

# *Tangeretin regulates platelet function through inhibition of phosphoinositide 3-Kinase and cyclic nucleotide signaling*

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**Full title:** Tangeretin regulates platelet function through inhibition of phosphoinositide 3-kinase and cyclic nucleotide signalling

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## **Tangeretin regulates platelet function through inhibition of phosphoinositide 3-kinase and cyclic nucleotide signalling**

*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 was explored on platelet function, signalling and haemostasis.

*Approach and Results-* Tangeretin inhibited agonist induced human platelet activation in a concentration-dependent manner. It inhibited agonist induced integrin  $\alpha\text{IIb}\beta\text{3}$  inside-out and outside-in signalling, intracellular calcium mobilisation 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 haemostasis in mice. Further characterisation to explore the mechanism by which tangeretin inhibits platelet function revealed distinctive effects of platelet signalling. Tangeretin was found to inhibit phosphoinositide 3-kinase (PI3K) mediated signalling and increase cGMP levels in platelets although phosphodiesterase activity was unaffected. Consistent with increased cGMP levels, tangeretin increased the phosphorylation of VASP at S239.

*Conclusions-* This study provides support for the ability and mechanisms of action of dietary flavonoids to modulate platelet signalling and function, which may impact on the risk of thrombotic disease.

Platelets, small circulating blood cells, are activated upon vessel wall damage and aggregate to form thrombi in order to prevent bleeding.<sup>1</sup> Inappropriate activation of platelets under some pathological conditions such as the rupture of atherosclerotic plaques leads to thrombosis, the formation of occlusive thrombi within circulation, which result in myocardial infarction or stroke.<sup>2</sup> 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.<sup>3-5</sup> Thus, the development of safer and more effective anti-thrombotic 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.<sup>6</sup> 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 tumour progression.<sup>7-10</sup> Several plant derived flavonoids such as quercetin and catechin have been reported to reduce platelet activation by inhibiting key platelet signalling enzymes (kinases), receptor antagonism (thromboxane  $A_2$  receptors) and anti-oxidant activities.<sup>11-13</sup>

Tangeretin, a flavonoid abundant in the peel of citrus fruits has been suggested to have several beneficiary roles in human health.<sup>8</sup> 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,<sup>14</sup> it is implicated in increasing glucose uptake by stimulating AMP activated protein kinase (AMPK) signalling,<sup>15</sup> and shown to modulate diet-induced hypercholesterolemia in mice.<sup>16</sup> Tangeretin has also been studied as an anti-cancer agent and reported to possess anti-invasive properties by inhibiting cell-cell adhesion and intercellular communication in oral and ovarian cancer cells *in vitro*.<sup>8, 17-19</sup> 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 following angioplasty.<sup>20</sup> A previous study has reported that tangeretin inhibits ADP and collagen induced human platelet aggregation but the detailed analysis of this was not explored.<sup>21</sup> In this report, we demonstrate the antithrombotic activities of tangeretin, which reduces platelet function and thrombus formation by modulation of platelet signalling, blocking AKT activation, and increasing cGMP levels leading to the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) upon agonist induced stimulation.

## Materials and Methods

Materials and Methods are available in the online-only Data 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 pre-incubated with vehicle (containing 0.01% DMSO) or different concentrations (5, 10, 15 and 20 $\mu$ M) of tangeretin for 3 minutes prior to activation with collagen (1 $\mu$ g/ml) for up to 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 $\mu$ M tangeretin at 5 minutes at this concentration of collagen. As collagen activates platelets by binding both GPVI and integrin  $\alpha$ 2 $\beta$ 1, a GPVI-selective agonist, CRP-XL was used in aggregation assays to identify if the inhibition of platelet aggregation occurred through the blockade of GPVI dependent signalling. CRP-XL (0.5 $\mu$ g/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 $\mu$ g/ml). Other flavonoids such as quercetin were shown to inhibit key kinases within the GPVI signalling pathway of platelets.<sup>11, 12</sup> 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 0.1U/ml thrombin was chosen to produce a similar level of aggregation to 1 $\mu$ g/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 $\mu$ M tangeretin inhibited thrombin (0.1U/ml) induced platelet aggregation by approximately 80% at 5 minutes, whereas 95% inhibition was obtained with 20 $\mu$ M tangeretin when stimulated with collagen (1 $\mu$ g/ml). Since dietary flavonoids have been shown to bind plasma proteins, the effect of tangeretin on platelet function in PRP was examined to compare its effects with washed platelets. As with washed platelets, collagen (1 $\mu$ g/ml) induced platelet (PRP) aggregation was inhibited by tangeretin at different concentrations (5, 10, 25, 50 and 100 $\mu$ M), but the level of inhibition was reduced (Figure 1I and 1J). For example, 5 $\mu$ M 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 $\mu$ M) noting the total tangeretin levels of around 6.2 $\mu$ M have been measured in rodents following oral supplementation of 50mg/kg tangeretin,<sup>22</sup> are able to cause significant levels of inhibition in PRP. These analyses together indicate that tangeretin predominantly inhibits collagen-stimulated platelet activation, although 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.<sup>11, 12</sup>

*Tangeretin inhibits integrin  $\alpha$ IIB $\beta$ 3 mediated inside-out signalling in human platelets-* Platelet aggregation is associated with the modulation of the conformation of integrin  $\alpha$ IIB $\beta$ 3 through inside-out signalling to enhance its affinity for fibrinogen binding which is necessary for platelet aggregation.<sup>23</sup> Thus, fibrinogen binding (a marker for  $\alpha$ IIB $\beta$ 3 inside-out signalling) was measured using flow cytometry in the presence or absence of tangeretin. Inhibition of CRP-XL- (0.5 $\mu$ g/ml) induced fibrinogen binding in washed platelets was observed at all concentrations of tangeretin tested (5, 10, 15 and 20 $\mu$ M) (Figure 2A; median fluorescence intensity is shown in Supplementary Figure IIA). Similarly, fibrinogen binding analysed using PRP upon stimulation with 0.5 $\mu$ g/ml CRP-XL was also inhibited at different concentrations (12.5, 25, 50 and 100 $\mu$ M) by approximately 70% inhibition with 50 and 100 $\mu$ M (Figure 2B; median fluorescence intensity is shown in Supplementary Figure IIB). Compared to 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 signalling to integrin  $\alpha$ IIB $\beta$ 3 that regulates platelet aggregation.

