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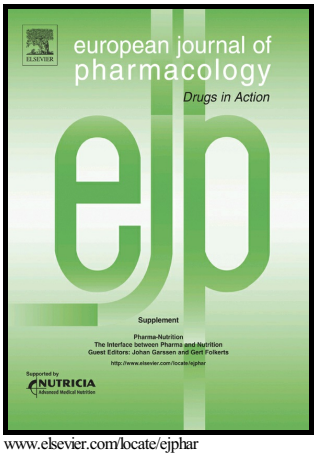

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The novel compound MP407 inhibits platelet aggregation through cyclic AMP-dependent processes

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Abstract:

Platelet hyperactivity plays a critical role for initiating several vascular diseases such as atherothrombosis. Therefore, development of effective antiplatelet agents is necessary for ameliorating platelet-related diseases. In this study, we investigated the effects of the new synthesized compound, MP407 on platelet aggregation and further elucidated the underlying mechanisms. Our results demonstrated that MP407 dose-dependently inhibited collagen-induced platelet aggregation, thromboxane B_2 (TXB_2) production, intracellular Ca^{2+} mobilization, platelet membrane GPIIb/IIIa expression, and the phosphorylation of Akt,

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GSK3β, p38MAPK, and phospho (Ser) PKC substrate (p47). Moreover, MP407 is able to increase the cyclic AMP formation both in resting and activated platelets. However, blocking cyclic AMP formation with 2'5'-ddAdo, an inhibitor of adenylate cyclase, greatly reversed the antiplatelet activity of MP407 and related platelet-activating pathways. MP407 also enhanced VASP phosphorylation at Ser157 in collagen-stimulated platelets, which was attenuated by addition of 2'5'-ddAdo. Therefore, the antiplatelet activity of MP407 may be modulated by cyclic AMP-dependent regulation of Akt, GSK3β, p38MAPK and VASP phosphorylation. Notably, treatment with MP407 markedly reduced the pulmonary thrombosis and the numbers of paralysis and death in mice induced by ADP injection, but did not affect the bleeding time. Taken together, MP407 may be a potential candidate or lead compound for developing novel antiplatelet or antithrombotic agents for platelet hyperactivity-triggered disease therapy.

Key words:

platelet aggregation; MP407; cyclic AMP; GSK3β; VASP; thromboxane B_2

1. Introduction

When blood vessels are damaged, platelets quickly adhere to the subcellular matrix at the sites of vascular injury, leading to initiation and formation of haemostatic plug. Platelet hyperactivity also plays a critical role in the development of circulatory disorders, including atherothrombosis, myocardial infarction and cerebrovascular diseases (Ruggeri, 2002). Several studies have reported that treatment with antiplatelet drugs can prevent and reduce the incidence of stroke in patients with vascular diseases (Willoughby et al., 2002). Therefore, inhibiting platelet hyperactivity has been regarded as a promising strategy for ameliorating

thrombosis-related cardiovascular diseases.

Platelet activation is a complex process modulated by a variety of molecules and signaling pathways (Lazarus et al., 2003). Under normal condition, collagen, the most potent physiological activator of platelets, is sequestered under the endothelium of blood vessel walls. When the vessels are injury, the subendothelial collagen is exposed and binds to platelet receptor GP-VI, resulting in Ca^{2+}-dependent phospholipase A_2 (PLA_2) activation and arachidonic acid (AA) release from membrane phospholipids. Then, AA is further converted to thromboxane A_2 (TXA_2), a strong stimulator of platelet aggregation, via the actions of cyclooxygenase (COX) and thromboxane synthase (Vezza et al., 2002). In response to agonists, phospholipase C (PLC) is activated, leading to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) production, which in turn elevates intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ and protein kinase C (PKC) activity that are essential for platelet aggregation and granule secretion (Jardin et al., 2008; Harper et al., 2007). Activation of phosphatidylinositol 3-kinase (PI3K)/Akt cascade is known to stimulate platelet aggregation through phosphorylation and inactivation of glycogen synthase kinase 3β (GSK3β), a negative regulator of platelet activation (O'Brien et al., 2011; Li, et al., 2008). Notably, there are endogenous aggregation-inhibiting molecules existing in platelets, such as cyclic adenosine monophosphate (cyclic AMP) that is produced by adenylate cyclase. Cyclic AMP is able to inhibit the adhesion, aggregation, release of granule contents, TXA_2 formation, and intracellular Ca^{2+} mobilization of platelets via a series of cellular signaling pathways (Jang et al., 2002). Several cyclic AMP-elevating agents have been confirmed to inhibit platelet aggregation (Hayashi et al., 2009; Liu et al., 2009). The major target of cyclic AMP is cyclic AMP-dependent protein kinase (PKA) that phosphorylates vasodilator-stimulated phosphoprotein (VASP), thereby attenuating the affinity of VASP for contractile protein filamentous actin and fibrinogen binding to platelet membrane glycoprotein IIb/IIIa (GPIIb/IIIa) (Laurent et al., 1999; Sudo, et al., 2003). Accordingly, the compounds with

