The naphthoquinone plumbagin suppresses ADP-induced rat platelet aggregation through $P2Y_1$-PLC signaling pathway

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Abstract: Plumbagin (PLB) isolated from *Plumbago zeylanica L* (Plumbaginaceae) was evaluated for the suppressive effect and mechanism on ADP induced rat platelet aggregation. Adult male SD rats were randomly divided into control group, clopidogrel group, PLB 25mg/kg group and PLB 50mg/kg group. Clopidogrel (13.5mg/kg per day) and PLB (25 and 50mg/kg per day) were orally given to experimental rats by gavage for seven consecutive days. The antiplatelet properties were assessed by measuring the ADP-induced platelet aggregation rate (Agg_{max}). The level of cAMP in platelets before aggregation was determined by ELISA. The protein expression of pAkt, Akt, pPLC β3 and PLC β3 in platelets was measured by western blot. Our data indicated that PLB (25 and 50mg/kg) significantly inhibited ADP-induced rat platelet aggregation as well as clopidogrel (13.5mg/kg) in a dose dependent manner compared with the control group. PLB (25 and 50mg/kg) remarkably reduced the ADP-induced PLC β3 phosphorylation but not Akt in platelets as compared with the control group. The present study suggests that PLB exerts a suppressive effect on ADP-induced rat platelet aggregation, at least in part, through $P2Y_1$-PLC signaling pathway.

Keywords: Plumbagin; ADP; platelet aggregation; Akt; PLC β3.

INTRODUCTION

Platelets are small blood anucleate cell fragments released by megakaryocytes responsible for maintaining hemostasis and play an important role in thrombosis, wound healing, atherosclerosis, inflammation, immunity, and tumor metastasis (Boilard *et al*., 2010; Leslie 2010). The primary physiological function of platelets is to form hemostatic thrombi during hemorrhaging (Wu *et al*., 2016). When vessel injury occurs, platelets in circulation adhere to the exposed collagen and become activated. A series of physiological agonists such as coagulation factors (thrombin), hormones (epinephrine), low-molecular-weight substances (adenosine diphosphate, ADP), lipid derivatives (platelet aggregating factor), thromboxane A_2 (TXA_2), and collagen activate platelets (Dumas *et al*., 2012). ADP is the most established platelet agonist that causes platelet shape change, aggregation and TXA generation (Kunapuli *et al*., 2003). ADP activates platelets through two G protein-coupled ADP receptors, G_q-linked $P2Y_1$ receptors and G_i-linked $P2Y_{12}$ receptors. G-protein-coupled receptors (GPCRs), a family of seven-transmembrane receptors, can transmit signals through heterotrimeric G proteins. The heterotrimeric G proteins are composed of an α-subunit interacting with a βγ complex. Based on the similarity of α subunits, G proteins can be classified into four families: $G_{i/o}$, G_s, $G_{q/11}$, and G_{12}, each of which is coupled with selective receptors and downstream effectors (Bologna *et al*., 2017). The $P2Y_1$ receptor is widely expressed throughout the body and couples with G_q, which leads to the activation of phospholipase C (PLC) β, increases cytosolic calcium levels, and activates protein kinase C. The $P2Y_{12}$ receptor couples with G_i to inhibit adenylyl cyclase (AC) and activates phosphoinositide 3-kinase (PI3K) (Li *et al*., 2010). Coactivation of the $P2Y_1$ and $P2Y_{12}$ receptors is essential for ADP-mediated platelet aggregation. Inhibiting one of the receptor signal pathways may reduce the activation and aggregation of the platelets (Kunapuli *et al*., 2003).