*Tangeretin inhibits  $\alpha$ -granule secretion-* Platelets contain different granule populations;  $\alpha$ -granules which are rich in proteins such as fibrinogen, vWF and P-selectin and dense granules which are rich in non-proteinaceous 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 analyse its inhibitory effects on platelet granule secretion by measuring P-selectin exposure (a marker for  $\alpha$ -granule secretion) in washed platelets and PRP using flow cytometry. Tangeretin inhibited the exposure of P-selectin upon CRP-XL (0.5 $\mu$ g/ml) activation at all the concentrations used in washed platelets (Figure 2C) and PRP (Figure 2D) while vehicle controls caused no inhibition. Maximal inhibition of approximately 50% was obtained with 10-15 $\mu$ M tangeretin in washed human platelets and 100 $\mu$ M in PRP.

*Tangeretin inhibits calcium mobilisation in platelets-* Elevation of intracellular calcium levels in platelets upon activation is important in regulating platelet function including thrombus formation, reorganisation of the actin cytoskeleton necessary for shape change,<sup>24</sup> degranulation and integrin  $\alpha$ IIb $\beta$ 3 affinity modulation.<sup>25</sup> In platelets, elevation of cytosolic Ca<sup>2+</sup> is mediated through release from intracellular stores and influx across the plasma membrane.<sup>26</sup> To assess the effects of tangeretin on elevation of calcium levels in platelets, intracellular calcium mobilisation was measured in human PRP by flow cytometry. Tangeretin (50 and 100 $\mu$ M) inhibited the level of cytosolic calcium elevation upon stimulation with CRP-XL (1 $\mu$ g/ml). Maximum inhibition of 70% was achieved with 100 $\mu$ M 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 to that for aggregation assays since calcium mobilisation was measured in PRP under non-stirring conditions. These data suggest that tangeretin affects the elevation of intracellular calcium through inhibition of upstream signalling.

*Tangeretin limits thrombus formation in vitro-* Since 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 labelled human blood perfused through collagen-coated biochips in the absence or presence of 10, 20 or 100 $\mu$ M 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 $\mu$ M. Tangeretin at 100 $\mu$ M reduced dramatically thrombus formation with only few platelets adhering to collagen surface (Figure 3D). These data suggest that tangeretin is able to modulate thrombus formation under arterial flow conditions in whole blood within a range that reported to be achievable in rat supplementation studies<sup>22</sup>.

#### *Tangeretin reduces clot retraction*

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

### *Tangeretin extends bleeding time in mice*

To analyse the effects of tangeretin on haemostasis, tail bleeding assays were performed on mice in the presence of vehicle (DMSO (0.01%)) or tangeretin (estimated 10 and 50 $\mu$ M based on the mouse weight and respective volume of blood). Vehicle treated mice bled for a mean time of 200 seconds (between 195s and 228s). As shown in Figure 3H, following administration of tangeretin bleeding was extended modestly at a concentration of 10  $\mu$ M (mean time of 252 seconds (between 210 and 294 seconds), and to a greater extent at 50  $\mu$ M 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 haemostasis.

### *Tangeretin blocks AKT activation*

Since collagen and CRP-XL stimulated platelet aggregation was more predominantly inhibited by tangeretin, the phosphorylation levels of various proteins involved in the GPVI pathway which is stimulated upon binding these agonists was analysed. Washed platelets were stimulated under non-aggregation conditions (in presence of 1mM EGTA, 10 $\mu$ M indomethacin and 2U/ml apyrase) with CRP-XL (1 $\mu$ g/ml) in the presence of tangeretin (10, 15, 20, 50, 100, 150 and 200 $\mu$ M) or vehicle control and lysates were prepared to analyse the phosphorylation of different proteins. Phosphotyrosine and phospho-specific antibodies against Syk (pY323), LAT (pY200), PLC $\gamma$ 2 (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 LAT (Figure 4B) tyrosine phosphorylation levels were unaffected following treatment with tangeretin. But phosphorylation of PLC $\gamma$ 2 was diminished at all the concentrations of tangeretin (particularly at 100, 150 and 200 $\mu$ M) used (Figure 4C). The serine phosphorylation of AKT (pS473) was also affected in a concentration-dependent manner by tangeretin (Figure 4D). Compared to the aggregation assays, the levels of inhibition obtained in the phosphorylation of proteins were small at lower concentrations of tangeretin. This is mainly due to the preparation of platelet lysates under conditions which disfavour aggregation and limitations of immunoblotting sensitivity. These data, however, suggest that although tangeretin may not influence the immediate effectors (LAT and Syk) within the GPVI pathway, it can modulate later signalling 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 PLC $\gamma$ 2 and AKT phosphorylation indicate its potential impact on PI3K mediated platelet signalling.