an ability to increase cyclic AMP formation and inhibit Akt activation may have a potential to inhibit platelet aggregation. Although many clinical antiplatelet drugs, such as aspirin, clopidogrel, and tirofiban, were used in treating thromboembolic diseases, they still have some defects and limitations (Tantry et al., 2005). After screening the effects of these new synthesized compounds on platelet aggregation, we found that the compound, MP407, exerts a potent antiplatelet activity mainly through elevation of cyclic AMP production, therefore regulating Akt/GSK3β cascade and VASP phosphorylation. Similarly, inhibition of ADP-induced pulmonary thrombosis in mice by MP407 was also observed.

2. Materials and methods

2.1.Materials

Collagen (type 1, equine tendon), AA, imidazole, indomethacin, 3-isobutyl-1-methylxanthine (IBMX), and Fura-2/AM were purchased from Sigma Chemical Company (St. Louis, MO, USA). The 2'5'-dideoxy-adeonsine (2'5'ddAdo) was purchased from Biomol company (Plymouth Meeting, PA, USA). Thromboxane B_2 (TXB_2), prostaglandin E_2 (PGE_2), and cyclic AMP enzyme-linked immunosorbent assay (ELISA) kits were purchased from Cayman Chemical Company, (Ann Arbor, MI, USA). The antibodies against p38MAPK, phospho-p38 MAPK(Thr180/Tyr182), phospho (Ser) PKC substrate (p47), AKT, phospho-AKT(Ser473), GSK3β, posopho-GSK3β, VASP, phospho-VASP(Ser157) were purchased from Cell Signaling (Danvers, MA, USA). The compound of MP407, 5-(2-hydroxy-3-(4-methoxybenzyl) aminopropoxy-3,3-dinethyl-1,3-dihydroindol-2-one, was provided by Prof, An-Rong Lee and it was synthesized as described previously (Lee et al. 1995). Briefly, *p*-Anisidine (**1**) was protected with benzyl bromide under basic conditions and then reacted with bromine in acetic acid at 10 – 15℃ to obtain **2** (90%) that was treated with equivalent amount of sodium hydride at 0-5℃ until the reaction was complete to give **3** (78%). The compound **3** was cyclized to get **4** in dry toluene by treatment with tributyltin hydride

(TBTH), catalyzed with azobisisobutyronitrile (AIBN) under a reflux condition (87%). Compound **4** was hydrolyzed by heating with 47% HBr to get **5** (66%). Treatment of **5** with excessive epichlorohydrin afforded **6** (42%) that was then reacted with 4-methoxybenzylamine under a reflux condition to obtain target MP407 (48%) (Fig.1). The crude MP407 was further purified by column chromatography (silica gel, CH_2Cl_2/THF/MeOH: 12:1:1). Recrystallization yielded pure MP407 that was characterized by mp (174) and specific spectra data (ir, nmr, and ms). The requirements for elemental analysis (purity) for C, H, and N were within ± 0.4% of the theoretical values and for HPLC chromatogram was essentially one peak. The MP407 was dissolved in dimethylsulfoxide (DMSO) for subsequent tests. All other chemicals and reagents were purchased from Sigma Chemical Company.