Thrombosis is the formation of a blood clot within a blood vessel or heart (Mackman 2012). Thrombotic diseases, such as heart attack and ischemic stroke, are a leading cause of mortality in the modern world. Platelets have a central role in cardio-vascular thrombosis (Furie and Furie 2008). In the process of thrombus formation, platelets adhere to the vascular wall, become activated, release agonists and finally aggregate at the injured vascular site (Kong *et al.*, 2009; Peng *et al*., 2011). Therefore, antiplatelet agents, including aspirin, ticlopidine and clopidogrel are used for the treatment and prevention of cardiovascular thrombotic diseases (Michelson 2010). However, these agents may produce hemorrhagic events or upper gastrointestinal bleeding, which limits their clinical application (Johnson 2008; Pan *et al*., 2012). Novel antiplatelet agents can be derived from various sources, including dietary and medicinal plants, which can contain natural compounds that exhibit antiplatelet/thrombotic properties or control cardiovascular diseases (Huang *et al*., 2010).

Plumbagin (PLB) is a natural naphthoquinone constituent found in many plants (fig. 1), especially *Plumbago*

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zeylanica L (Plumbaginaceae) which is widely used in traditional Chinese medicine as an antifungal, antibacterial and anti-inflammatory agent (Munday and Munday 2000; Xue *et al*., 2010). PLB has potent pharmacologic activities including antimicrobial, proapoptotic, antiangiogenic, and anti-fibrotic effects (Xu *et al*., 2013; Sinha *et al*., 2013; Wei *et al*., 2015). However, the possible effect of PLB on blood circulation system remains unclear. The present study was attempted to investigate the effect of PLB on ADP induced rat platelet aggregation and the possible mechanisms involved.

MATERIALS AND METHODS

Materials

Plumbagin (purity≥95%, dissolved in 1% Tween-20 saline) was purchased from Sigma (St. Louis, MO). ADP (purity≥95%) was purchased from Sigma (St. Louis, MO). cAMP ELISA kit was purchased from ENZO life sciences (NY, USA). Anti-pAkt and anti-Akt antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-pPLC β3, anti-PLC β3 and anti-β-tubulin antibodies were purchased from Santa Cruz Biotechnology Inc (California, USA). All other chemicals used in this study were purchased from Sigma (St. Louis, MO).

Animals

This study was carried out in strict accordance with the guideline of the Council on Animal Care of Academia Sinica. The protocol was approved by the Ethical Committee on Animal Experimentation of General Hospital of the Yangtze River Shipping, Wuhan, China. Thirty-two male SD rats weighing 250-280g were obtained from the Center of Experimental Animal of Hubei Province (Wuhan, China). All animals were kept under the same laboratory conditions of temperature (25± 2°C) and lighting (12:12h light: dark cycle), and were given free access to standard laboratory chow and tap water. All rats were allowed to acclimatize for one week before experiment.

Experimental design

The animals were randomly divided into four experimental groups (n=8): (1) control group, rats were given saline by gavage once per day for consecutive seven days; (2) clopidogrel group, rats were given clopidogrel dissolved in 1% Tween-20 saline (13.5mg/kg B.W.) by gavage once a day for consecutive seven days; (3) PLB 25mg/kg group, rats were given PLB dissolved in 1% Tween-20 saline (25mg/kg B.W.) by gavage once a day for consecutive seven days; (4) PLB 50mg/kg group, rats were given PLB dissolved in 1% Tween-20 saline (50 mg/kg B.W.) by gavage once a day for consecutive seven days.

Preparation of rats' platelets

Rats were put on a fast for 12 h after the last dose of agents before they were anesthetized with pentobarbital sodium (30mg/kg B.W., intraperitoneally). Blood was drawn from the abdominal aorta and collected in a vacutainer containing sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation at 180 ×g for 20 minutes. An equal volume of buffer (140mM NaCl; 2.7 mM KCl; 2.1mM $MgCl_2$.$6H_2O$; 0.42mM NaH_2PO_4.$2H_2O$; 1.8mM $CaCl_2$; 5.6mM glucose; 255.9 mM $NaHCO_3$ and 2000 U/L Heparin, pH 7.4) was added into PRP followed by a centrifugation at 360 ×g for 10 minutes. Then, the pellet was collected and used for assay.

