in a concentration-dependent manner on stimulation with CRP-XL (1 µg/mL) but only modest changes (at higher concentrations) in phosphorylation of position S157 (Figure 6A and 6C). Increase in phosphorylation of VASP at S239 position was inhibited when platelets were treated with 30 µmol/L PKG inhibitor (Rp-8-Br-PET-cGMPS) before incubation with tangeretin (Figure 6D). Incubation of platelets with either protein kinase A (H89; 5 µmol/L) or protein kinase C (GF109203X; 5 µmol/L) inhibitors before treatment with tangeretin did not affect the phosphorylation of VASP at S239 (Figure 6D), indicating that tangeretin induces phosphorylation of this site in VASP through the elevation of cGMP- and PKG-dependent signaling. These data suggest that tangeretin may modulate the inhibitory signaling mediated by protein kinase G on activation by agonists such as CRP-XL.

Discussion

Cardiovascular diseases, such as heart attack and stroke, are the most common causes of death in the Western world. Inappropriate activation of platelets in the circulation is the major cause for atherothrombosis. Existing antithrombotic/platelet drugs, such as aspirin and clopidogrel, are associated with side effects, such as intestinal bleeding, resulting in morbidity or mortality, and also are ineffective in some patients. Hence, alternative therapies and preventive measures to reduce the burden of cardiovascular diseases are a priority. Diet is one of the modifiable factors that influence hemostasis and indeed, several dietary flavonoids have been found to have inhibition of platelet function by tangeretin is partially regulated by the increased synthesis of cGMP.
Tangeretin is a polymethoxylated flavone present in tissues and most abundantly in the peel of citrus fruits and has been suggested to have several beneficial effects on human health. In this current study, we evaluated the effects of tangeretin on platelet function and thrombus formation and characterized its mechanism of action in platelets.

Tangeretin was found to inhibit collagen- or CRP-XL–induced platelet aggregation in a concentration-dependent manner, although lower levels of inhibition were obtained with other agonists such as thrombin. A previous review indicated that tangeretin inhibits ADP and collagen-induced platelet aggregation at 30 μmol/L, but the detailed analysis was not reported. In our study, tangeretin was found to inhibit integrin αIIbβ3–mediated outside-in and outside-in signaling, granule secretion, and thrombus formation in human platelets. The ability of tangeretin to inhibit integrin αIIbβ3–mediated outside-in signaling is consistent with its ability to lower potency to inhibit platelet function stimulated by other agonists such as thrombin. Similarly, quercetin, another flavonoid, and its metabolites were shown to inhibit collagen-induced platelet activation through inhibition of GPVI-mediated signaling in platelets. Some flavonoids, such as apigenin and luteolin, have been shown to antagonize thromboxane A2 receptors to inhibit platelet functions. In addition, purple grape juice and its flavonoid derivatives have shown to inhibit platelet function by increasing platelet-derived NO release and decreasing superoxide production.

Flavonoids have been shown to inhibit the functions of several enzymes involved in platelet signaling, such as cyclooxygenases, lipoygenases, phosphodiesterases, tyrosine kinases, and phospholipases. Although tangeretin did not inhibit the tyrosine phosphorylation of signaling proteins, Syk and linker for activation of T cells, it inhibited the tyrosine phosphorylation of PLCγ2 involved in GPVI pathway on stimulation with CRP-XL. Consistent with the inhibition of PLCγ2 signaling tangeretin inhibited the elevation of intracellular calcium levels in platelets, and intracellular calcium mobilization was recognized to be vital for platelet secretion and aggregation. Within the GPVI signaling pathway, PLCγ2 is at least, in part, dependent on the upstream activation of PI3K, a family of enzymes whose activities are well documented to be modulated by flavonoids, such as quercetin, and its structural analogues. Indeed, the widely used PI3K inhibitor, LY294002, was designed based on the structure of quercetin. In our study, tangeretin was found to inhibit the serine phosphorylation of AKT at position S473, an established marker of PI3K-mediated signaling. Tangeretin was shown to inhibit the phosphorylation of AKT in vascular smooth muscle cells previously. Cathechin and epigallocatechin gallate, flavonoids present in green tea, have been shown to inhibit the phosphorylation of p38 mitogen-activated protein kinase and extracellular signal–related kinase 1/2 in platelets. Similarly, quercetin and catechin are able to inhibit the phosphorylation of serine/threonine kinases, extracellular signal–related kinase 1/2, c-Jun N-terminal kinase, mitogen-activated protein kinase, and protein kinase C (AKT) in vascular smooth muscle and endothelial cells. Purple grape juice products were shown to partially inhibit the phosphorylation of protein kinase C in platelets. The potential effects of flavonoids on the platelet signaling molecules may not be restricted to kinases within the GPVI signaling pathway, and therefore we explored the possibility of whether well-established endogenous platelet inhibition mechanisms, such as those stimulated by NO, might also be modulated by tangeretin.