### *Tangeretin elevates platelet cGMP levels but does not inhibit phosphodiesterase activity*

Under normal physiological conditions endothelium-derived nitric oxide (NO) and prostaglandin, PGI<sub>2</sub> inhibit platelet function by elevating cGMP and cAMP respectively. Since plant flavonoids have shown to act on cyclic nucleotide signalling in other cell types previously,<sup>27</sup> the effects of tangeretin on the levels of cGMP and cAMP upon agonist stimulation in platelets (at the density of 4x10<sup>8</sup>) were analysed in presence of vehicle control or different concentrations (10, 25, 50 and 100 $\mu$ M) of tangeretin. The levels of cGMP (Figure 5A) and cAMP (Figure 5B) were reduced slightly upon stimulation with CRP-XL (0.5 $\mu$ g/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 immunoassay (Supplementary Figure III). 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  $\mu$ M) 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 previously been shown to inhibit phosphodiesterases<sup>6</sup> which hydrolyse cAMP and cGMP to terminate cyclic nucleotide signalling. Since 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 100µM) of tangeretin used in these assays did not show any inhibitory effects of phosphodiesterase activity on either the hydrolysis of cGMP (Figure 5D) or cAMP (Figure 5E). The phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) inhibited phosphodiesterase activity by 30% at 10µM and around 50% at 40µM 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 presence of soluble guanylate cyclase inhibitor, 1*H*-[1,2,4] oxadiazolo [3,4-*a*] quinoxalin-1-one (ODQ) and cGMP dependent protein kinase (PKG) inhibitor [Rp-8-Bromo-β-phenyl-1,N2-ethenoguanosine 3',5'-cyclic monophosphorothioate sodium salt (*Rp*-8-Br-PET-cGMPS)]. Although, ODQ (20µM) delayed slightly the acceleration of aggregation, it did not significantly affect the final extent of platelet aggregation, but its pre-incubation at this concentration prior to treatment with tangeretin (20µM) restored platelet aggregation by approximately 60% (Figure 5F-5G). Similarly, the PKG inhibitor (30µM) did not affect the level of platelet aggregation but its pre-incubation restored aggregation levels to around 70% when incubated with tangeretin (Figure 5H-5I). The prior treatment of platelets with ODQ (20µM) also significantly reduced the tangeretin mediated elevation of cGMP levels in resting (Figure 5J) and CRP-XL (0.5µg/ml) (Figure 5K) stimulated platelets. These data suggest that the inhibition of platelet function by tangeretin is partially regulated by the increased synthesis of cGMP.

#### *Tangeretin increases VASP phosphorylation*

VASP is a substrate for cAMP- and cGMP- dependent protein kinases which mediate cAMP and cGMP dependent inhibitory signalling in platelets, respectively. Upon elevation of cAMP, the cAMP-dependent protein kinase (PKA) phosphorylates VASP at position S157 whereas increased level of cGMP results in phosphorylation of S239 in VASP by cGMP- dependent protein kinase (PKG) although some debate exist within the literature as to whether this site (S239) may also be phosphorylated by a cAMP dependent mechanism in platelets.<sup>28, 29-31</sup> To further analyse if tangeretin is involved in cAMP or cGMP mediated inhibitory signalling, the phosphorylation of VASP at these two sites were assessed using phospho-specific antibodies upon stimulation with agonist, CRP-XL (1µg/ml). Immunoblot analysis showed that tangeretin increased the phosphorylation of VASP at S239 (Figure 6A-6B) in a concentration-dependent manner upon stimulation with CRP-XL (1µg/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 30µM PKG inhibitor (*Rp*-8-Br-PET-cGMPS) prior to incubation with tangeretin (Figure 6D). Incubation of platelets with either PKA (H89) (5µM) or PKC (GF109203X) (5µM) inhibitors prior to 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 signalling. These data suggest that tangeretin may modulate the inhibitory signalling mediated by protein kinase G upon 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.<sup>1</sup> Existing anti-thrombotic/platelet drugs such as aspirin and clopidogrel are associated with side effects such as intestinal bleeding resulting in morbidity or mortality<sup>4</sup> 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



haemostasis and indeed, several dietary flavonoids have been found to have beneficial effects in the prevention of cardiovascular diseases.<sup>6</sup> 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.<sup>8</sup> In this current study, we evaluated the effects of tangeretin on platelet function and thrombus formation, and characterised 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 $\mu$ M but the detailed analysis was not reported.<sup>21</sup> In our study tangeretin was found to inhibit integrin  $\alpha$ IIb $\beta$ 3-mediated inside-out and outside-in signalling, granule secretion and thrombus formation in human platelets. The ability of tangeretin to inhibit integrin  $\alpha$ IIb $\beta$ 3-mediated outside-in signalling is consistent with its ability at 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 signalling in platelets.<sup>12, 32</sup> Some flavonoids such as apigenin and luteolin have been shown to antagonise thromboxane A<sub>2</sub> receptors in order to inhibit platelet functions.<sup>33, 34</sup> 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.<sup>35</sup>

Flavonoids have been shown to inhibit the functions of several enzymes involved in platelet signalling such as cyclooxygenases, lipoxygenases, phosphodiesterases, tyrosine kinases and phospholipases.<sup>6</sup> Although tangeretin did not inhibit the tyrosine phosphorylation of signalling proteins, Syk and LAT, it inhibited the tyrosine phosphorylation of PLC $\gamma$ 2 involved in GPVI pathway upon stimulation with CRP-XL. Consistent with the inhibition of PLC $\gamma$ 2 signalling tangeretin inhibited the elevation of intracellular calcium levels in platelets, and intracellular calcium mobilisation is recognised to be vital for platelet secretion and aggregation. Within the GPVI signalling pathway PLC $\gamma$ 2 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.<sup>36</sup> Indeed, the widely used PI3K inhibitor, LY294002 was designed based on the structure of quercetin.<sup>37</sup> In our study tangeretin was found to inhibit the serine phosphorylation of AKT at position S473, an established marker of PI3K-mediated signalling. Tangeretin was shown to inhibit the phosphorylation of AKT in vascular smooth muscle cells previously.<sup>20</sup> Catechin and epigallocatechin gallate, flavonoids present in green tea have been shown to inhibit the phosphorylation of p38 mitogen activated protein kinase (MAPK) and extracellular signal related kinase (ERK) 1/2 in platelets.<sup>13</sup> Similarly, quercetin and catechin are able to inhibit the phosphorylation of serine/threonine kinases, ERK1/2, c-Jun N-terminal kinase, MAPK and protein kinase B (AKT) in vascular smooth muscle<sup>38</sup> and endothelial cells.<sup>6, 39</sup> Purple grape juice products were shown to partially inhibit the phosphorylation of PKC in platelets.<sup>35</sup> The potential effects of flavonoids on the platelet signalling molecules may not be restricted to kinases within the GPVI signalling 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 increased the phosphorylation of VASP at position S239 which is regulated by cGMP-mediated signalling in platelets. Tangeretin exhibited only modest effects on the phosphorylation of VASP at S157 and did not show 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 production and to inhibit platelet function.<sup>35</sup> Elevated levels of NO in platelets have been shown to increase cGMP levels which in turn activate cGMP kinase to phosphorylate its substrate VASP at position S239.<sup>40</sup> It is presently unclear whether the effects that we observed are mediated by direct actions of tangeretin