Antiplatelet aggregation studies

Agonist-induced platelet aggregation was measured using a platelet aggregometer (QX200, Shanghai Yida Instrument Co Ltd, Shanghai, China) according to the instruction of manufacturer. Briefly, after calibration of the platelet aggregometer, anticoagulant whole blood (0.5 mL) was incubated with buffer (0.5mL) for 5 min at 37 °C without stirring before the addition of ADP. Then, the ADP (25μL, 4mM) was added with stirring to record the platelet aggregation strength (resistance value, Ω) in 5 min, and the data were expressed as aggregation inhibition ratio.

Measurement of cAMP levels in platelets before aggregation

After washing twice with PBS, isolated platelets from experimental rats were mixed with 0.1N HCl, scraped, and collected by centrifugation. Levels of cAMP in the supernatants were determined using a cAMP ELISA kit according to the manufacturer's instructions (ENZO life sciences, NY, USA).

Western blot analysis

After putting on the ice to terminate the aggregation reaction, the platelet lysate was prepared to evaluate the expression levels of pAkt, Akt, pPLC β3 and PLC β3. The protein concentration was determined using the bicinchoninic acid (BCA) assay. Equal amounts of protein were separated with 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. After electrophoresis, the gels were transferred onto poly vinylidene difluoride (PVDF) membranes, which were blocked with Tris-buffered saline containing 5% non-fat milk at 4°C. Afterwards, the membranes were incubated overnight at 4°C in a solution containing 0.1% Tween 20, 5% non-fat milk and the following primary antibodies: pAkt (1:1000); Akt (1:1000), pPLC β3 (1:400); PLC β3 (1:500); beta-tubulin (1:10000). After incubation with the corresponding secondary antibodies for 2 h at room temperature, densitometric band scanning was performed using image processing and analysis with Quantity One v4.62 (Bio-Rad Laboratories, Hercules, CA). Tubulin was used as an internal index.

STATISTICAL ANALYSIS

Data were expressed as mean ± S.D. The significant differences between groups were assessed with SPSS version 13.0. The differences between group means were calculated by one-way ANOVA with LSD post hoc analysis. Difference was considered statistically significant when $p<0.05$ and extremely significant when $p<0.01$.

RESULTS

Effect of PLB on ADP-induced platelet aggregation

To realize the effect of PLB on platelet aggregation, we analyzed PLB-treated rats to evaluate possible effects on platelet aggregation. The ADP-induced platelet aggregation was significantly suppressed by clopidogrel ($p < 0.01$). Treatment of rats with different doses of PLB resulted in a concentration-dependent inhibition of ADP-induced platelet aggregation ($p<0.05$ and $p<0.01$). These results showed that aggregation strength was decreased in blood of rats treated with PLB (table 1).

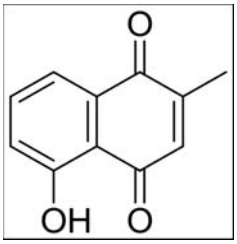

Fig. 1: Chemical structure of plumbagin.

Effect of PLB on cAMP content in platelets before aggregation

The best-known inhibitor and turn off signaling in platelet activation is cAMP To explore the possible effects of clopidogrel or PLB treatment on intraplatelet cAMP levels, we determined the cAMP content in platelets with ELISA kit. Our data revealed that clopidogrel (13.5mg/kg) or PLB (25 and 50mg/kg) treatment did not significantly influence the cAMP content in platelets before aggregation in comparison to the control group (fig. 2).

Effect of PLB on pAkt and Akt expression in platelets

To investigate the possible intracellular signaling target of PLB, we evaluated the important platelet signaling pathway, the PI3K pathway (Akt Ser473 phosphorylation as the activation marker). The pAkt and Akt levels in platelets after ADP-induced aggregation were examined using immunoblotting method. The results showed that PLB did not significantly influence ADP-induced Akt phosphorylation in platelets, which indicated that PLB did not affect PI3K signaling in platelets. On the contrary, clopidogrel treatment significantly ($p<0.01$) reduced ADP-induced Akt phosphorylation (fig. 3).