2.2.Platelet aggregation

The present study and all procedures of mice were approved by the Ethical Committee of Animal Experiments (102069-A), Tzu Chi University. Blood was withdrawn from rabbit marginal vein mixed with anticoagulant, EDTA (100 mM ,14:1 v/v) and centrifuged at 160 g, 25°C for 10 min to obtain platelet rich plasma (PRP). Then, platelet suspension was prepared from the PRP according to the washing procedures described previously (Lai et al., 2010). The platelet pellets were finally suspended in Tyrode's solution containing $CaCl_2$ (1 mM), NaCl (136.8 mM), KCl (2.7 mM), $NaHCO_3$ (11.9 mM), $MgCl_2$ (2.1 mM), NaH_2PO_4 (0.4 mM), glucose (10 mM), and bovine serum albumin (0.35%). Platelet concentration was counted by Coulter counter (Model ZM) and adjusted to $3.0x10^8$ platelets/ml. The platelet aggregation was measured turbidimetrically at 37°C with constant stirring at 1000 rpm by using an aggregometer (Model 560, Chrono-Log Corporation, Havertown, PA, USA). The platelet suspension was preincubated with drugs or solvent control (DMSO) for 3 min followed by addition of collagen (10 μg/ml) or AA (100 μM) for 6 min. The platelet aggregation was evaluated by measuring the peak height of the aggregation curves and expressed as the

percentage of maximal aggregation. To avoid the effect of the solvent on the platelet aggregation, the final concentration of DMSO was fixed at 0.5% (v/v).

2.3.Lactate dehydrogenase assay

Level of lactate dehydrogenase (LDH), acting as an index of platelet damage, was measured. Briefly, platelets were preincubated with MP407 (10, 25 μM) for 10 min and centrifugalized at 10,000g for 5 min. Then, the supernatant was incubated with phosphate buffer, containing 0.2 mg β-NADPH for 20 min at room temperature followed by addition of 100 μl pyruvate solution and the absorbance wavelength was read at 340 nm. LDH released was compared with the total LDH activity of platelets dissolved in 0.1% Triton X-100.

2.4.Measurement of cyclic AMP and TXB_2 levels

Platelet suspensions (3×10^8/ml) were incubated with drugs or solvent control at 37°C for 3 min, and the reaction was stopped by adding 10 mM EDTA and immediately boiling for 3 min. After centrifugation at 10,000*g* for 5 min, the levels of cyclic AMP in the supernatants were determined by ELISA kits, respectively. For determination of TXB_2 formation, platelets were preincubated with drugs or solvent control at 37°C for 3 min followed by addition of agonists for 6 min. After boiling for 6 min, the amounts of TXB_2 in the supernatants were measured by using ELISA kit.

2.5.Measurement of COX activity

Various concentrations of MP407 were preincubated with platelets at 37℃ for 3 min in the presence of imidazole (1 mM), an inhibitor of thromboxane synthase, then AA (100 μM) was added for another 6 min and boiled for 3 min. The amount of PGE_2 in the samples was measured using an ELISA kit to reflect the COX activity.

2.6.Western blotting

The isolated platelet proteins (20 μg) of different groups were separated in 8 % SDS-polyacrylamide gels and electrotransferred using semi dry transfer (Bio-Rad Laboratories

Inc., Hercules, California, USA). Then, various primary antibodies were incubated with the transferred membranes for 1.5 h. A peroxidase-conjugated secondary antibody was then added in a PBS-Tween 20 and incubation for 1 h. The protein bands were detected by the enhanced chemiluminescence chemiluminescence (ECL) reagent (Amersham International Plc., Buckinghamshire, UK).

2.7.Measurement of Ca^{2+} mobilization in platelets

The PRP ($3.0x10^8$ platelets/ml) was incubated with Fluo-2 AM (5 μM) for 30 min at 37°C in the dark followed by centrifugation at 500g for 10 min. Then, the pellets were suspended in 2 ml Tyrode solution. The fluorescence intensity of 20,000 platelets per sample was analyzed using a flow cytometer (FACScan, Becton Dickinson, Heidelberg, Germany).

2.8.Measurement of platelet surface glycoprotein IIb/IIIa expression

After platelet suspension was incubated with CD41/CD61-FITC that was raised against a platelet glycoprotein IIb/IIIa (GPIIb/IIIa) complex, various drugs and collagen (10 μg/ml) was added for 10 min. The reaction was stopped by adding 500 μl of 1% paraformaldehyde, and the fluorescence intensity of 20,000 platelets of samples was analysed using a flow cytometer.

2.9.ADP-induced pulmonary thrombosis in mice

The thrombotic mice model was performed as described previously (Chen et al., 2015). The ICR mice (20-25g) were randomly divided into 3 groups, including (1) the DMSO (solvent control), (2) MP407 (2 mg/kg, i.p.)-treated mice, (3) MP407 (4 mg/kg, i.p.)-treated mice, and each group contained 10 mice. After administration of solvent or MP407 for 30 min, the thrombi occluded in the pulmonary vessels was induced by intravenous injection of ADP (300 mg/kg). The numbers of paralysis and death of each group were recorded at 24 h after ADP injection.