on protein kinase G, NO production, or cGMP levels. In this study, however, we further confirmed that the elevation of cGMP levels by tangeretin is mediated through soluble guanylate cyclase activity, and that the phosphorylation of VASP is mediated through protein kinase G.

The observed elevated cGMP levels may affect several signalling pathways such as cGMP-dependent effectors including protein kinases.<sup>41, 42</sup> Increased cGMP levels/PKG activation is also accompanied by decreased intracellular calcium mobilisation<sup>43, 44</sup> mediated through phospholamban phosphorylation and SERCA activation.<sup>45, 46</sup> Increased levels of cGMP have also been shown to inhibit thrombin-mediated PI3K activity which results in reduced irreversible association of fibrinogen with integrin  $\alpha$ IIb $\beta$ 3 leading to platelet disaggregation.<sup>47</sup> Decreased calcium levels,<sup>48</sup> cGMP-dependent inhibition of PI3K<sup>47</sup> and cGMP-dependent phosphorylation of VASP<sup>49</sup> reduce the conformational changes in  $\alpha$ IIb $\beta$ 3 and subsequent fibrinogen binding. Furthermore, cGMP-dependent protein kinase phosphorylates thromboxane (TXA<sub>2</sub>) receptors to suppress platelet activation.<sup>50</sup> 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.<sup>27</sup> Thus, tangeretin may modulate platelet functions through inhibition of phosphorylation of signalling proteins and the modulation of cyclic nucleotide signalling. It cannot be ruled out that tangeretin may also regulate platelet functions through mechanisms other than reported in this study. Quercetin-3-rutinoside was recently demonstrated as an inhibitor for protein disulphide isomerase on platelet surfaces to inhibit platelet function.<sup>51</sup> In similar ways, tangeretin may have other targets in addition to the above described and together act to inhibit platelet function.

Flavonoids are recognised to be promiscuous modulators of cell signalling,<sup>6</sup> and it is possible therefore that the observed effects of tangeretin are not causally linked. The recognised abilities of some flavonoids to inhibit PI3K are, however, consistent with tangeretin inhibiting AKT, PLC $\gamma$ 2 and calcium mobilisation. Similarly cGMP is implicated in the modulation of PI3K signalling in platelets,<sup>47</sup> and therefore these events may be causally linked. Further work will be required to explore this in detail. PI3K is an important regulator of eNOS in endothelial cells, and thus the stimulation of cGMP dependent signalling. Therefore in the *in vivo* situation it is possible that tangeretin modulates alternative mechanisms that impact upon platelet regulation via cGMP. The presence of eNOS in platelets is a subject of considerable debate<sup>52</sup> with some studies reporting its presence and function,<sup>53, 54</sup> and others its absence.<sup>55, 56</sup> If present, this may represent an additional explanation for the effects of tangeretin on cGMP signalling in platelets. The broad spectrum inhibitory effects of tangeretin in various cell types indicate the value of further research to scrutinise its potential for reducing cardiovascular disease risk. The less polar and planar structure of tangeretin compared to other flavonoids has been suggested to play roles in its biological activity by enhancing permeability to biological membranes and its binding properties.<sup>8, 57</sup>

While little detail is available with respect to the significance of metabolites of tangeretin, one primary metabolite, 4'-hydroxy-5,6,7,8-tetramethoxyflavone, was shown to inhibit cell cycle progression in hepatocytes.<sup>58</sup> A pharmacokinetic study also identified two metabolites in plasma upon oral or intraperitoneal administration of tangeretin in rodents,<sup>22</sup> but detailed research to analyse the effects of different metabolites of tangeretin on platelets and other cell types will be required in order to assess potential roles and mechanism of action *in vivo*.

This study demonstrates that 10 $\mu$ M tangeretin is able to moderately inhibit platelet function *in vitro* and *in vivo*. A previous pharmacokinetic study reported to achieve a total tangeretin concentration of around 6.2 $\mu$ M (including known metabolites) in rodents through oral supplementation of 50mg/kg.<sup>22</sup> Although it is unlikely that levels of between 5 and 10 $\mu$ M 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 $\mu$ M) levels through supplementation in humans.<sup>9, 32</sup> Dietary sources were similarly found to be beneficial following absorption, affecting platelet signalling 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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### **Disclosures**

None.

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### **Significance**

Platelets are small circulating blood cells that play paramount roles in haemostasis and trigger thrombosis under pathological conditions. Existing anti-thrombotic 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 anti-thrombotic 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 haemostasis through suppression of platelet signalling mechanisms. This study provides additional insights into the relationship between dietary components and cardiovascular function. Given the dual inhibitory effects of tangeretin on platelet function, it may pave for the development of novel anti-thrombotic strategies.

## Figure legends

**Figure 1: Tangeretin inhibits agonist induced platelet aggregation.** Human platelet aggregation performed in the presence or absence of various concentrations of tangeretin was recorded following stimulation with 1µg/ml collagen (A and B), CRP-XL [0.5µg/ml (C and D) and 1 µg/ml (E and F)] or 0.1U/ml thrombin (G and H). The effects of tangeretin on platelet-rich plasma were analysed upon stimulation with 1µg/ml collagen (I and J). Cumulative data represent mean values ± S.D (n=4), where the level of aggregation obtained at 5 minutes with vehicle-treated control (0µM) was taken as 100%. The *p*-values shown in figure are as calculated by students T-test (\**p*<0.05, \*\**p*<0.01 and \*\*\**p*<0.001).