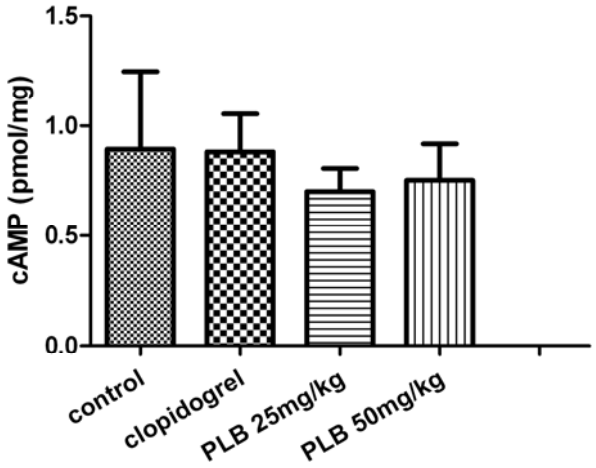

Fig. 2: Effects of PLB on cAMP content in platelets before aggregation. The blood was collected 30 min after last dose of PLB or clopidogrel. The normal control rats received saline. After washing twice with PBS, isolated platelets from experimental rats were with 0.1N HCl, scraped, and collected by centrifugation. Data are represented as means ± S.D. for six animals per group. $^{*}p<0.05$ versus control, $^{**}p<0.01$ versus control by one- way ANOVA and LSD post hoc test.

Effect of PLB on pPLC β3 and PLC β3 expression in platelets

We further investigated the expression of PLC β3, an upstream component of ADP-induced platelet aggregation. To determine whether PLC β3 is a target of PLB for repression of platelet aggregation, the platelets were treated with ADP to trigger aggregation, then the pPLC β3 and PLC β3 levels were examined using immunoblotting method. The results showed that PLB significantly increased ADP-induced PLC β3 (Ser1105) phosphorylation, thus increased the pPLC β3/PLC β3 ratio. Clopidogrel did not significantly change pPLC β3 and PLC β3 levels (fig. 4). It is known that phosphorylation of PLC β3 leads to the inhibition of G-protein-activated PLC β3 activity. The results indicated that PLB might inhibit the activity of PLC β3 induced by ADP.

DISCUSSION

In the present study, we demonstrated that PLB, a main bioactive component in *Plumbago zeylanica L* (Plumbaginaceae), significantly inhibits ADP-induced rat platelet aggregation. Platelets play a critical role in the process of hemostasis and thrombosis through adhesion, activation, and aggregation (McNicol and Israels 2008). Under some pathological conditions, above functions of platelets may mediate atherosclerotic arteries, thrombus formation, and cause vascular occlusions and result in stroke or myocardial infarction (Davi and Patrono 2007;

Table 1: Effect of PLB on platelet aggregation in rats ($\bar{X}$ ±S.D., n=6)

Group	Dose / mg·kg^{-1}	Platelet aggregation strength)/ Ω	Aggregation Inhibition ratio)/ %
control	-	5.24 ± 0.83	-
clopidogrel	13.5	3.03 ± 0.40**	42.18
PLB	25	4.13± 1.19*	21.18
PLB	50	3.07 ± 0.47$^{**\#}$	41.41

$^{*}p$<0.05 versus control, $^{**}p$<0.01 versus control; $^{\#}p$<0.05 versus PLB 25mg/kg.

Stegner and Nieswandt 2011). Aspirin and clopidogrel are used frequently to prevent platelet aggregation and thrombus formation in stroke patients. However, both drugs show significant side effects, such as gastrointestinal irritation and hemorrhages. It is necessary to develop new antiplatelet agents with low risk of bleeding (Gachet 2015). Some natural compounds from traditional Chinese medicine showed potent antiplatelet effects, representing a strategy to develop novel antiplatelet medicines (Tang *et al*., 2014; Su *et al*., 2016).