2.10. The bleeding time assay

After the mice were treated with solvent or MP407 (2 or 4 mg/kg, i.p.) for 15 min, the tail

was transected 3 mm from the tip and immersed into saline at 37℃. The accumulated bleed time was recorded over a 20-min period.

2.11. Statistical analysis

The data was expressed as means and their standard errors. One-way ANOVA with *post hoc* Bonferroni test was used for statistical analysis. Results were considered significant difference at a value of $P<0.05$.

3. Results

3.1.MP407 increased cyclic AMP formation but inhibited platelet aggregation

Treatment with MP407 significantly increased cyclic AMP formation both in resting and collagen-activated platelets, but did not affect the cyclic GMP level (data not shown). The elevation of cyclic AMP formation caused by MP407 in activated platelets was markedly enhanced by forskolin, an adenylate cyclase activator. However, simultaneous addition of 3-Isobutyl-1-methylxanthine (IBMX), a cyclic AMP phosphodiesterase inhibitor, did not further enhance the cyclic AMP formation (Table 1). Moreover, MP407 dose-dependently inhibited the platelet aggregation induced by collagen or AA with IC_{50} values of 7.2±0.6 and 2.5±0.3 μM, respectively. However, blocking cyclic AMP synthesis with 2'5'ddAdo (200 μM), an adenylate cyclase inhibitor, greatly diminished the antiplatelet activity of MP407 (Fig. 2), suggesting the involvement of cyclic AMP in its antiplatelet activity. The effects of MP407 were not due to the cytotoxicity evidenced by no significant release of LDH (data not shown).

3.2.Effects of MP407 on TXB_2 production and COX activity

To investigate the antiplatelet mechanisms of MP407, the TXB_2 production was measured. Our results revealed that MP407 markedly inhibited collagen-induced TXB_2, a stable metabolite of TXA_2, production, which was reduced by 2'5'-ddAdo. However, MP407 did not affect AA-induced TXB_2 production (Table 2). In the presence of imidazole, MP407 had no significant effect on the PGE_2 production in AA-stimulaed platelets. On the contrary, addition

of indomethacin, a COX inhibitor, strongly decreased the PGE_2 production in AA-stimulated platelets (Table 3). Thus, the inhibition of TXB_2 production by MP407 may be not due to directly suppressing COX activity.

3.3.Effects of MP407 on Akt, GSK3β and VASP phosphorylation

To explore the molecular mechanisms of MP407, the important mediators for regulating platelet aggregation, such as Akt, GSK3β, VASP and p38MAPK were examined. As shown in Fig. 3A, MP407 inhibited Akt and GSK3β phosphorylation and increased the VASP phosphorylation(Ser157) in collagen-stimulated platelets, and the effects of MP407 were reversed by addition of 2'5'ddAdo. Similarly, the inhibitory effects of MP407 on collagen-induced p38MAPK and p47 phosphorylation were attenuated by 2'5'ddAdo (Fig. 3B). These findings suggest that cyclic AMP involves MP407-regulaed platelet signaling.

3.4.MP407 reduced Ca^{2+} mobilization and platelet surface GPIIb/IIIa expression

The increase of intracellular Ca^{2+} mobilization and the GPIIb/IIIa expression is essential for platelet aggregation. In this study, we confirmed that collagen-evoked Ca^{2+} mobilization in Fluo-2-loaded platelets was inhibited by MP407 (Fig. 4A). Moreover, MP407-mediated reduction of Ca^{2+} mobilization was similar in the presence of external Ca^{2+} (1 mM) or in the Ca^{2+}-free solution (data not shown). Compared to collagen-stimulated alone platelets, MP407 dose-dependently reduced platelet surface GPIIb/IIIa expression, and it was attenuated by 2'5'-ddAdo (Fig. 4B). This highlights the importance of cyclic AMP on the inhibition of platelet activation by MP407.