**Figure 2: Tangeretin inhibits inside-out signalling, granule secretion and calcium mobilisation.** The effect of tangeretin on CRP-XL (0.5µg/ml)-induced fibrinogen binding in washed platelets (A) or PRP (B) was measured by flow cytometry. Similarly, the effect of tangeretin on P-selectin exposure upon stimulation with CRP-XL (0.5µg/ml) was measured in washed platelets (C) and PRP (D). The median fluorescent intensity values were converted into percentages for comparison. The level of fibrinogen binding or P-selectin exposure obtained with vehicle was taken as 100%. Data represent mean ± S.D (n=4). Fluo4-NW dye labelled human PRP was stimulated with 1µg/ml CRP-XL in the presence of 50 or 100µM tangeretin or vehicle control and the intracellular calcium levels were measured by flow cytometry (E). Traces shown are representative of four separate experiments. The inhibition of intracellular calcium levels was calculated by comparing the levels obtained at 90 seconds (F) in the presence or absence (taken as 100%) of tangeretin. 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).

**Figure 3: Tangeretin inhibits thrombus formation *in vitro*, outside-in signalling and extends tail bleeding in mice.** DiOC<sub>6</sub> labelled human blood was pre-incubated with vehicle (A) or tangeretin [10 (B), 20 (C) and 100µM (D)] and perfused over a collagen-coated Vena8 BioChips. Images were obtained every 30 seconds for up to 10 minutes and representative images (at 10 minutes) are shown in figure. The fluorescence intensity of thrombi obtained in absence of tangeretin was taken as 100% and compared to the treated thrombi levels (E). Data represent mean ± S.D (n=3). The *p*-values shown in figure are as calculated by students T-test (\**p*<0.05 and \*\**p*<0.01). Effect of tangeretin (12.5, 25, 50 and 100µM) on clot retraction was analysed *in vitro* (F and G). Figure F shows a representative image of clot retraction at 90 minutes in the presence and absence of different concentrations of tangeretin. Data represent mean ± S.D (n=4) of clot weights measured at 2 hours. The *p*-values calculated by students T-test (\**p*<0.05 and \*\**p*<0.01). The effect of tangeretin (estimated 10 and 50µM) on haemostasis of mice was analysed by measuring the bleeding time after tail tip excision. The bleeding time obtained with vehicle treated group was compared with tangeretin treated mice (H). Data represent mean ± S.D (n=5 mice for vehicle control, n=4 for 10µM and n=8 mice for 50µM tangeretin treated). The significance between control and treated groups, and P values (as shown) were calculated by nonparametric Kruskal-Wallis global test using GraphPad Prism.

**Figure 4: Tangeretin inhibits phosphorylation of PLCγ2 (pY759) and AKT (pS473).** Washed human platelets stimulated with CRP-XL (1µg/ml) in the presence of tangeretin (10, 15, 20, 50, 100, 150 and 200µM) or vehicle control were analysed by immunoblotting using anti-phosphotyrosine antibody (A) and phospho-specific antibodies for proteins involved in GPVI pathway such as Syk pY323 (B), LAT pY200 (B), PLCγ2 pY759 (C) and AKT pS473 (D). Total level of 14-3-3ζ was measured on each sample as a loading control. The blots shown in figure are representative of four separate experiments. The level of phosphorylation was quantified using Qunatity One software (GE Healthcare, UK) and converted into percentages for comparison. The level of phosphorylation obtained with vehicle control was taken 100%. R- represents the level of phosphorylation in resting

platelets. The *p*-values shown in figure are as calculated by students T-test (\**p*<0.05, \*\**p*<0.01 and \*\*\**p*<0.001).

**Figure 5: Tangeretin elevates cGMP levels and does not inhibit phosphodiesterase activity.** The levels of cGMP (A) and cAMP (B) were measured in platelets upon stimulation with CRP-XL (0.5µg/ml) using ELISA kits in 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 concentration of tangeretin on phosphodiesterase activity were measured using assay kits upon the hydrolysis of cGMP (D) and cAMP (E). I- represent the phosphodiesterase inhibitor, IBMX at 10 and 40µM concentration. In figure D and E, the level of phosphodiesterase activity obtained in absence of inhibitor or tangeretin was taken as 100%. Human platelet aggregation was measured in the presence or absence of 20µM ODQ, a soluble guanylate cyclase inhibitor (F and G) or 30µM PKG inhibitor (Rp-8-Br-PET-cGMPS) (H and I), following stimulation with CRP-XL (0.5µg/ml) for 5 minutes. Similarly, following incubation with 20µM ODQ (F and G) or 30µM PKG inhibitor (H and I), platelets were treated with 20µM tangeretin or vehicle control prior to stimulation with CRP-XL (0.5µg/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 (0.5µg/ml) (K) stimulated platelets after the treatment with 20µM ODQ prior to the addition of tangeretin. The level of cGMP released was calculated using standard curves. Cumulative data represent mean values ± S.D (*n*=3). *p*-values shown are calculated using student T-test (\*\**p*<0.01, \*\**p*<0.01 and \*\*\**p*<0.001). C-CRP-XL (0.5µg/ml), T-tangeretin (20µM), O-ODQ (20µM) and P-30µM PKG inhibitor.