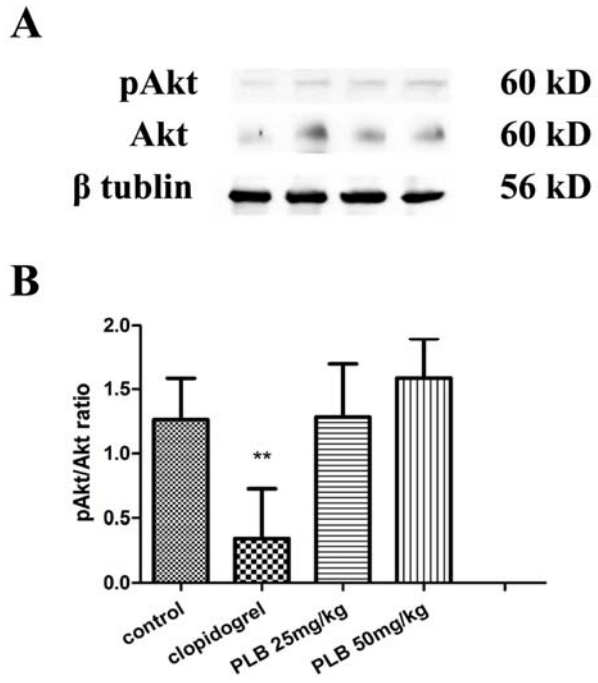

Fig. 3: Effects of PLB on pAkt (ser473) and Akt protein expressions in rat platelets after treatment of ADP. A: Representative Western blotting images of pAkt and Akt in platelets. B: The ratios of pAkt/Akt were showed in PLB treated rats and clopidogrel-treated rats. Data are represented as means ± S.D. for 3-4 animals per group. * p < 0.05 versus control, ** p < 0.01 versus control by one-way ANOVA and LSD post hoc test.

ADP mediates platelet aggregation by stimulating two G-protein-coupled receptors on platelets, P2Y$_1$ and P2Y$_{12}$ (Offermanns 2006). The P2Y$_1$ receptor couples to G$_q$ protein. When ADP stimulates P2Y$_1$ receptor, the G protein activates PLC β, and PLC catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to generate diacylglycerol (DAG, may activate protein kinase C) and inositol 1,4,5-triphosphate (IP3), both leading to the mobilization of intracellular Ca^{2+} (Purvis *et al*., 2008). The P2Y$_{12}$ receptor couples to G$_i$ protein. The P2Y$_{12}$ receptor activation results in G$_i$-mediated inhibition of stimulated AC and Gβγ-mediated activation of PI3K, Akt and Rap1b (Woulfe *et al*., 2002; Kim *et al*., 2004). It subsequently leads to the increase of Ca^{2+} level in platelets. Clopidogrel is a commonly used P2Y$_{12}$ receptor antagonist and potently inhibits platelet aggregation (Secco *et al*., 2013). Therefore, we chose clopidogrel as a positive control in the *in vivo* experiments. So far, no P2Y$_1$ receptor antagonist reported as antiplatelet agent has been used in clinic.