Increasing concentrations of tangeretin were found to elevate platelet cGMP levels and increase the phosphorylation of VASP at position S239, which is regulated by cGMP-mediated signaling in platelets. Tangeretin exhibited only modest effects on the phosphorylation of VASP at S157, which is not shown any effects in the elevation of cAMP levels. Our data suggest that the elevation of cGMP levels is not mediated through the inhibition of phosphodiesterases in platelets. Purple grape juice and its flavonoid derivatives were previously shown to increase platelet-derived NO release and decreasing superoxide production.
protein kinases.\textsuperscript{51,42} Increased cGMP levels/PKG activation is also accompanied by decreased intracellular calcium mobilization\textsuperscript{53,44} mediated through phospholamban phosphorylation and sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase activation.\textsuperscript{55,46} Increased levels of cGMP have also been shown to inhibit thrombin-mediated PI3K activity, which results in reduced irreversible association of fibrinogen with integrin \alpha\text{IIb}β3 leading to platelet disaggregation.\textsuperscript{45} Decreased calcium levels,\textsuperscript{46} cGMP-dependent inhibition of PI3K,\textsuperscript{47} and cGMP-dependent phosphorylation of VASP\textsuperscript{49} reduce the conformational changes in \alpha\text{IIb}β3 and subsequent fibrinogen binding. Furthermore, cGMP-dependent protein kinase phosphatases thromboxane \text{A}_3 receptors to suppress platelet activation.\textsuperscript{50} Recently, epigallocatechin-3-gallate, a catechin analogue from green tea, was shown to increase cAMP levels and increase the phosphorylation of VASP at S157 to inhibit platelet function.\textsuperscript{27} Thus, tangeretin may modulate platelet functions through inhibition of phosphorylation of signaling proteins and the modulation of cyclic nucleotide signaling. It cannot be ruled out that tangeretin may also regulate platelet functions through mechanisms other than reported in this study. Quercetin-3-rutinoside was demonstrated recently as an inhibitor for protein disulphide isomerase on platelet surfaces to inhibit platelet function.\textsuperscript{61} In similar ways, tangeretin may have other targets in addition to the above described and together act to inhibit platelet function.

Flavonoids are recognized to be promiscuous modulators of cell signaling,\textsuperscript{6} and it is possible therefore that the observed effects of tangeretin are not causally linked. The recognized abilities of some flavonoids to inhibit PI3K are, however, consistent with tangeretin inhibiting AKT, PLC\textgamma;2, and calcium mobilization. Similarly, cGMP is implicated in the modulation of PI3K signaling in platelets,\textsuperscript{47} and therefore these events may be causally linked. Further work will be required to explore this in detail. PI3K is an important regulator of endothelial nitric oxide synthase in endothelial cells and thus the stimulation of cGMP-dependent signaling. Therefore, in the in vivo situation, it is possible that tangeretin modulates alternative mechanisms that affect platelet regulation via cGMP. The presence of endothelial nitric oxide synthase in platelets is a subject of considerable debate\textsuperscript{52} with some studies reporting its presence and function,\textsuperscript{53,54} and others its absence.\textsuperscript{55,56} If present, this may represent an additional explanation for the effects of tangeretin on cGMP signaling in platelets. The broad spectrum inhibitory effects of tangeretin in various cell types indicate the value of further research to scrutinize its potential for reducing cardiovascular disease risk. The less polar and planar structure of tangeretin compared with other flavonoids has been suggested to play roles in its biological activity by enhancing permeability to biological membranes and its binding properties.\textsuperscript{57,58}

Although little detail is available with respect to the significance of metabolites of tangeretin, 1 primary metabolite, 4′-hydroxy-5,6,7,8-tetramethoxyflavone, was shown to inhibit cell cycle progression in hepatocytes.\textsuperscript{56} A pharmacokinetic study also identified 2 metabolites in plasma on oral or intraperitoneal administration of tangeretin in rodents,\textsuperscript{22} but detailed research to analyze the effects of different metabolites of tangeretin on platelets and other cell types will be required to assess potential roles and mechanism of action in vivo.

This study demonstrates that 10 µmol/L tangeretin is able to inhibit moderately platelet function in vitro and in vivo. A previous pharmacokinetic study reported to achieve a total tangeretin concentration of ≈6.2 µmol/L (including known metabolites) in rodents through oral supplementation of 50 mg/kg.\textsuperscript{22} Although it is unlikely that levels of between 5 and 10 µmol/L tangeretin would be achieved through regular dietary intake, this is not the only flavonoid present in most diets and higher levels may also be attainable by supplementation, as indicated from previous study in rodents. Indeed, other flavonoids, such as quercetin, have been shown to reach similar (10 µmol/L) levels through supplementation in humans.\textsuperscript{5,32} Dietary sources were similarly found to be beneficial after absorption, affecting platelet signaling and aggregation. Thus, tangeretin together with other bioactive flavonoids may collectively modulate platelet function with beneficial effects through reduction of thrombosis risk. The effects of tangeretin, however, measured below the threshold of detection in vivo may still be relevant under physiological conditions. Also, chronic ingestion of tangeretin-rich diets may result in a cumulative effect on reduction of platelet function over time. A diet rich in sources of flavonoids results in intake of a range of complex mixture of various flavonoids (such as quercetin) that may also be beneficial in reducing cardiovascular disease incidence. Thus, understanding the mechanisms of action of specific flavonoids on platelet function may be important to further determine the basis of the relationship between diet and cardiovascular disease risk.