**Figure 6: Tangeretin increases VASP phosphorylation.** The effect of various concentrations of tangeretin on phosphorylation of VASP [at positions pS239 (A and B) and pS157 (A and C)], a substrate for cyclic nucleotide dependent protein kinases was analysed (A) in washed platelets upon stimulation with CRP-XL (1µg/ml) by immunoblotting. The level of phosphorylation obtained in absence of tangeretin (vehicle control) was taken as 100% (B and C). VASP phosphorylation at S239 was also analysed in presence of 30µM PKG, 5µM PKA (H89) and 5µM PKC (GF109203X) inhibitors (D) prior to the incubation with different concentrations of tangeretin. R-represents the phosphorylation levels in resting platelets. The blots shown in figure are representative of four separate experiments. The *p*-values shown in figure were calculated by comparing the phosphorylation levels in resting platelets with tangeretin treated samples using students T-test (\**p*<0.05, \*\**p*<0.01 and \*\*\**p*<0.001).



Figure 1

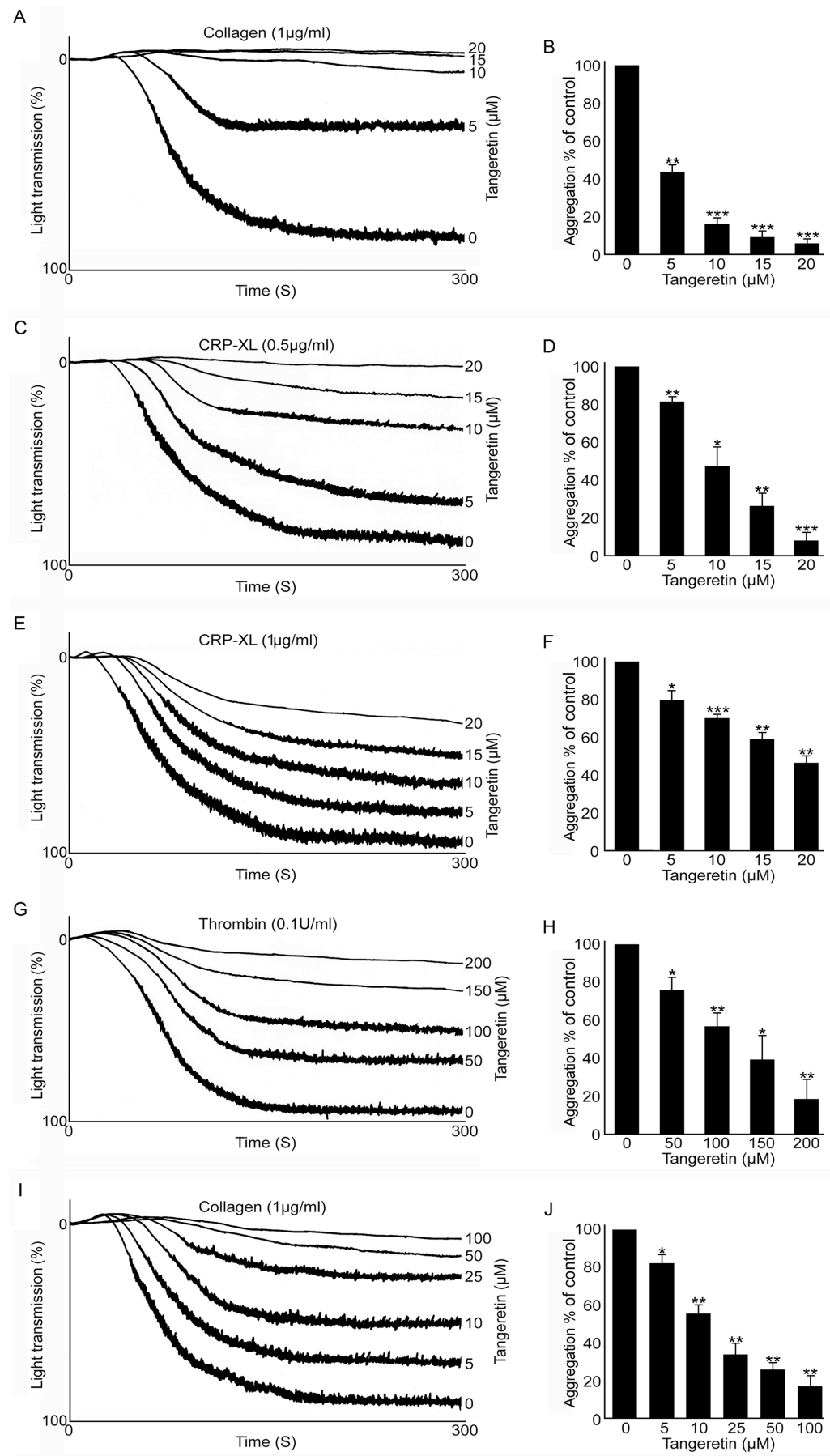


Figure 2

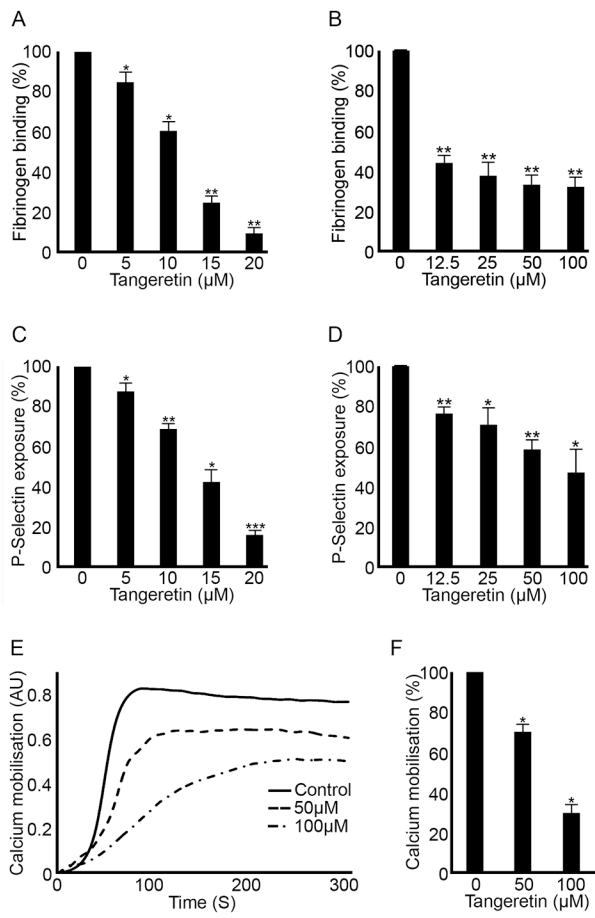


Figure 3

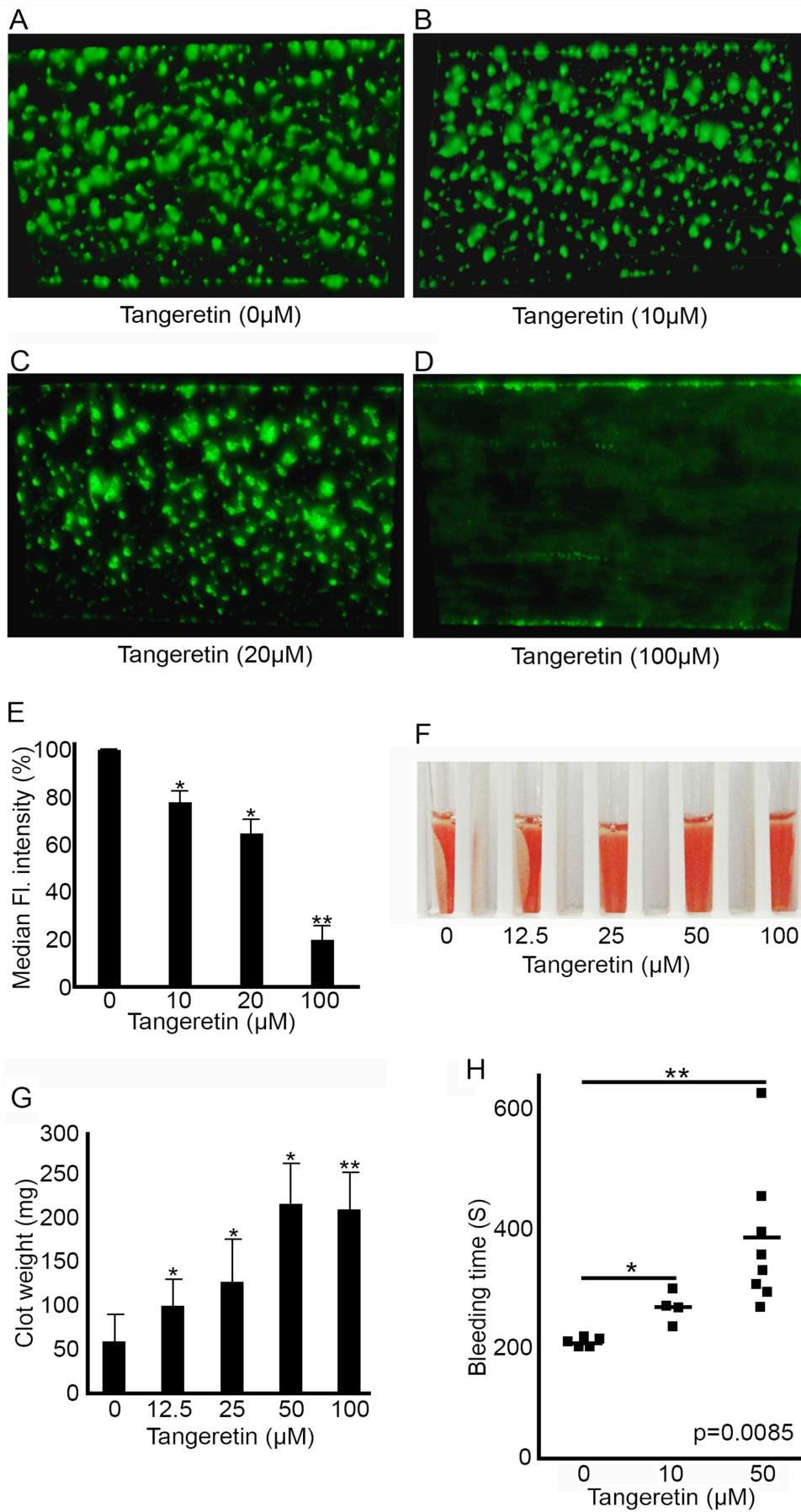


Figure 4

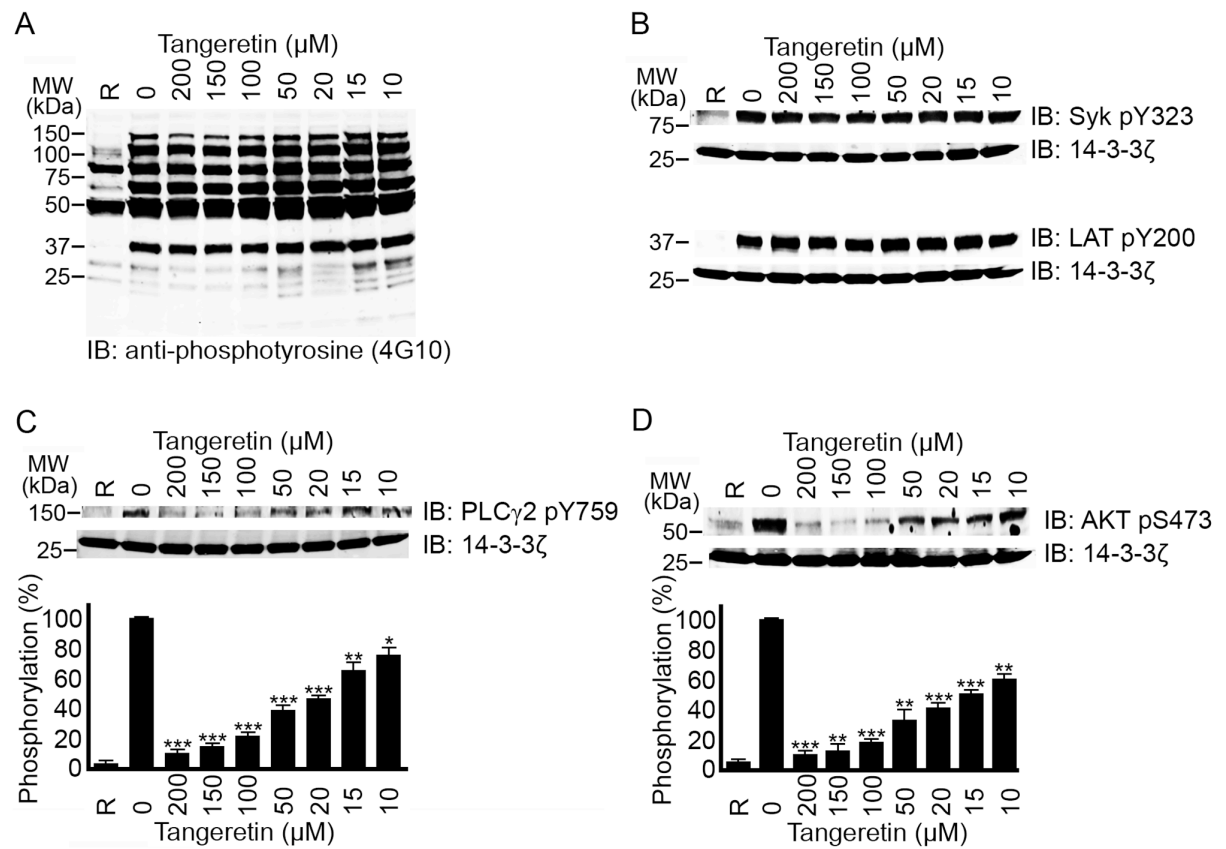


Figure 5

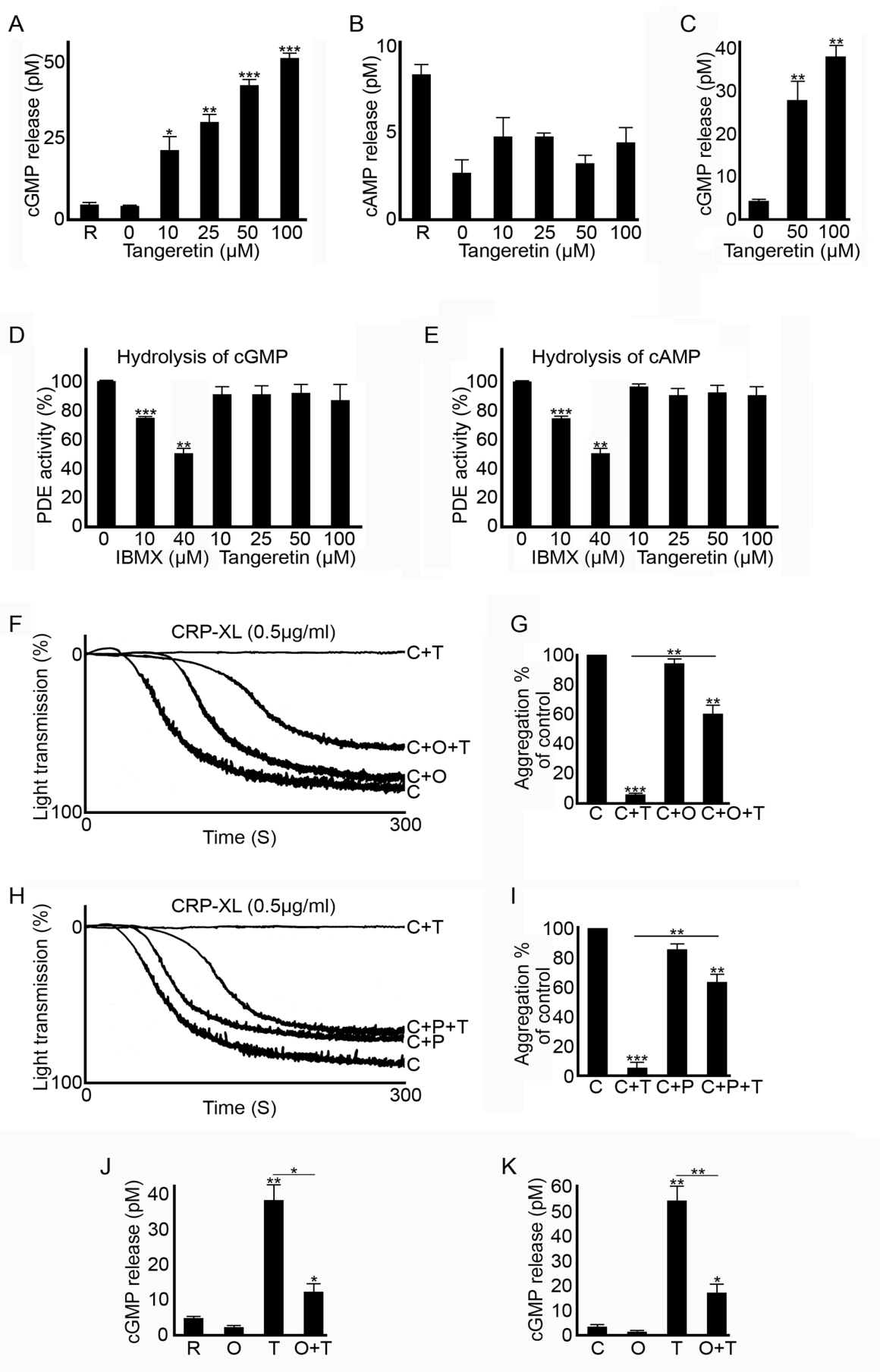


Figure 6