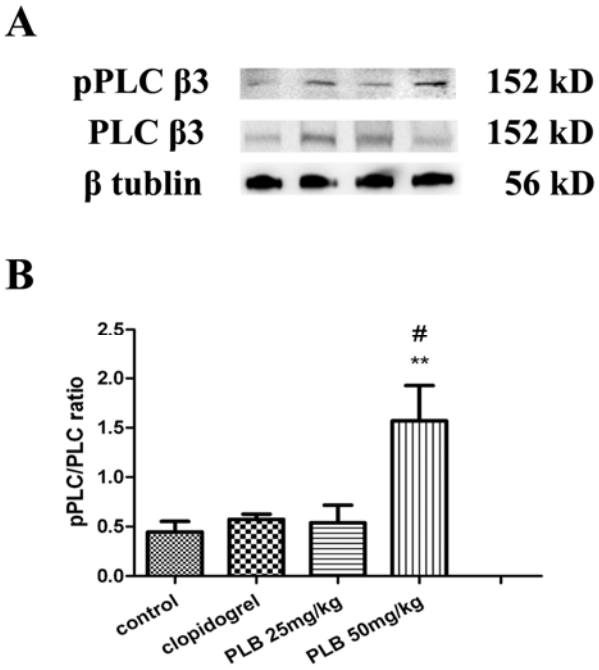

Fig. 4: Effects of PLB on pPLC (ser1105) and PLC protein expressions in rat platelets after treatment of ADP. A: Representative Western blotting images of pPLC and PLC in platelets. B: The ratios of pPLC/PLC were showed in PLB treated rats and clopidogrel-treated rats. Data are represented as means ± S.D. for 3-4 animals per group. * p<0.05 versus control, $^{**}p$<0.01 versus control; $^{\#}$ p<0.05 versus PLB 25mg/kg by one- way ANOVA and LSD post hoc test.

PI3K pathways downstream of G_i are important for ADP-induced platelet aggregation (Kauffenstein *et al*., 2001). PI3K/Akt signaling has been proved to be a valuable antithrombotic therapy target without a significant effect on primary hemostasis (Jackson *et al*., 2004). PI3Ks are activated upon $P2Y_{12}$ receptor stimulation and generate pro-aggregatory signals (Garcia *et al*., 2010). A recent study has indicated that ADP-induced Akt phosphorylation is regulated by both PI3Kβ and PI3Kγ (Canobbio *et al*., 2009). In this study, we investigated the effect of PLB on pAkt and Akt expression in platelets to clarify the mechanism of PLB treatment. Our data showed that PLB failed to significantly affect the pAkt expression induced by ADP. Moreover, it did not affect the content of cAMP in platelets which responses to the activity of $P2Y_{12}$-G_i-AC. All these results suggest that PLB might not repress ADP-induced platelet aggregation via $P2Y_{12}$-coupled G_i signaling.

Furthermore, the expression of PLC β was determined to elucidate the potential effects of PLB through $P2Y_1$ receptor-G_q-PLC pathway. It is well known that phosphorylation of PLC β3 leads to the inhibition of G-protein-activated PLC β activity (Yang *et al*., 2015). In this study, our data indicated that PLB could notably abrogate the effect of ADP by increasing PLC β3 phosphorylation. And clopidogrel showed no significant effect on it.

CONCLUSION

Taken together, our study showed that PLB might repress ADP-induced rat platelet aggregation, at least in part, by inhibiting $P2Y_1$ receptor-G_q-PLC signaling pathway, but not the $P2Y_{12}$ receptor-G_i-AC pathway. Our results imply that blocking $P2Y_1$ receptor or inhibiting its signaling pathway may be used as a new antiplatelet therapy. However, there are still some matters remain to be elucidated. One of question is that chronic PLB treatment would change the expression level of $P2Y_1$ or $P2Y_{12}$ receptors? Additionally, would PLB treatment lower intracellular Ca^{2+} content by reducing IP3 production or by attenuating Ca^{2+} release from intracellular storage? Further studies on those topics are still ongoing.

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REFERENCES

Boilard E, Nigrovic PA, Larabee K, Watts GF, Coblyn JS, Weinblatt ME, Massarotti EM, Remold-O'Donnell E, Farndale RW, Ware J and Lee DM (2010). Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. *Science*, **327**: 580-583.

Bologna Z, Teoh JP, Bayoumi AS, Tang Y and Kim IM (2017). Biased G Protein-Coupled Receptor Signaling: New Player in Modulating Physiology and Pathology. *Biomol. Ther. (Seoul)*, **25**: 12-25.

Canobbio I, Stefanini L, Cipolla L, Ciraolo E, Gruppi C, Balduini C, Hirsch E and Torti M (2009). Genetic evidence for a predominant role of PI3Kbeta catalytic activity in ITAM- and integrin-mediated signaling in platelets. *Blood*, **114**: 2193-2196.