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J.M. Gibbins is a visiting professor at King Saud University, Riyadh, Saudi Arabia. The other authors report no conflicts.

References


Platelets are small circulating blood cells that play paramount roles in hemostasis and trigger thrombosis under pathological conditions. Existing antithrombotic drugs, such as aspirin and clopidogrel, cause serious side effects, such as bleeding, and are frequently ineffective. Hence, the development of safer and more effective antithrombotic drugs is a priority. Modification of dietary habits is a proven method to reduce risk factors associated with cardiovascular diseases. In this study, we report the ability of the dietary flavonoid, tangeretin, to inhibit platelet function, thrombus formation, and hemostasis through suppression of platelet signaling mechanisms. This study provides additional insights into the relationship between dietary components and cardiovascular function. Given the inhibitory effects of tangeretin on platelet function, it may pave the way for the development of novel antithrombotic strategies.
Tangeretin Regulates Platelet Function Through Inhibition of Phosphoinositide 3-Kinase and Cyclic Nucleotide Signaling
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Supplemental Figure I: **Effect of 0.01% DMSO on platelet aggregation.** Human platelet aggregation was measured upon stimulation with 0.5µg/ml CRP-XL with (A) and without (B) 0.01% DMSO (the concentration that was used in all experiments in this study) and for 5 minutes. The traces shown are representative of three separate experiments.
**Supplemental Figure I**: Tangeretin inhibits fibrinogen binding in platelets. Median fluorescence intensity of fibrinogen binding in absence and presence of different concentrations of tangeretin in washed platelets (A) and PRP (B) is shown. Data represent mean ± S.D. (n=4). The p-values shown in figure are as calculated by students T-test (*p=<0.05, **p=<0.01 and ***p=<0.001).
Supplemental Figure III: Effect of tangeretin on cGMP immunoassay. The effect of tangeretin alone was assessed in cGMP assay without platelets and observed the level of absorbance obtained. The value obtained after deducting the background was used to calculate the cGMP in comparison to resting and platelets treated with tangeretin. T- tangeretin and P- resting platelets.
Materials and Methods

Platelet preparation, aggregation and immunoblotting

Human platelet preparation and aggregation assays were performed as described previously. Briefly, blood was obtained from healthy, aspirin-free, human volunteers with informed consent. Platelet-rich plasma (PRP) was prepared by centrifuging the blood for 20 minutes at 102g at room temperature for aggregation and flow cytometry assays. Washed platelets were prepared and re-suspended in modified Tyrodes-HEPES buffer (134mM NaCl, 2.9mM KCl, 0.34mM Na2HPO4, 12mM NaHCO3, 20mM HEPES and 1mM MgCl2, pH 7.3) to the final density of 4x10^8 cells/ml for aggregation assays. Contaminating blood cells were counted by light microscopy and were mainly erythrocytes; leukocytes were rarely encountered with total cell contaminant level was <1 per 13000 platelets. Aggregation assays were performed using collagen-related peptide [(CRP-XL), a selective agonist for the platelet collagen receptor glycoprotein (GP) VI, from Prof R Farndale (University of Cambridge, UK)], thrombin (Sigma Aldrich, UK) and collagen (Horm collagen, Nycomed, Austria) in the presence or absence of various concentrations of tangeretin (Sigma Aldrich, UK), dissolved in DMSO. The final concentration of DMSO used where required as diluent was 0.01% (v/v) which does not affect platelet function (Supplementary Figure S1), and assays incorporated appropriate vehicle controls. SDS-PAGE and immunoblotting were performed using standard protocols as described previously. Rabbit anti-human 14-3-3ζ (Santa Cruz Biotechnology, USA) was used to detect 14-3-3ζ to ensure equivalent levels of protein loading in immunoblots. The anti-phosphotyrosine antibody (4G10) was obtained from Millipore, USA and phospho-specific antibodies against various signalling proteins (Syk, PLCγ2, LAT and Lyn) were obtained from Epitomics, USA. Anti-phospho VASP antibodies were obtained from Cell signalling technology, USA. The secondary antibodies for immunoblotting; Cy5® goat anti-rabbit IgG and Cy3® goat anti-mouse IgG antibodies were obtained from Invitrogen, UK. cGMP and cAMP ELISA detection and, cyclic nucleotide phosphodiesterase assays were obtained from Enzo lifesciences, UK and performed according to manufacturer’s protocols.

Flow cytometry

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

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

Clot retraction

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

**Thrombus formation in vitro**

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

**Assessment of haemostasis**

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

**Statistical analysis**

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

**References**