Davi G and Patrono C (2007). Platelet activation and atherothrombosis. *N. Engl. J. Med.*, **357**: 2482-294.

Dumas M, Nadal-Wollbold F, Gaussem P, Perez M, Mirault T, Létienne R, Bourbon T, Grelac F, Le Grand B and Bachelot-Loza C (2012). Antiplatelet and antithrombotic effect of F 16618, a new thrombin proteinase-activated receptor-1 (PAR1) antagonist. *Br. J. Pharmacol*., **165**: 1827-1835.

Furie B and Furie BC (2008). Mechanisms of thrombus formation. *N. Eng. J. Med.*, **359**: 938-949.

Gachet C (2015). Antiplatelet drugs: which targets for which treatments? *J. Thromb. Haemost.*, **Suppl 1**: S313-S322.

Garcia A, Kim S, Bhavaraju K, Schoenwaelder SM and Kunapuli SP (2010). Role of phosphoinositide 3-kinase beta in platelet aggregation and thromboxane A2 generation mediated by G_i signalling pathways. *Biochem. J.*, **429**: 369-377.

Huang ZS, Zeng CL, Zhu LJ, Jiang L, Li N and Hu H (2010). Salvianolic acid A inhibits platelet activation and arterial thrombosis via inhibition of phosphoinositide 3-kinase. *J. Thromb. Haemost.*, **8**: 1383-1393.

Jackson S, Yap C and Anderson K (2004). Phosphoinositide 3-kinases and the regulation of platelet function. *Biochem. Soc. Trans.*, **32**: 387-392.

Johnson S (2008). Known knowns and known unknowns: Risks associated with combination antithrombotic therapy. *Thromb. Res.*, **Suppl 1**: S7-S11.

Kauffenstein G, Bergmeier W, Eckly A, Ohlmann P, Léon C, Cazenave JP, Nieswandt B and Gachet C (2001). The P2Y(12) receptor induces platelet aggregation through weak activation of the alpha(IIb)beta(3) integrin-a phosphoinositide 3-kinase-dependent mechanism. *FEBS Lett.*, **505**: 281-290.

Kim S, Jin J and Kunapuli SP (2004). Akt activation in platelets depends on Gi signaling pathways. *J. Biol. Chem*, **279**: 4186-4195.

Kong Y, Huo JL, Xu W, Xiong J, Li YM and Wu WT (2009). A novel anti-platelet aggregation tripeptide from Agkistrodon acutus venom: isolation and characterization. *Toxicon*, **54**: 103-109.

Kunapuli SP, Ding Z, Dorsam RT, Kim S, Murugappan S and Quinton TM (2003). ADP receptors--targets for developing antithrombotic agents. *Curr. Pharm. Des.*, **9**: 2303-2316.

Kunapuli SP, Dorsam RT, Kim S and Quinton TM (2003). Platelet purinergic receptors. *Curr. Opin. Pharmacol.*, **3**: 175-180.

Leslie M (2010). Cell biology. Beyond clotting: the powers of platelets. *Science*, **328**: 562-564.

Li Z, Delaney MK, O'Brien KA and Du X (2010). Signaling during platelet adhesion and activation. *Arterioscler. Thromb. Vasc. Biol.*, **30**: 2341-2349.

Mackman N (2012). New insights into the mechanisms of venous thrombosis. *J. Clin. Invest*, **122**: 2331-2336.

McNicol A and Israels SJ (2008). Beyond hemostasis: The role of platelets in inflammation, malignancy and infection. *Cardiovasc. Hematol. Disord. Drug Targets*, **8**: 99-117.

Michelson AD (2010). Antiplatelet therapies for the treatment of cardio-vascular disease. *Nat. Rev. Drug Discov.*, **9**: 154-169.

Munday R and Munday CM (2000). Induction of quinone reductase and glutathione transferase in rat tissues by juglone and plumbagin. *Planta. Med.*, **66**: 399-402.

Offermanns S (2006). Activation of platelet function through G protein-coupled receptors. *Circ. Res.*, **99**: 1293-1304.

Pan C, Wei X, Ye J, Liu G, Zhang S, Zhang Y, Du H and Ding Z (2012). BF066, a novel dual target antiplatelet agent without significant bleeding. *PLoS One*, **7**: e40451.

Peng L, Xu X, Shen D, Zhang Y, Song J, Yan X and Guo M (2011). Purification and partial characterization of a novel phosphodiesterase from the venom of Trimeresurus stejnegeri: Inhibition of platelet aggregation. *Biochimie.*, **93**: 1601-1609.

Purvis JE, Chatterjee MS, Brass LF and Diamond SL (2008). A molecular signaling model of platelet phosphoinositide and calcium regulation during homeostasis and P2Y1 activation. *Blood*, **112**: 4069-4079.

Sinha S, Pal K, Elkhanany A, Dutta S, Cao Y, Mondal G, Iyer S, Somasundaram V, Couch FJ, Shridhar V, Bhattacharya R, Mukhopadhyay D and Srinivas P (2013). Plumbagin inhibits tumorigenesis and angiogenesis of ovarian cancer cells *in vivo*. *Int. J. Cancer*, **132**: 1201-1212.

Secco GG, Parisi R, Mirabella F, Fattori R, Genoni G, Agostoni P, Luca GD,Marino PN, Lupi A and Rognoni A (2013). P2y12 inhibitors: pharmacologic mechanism and clinical relevance. *Cardiovasc. Hematol. Agents Med. Chem.*, **11**: 101-105.

Stegner D and Nieswandt B (2011). Platelet receptor signaling in thrombus formation. *J. Mol. Med. (Berl)*, **89**: 109-121.

Su XL, Su W, Wang Y, Wang YH, Ming X and Kong Y (2016). The pyrrolidinoindoline alkaloid Psm2 inhibits platelet aggregation and thrombus formation by affecting PI3K/Akt signaling. *Acta. Pharmacol. Sin*, **37**: 1208-1217.

Tang XL, Liu JX, Dong W, Li P, Li L, Lin CR, Zheng YQ, Cong WH and Hou JC (2014). Cardioprotective effect of protocatechuic acid on myocardial ischemia/reperfusion injury. *J. Pharmacol. Sci.*, **125**: 176-183.

Wei Y, Huang M, Liu X, Yuan Z, Peng Y, Huang Z, Duan X and Zhao T (2015). Anti-fibrotic effect of plumbagin on CCl_4-lesioned rats. *Cell Physiol. Biochem.*, **35**: 1599-608.

Woulfe D, Jiang H, Mortensen R, Yang J and Brass LF (2002). Activation of Rap1b by Gi family members in platelets. *J. Biol. Chem.*, **277**: 23382-23390.

Wu CC, Tsai FM, Chen ML, Wu S, Lee MC, Tsai TC, Wang LK and Wang CH (2016). Antipsychotic Drugs Inhibit Platelet Aggregation via $P2Y_1$ and $P2Y_{12}$ Receptors. *Biomed. Res. Int.*, 2016: 2532371.

Xu TP, Shen H, Liu LX and Shu YQ (2013). Plumbagin from Plumbago Zeylanica L induces apoptosis in human non-small cell lung cancer cell lines through NF- κB inactivation. *Asian Pac. J. Cancer Prev.*, **14**: 2325-2331.

Xue YL, Meng XQ, Ma LJ and Yuan Z (2016). Plumbagin exhibits an anti-proliferative effect in human osteosarcoma cells by down regulating FHL2 and interfering with Wnt/β-catenin signaling. *Oncol. Lett.*, **12**: 1095-1100.

Yang H, Xu S, Li J, Wang L and Wang X (2015). Potassium 2-(1-hydroxypentyl)-benzoate inhibits ADP-induced rat platelet aggregation through P2Y1-PLC signaling pathways. *Naunyn Schmiedebergs Arch Pharmacol.*, **388**: 983-990.