3.5.Effects of MP407 on ADP-induced pulmonary thrombosis and bleeding time

Compared to vehicle-treated group, pre-treatment with MP407 greatly reduced the numbers of paralysis and death caused by ADP injection (Table 4), supporting that MP407 exhibits an antithrombotic effect *in vivo*. No significant difference was observed between MP407 (2 or 4 mg/kg, i.p.)-treated mice and the normal mice (11.9±1.8 min, 10.8±1.7 min versus 10.5 ±1.4

min), suggesting that MP407 may not have an obvious bleeding risk.

4. Discussion

Platelets are regarded as major cells accounting for the formation of primary hemostasis to avoid blood loss. However, it has been demonstrated that platelet-derived pro-coagulant and pro-inflammatory molecules also trigger thrombosis, atherogenesis and inflammatory responses (Davi et al. 2007). Therefore, attenuating the platelet hyperactivity occurred in patients with vascular diseases may be a potential way to alleviate cardiovascular disorders. It is well known that cyclic AMP-elevating agents have an antiplatelet activity (Hayashi et al., 2009), implying that development of this type of compounds is a promising strategy to discover novel antiplatelet agents. In this study, we confirmed that MP407 exhibits a potent antiplatelet activity due to elevation of cyclic AMP formation in collagen-stimulated platelets. The amount of cyclic AMP is controlled by its synthesis and degradation mediated by adenylate cyclase and cyclic AMP phosphodiesterase, respectively. Based on the fact that the increase of cyclic AMP formation caused by MP407 was markedly enhanced by simultaneous addition of forskolin but not by IBMX, we propose that inhibiting cyclic AMP phosphodiesterase activity may be a major mechanism accounting for the increased cyclic AMP formation.

Blocking cyclic AMP formation with 2’5’-ddAdo greatly diminished the antiplatelet activity of MP407, indicating that elevation of cyclic AMP involves its actions on platelet aggregation. Next, the role of cyclic AMP and its-regulated signaling on the antiplatelet activity of MP407 were investigated. The PI3K/Akt cascade modulates several cellular functions, including metabolism, proliferation, survival, angiogenesis, and platelet aggregation (Sussman et al., 2011; Guidetti et al., 2015; Chen et al., 2004). Upon PI3K activation, pyruvate dehydrogenase kinase isozyme-1 (PDK1) and Akt, a family of serine/threonine kinases, are recruited to the membrane, therefore stimulating Akt phosphorylation (activation) by PDK1, which in turn induces platelet aggregation (Kim et al., 2001). Importantly, cyclic AMP can

suppress Akt phosphorylation by inhibiting PDK1 activity via preventing PDK1 translocation to the plasma membrane (Kim et al., 2001). The collagen-induced phosphorylation of Akt(Ser473) was inhibited by MP407, which is consistent with other cyclic AMP-elevating agents (Hayashi et al., 2009). Similarly, the above effects of MP407 were markedly reversed by 2'5'-ddAdo. These findings indicate that cyclic AMP-dependent responses are closely linked to the inhibition of MP407 on Akt activity and platelet aggregation. Moreover, attenuating Akt-induced phosphodiesterase 3A (PDE3A) activation may be another possible mechanism for enhancing platelet cyclic AMP level by MP407 (Zhang et al., 2007) by preventing cyclic AMP degradation.

The GSK3, a serine-threonine kinase, has two isoforms (α and β), and the GSK3β, an active form, is expressed predominantly in platelets. The platelets come from GSK3β$^{+/-}$ mice display increased platelet aggregation, dense granule secretion, fibrinogen binding, and thrombi formed (Li et al., 2008), suggesting that GSK3β is capable of inhibiting platelet aggregation. Importantly, Akt also inhibits GSK3β activity by phosphorylating GSK3β at Ser9 (van Weeren et al., 1998). Thus, inhibition of GSK3β(Ser9) phosphorylation by MP407 may be associated with suppression of Akt activity, while the event was abolished by 2'5'-ddAdo. The functions of VASP, an actin- and profilin-binding protein, are largely controlled by the phosphorylation at the specific sites. The serine 157 and serine 239 of VASP are preferentially phosphorylated by cyclic AMP and cyclic GMP-dependent kinases (PKA and PKG), respectively (Aszodi et al., 1999), which ultimately suppresses the affinity of VASP for contractile protein filamentous actin and fibrinogen binding to GPIIb/IIIa, thereby inhibiting platelet aggregation (Horstrup et al., 1994). Our data showed that MP407 increased the VASP (Ser157) phosphorylation without affecting the phosphorylation of VASP (Ser239) (data not shown). Collectively, MP407-regulated the activity of GSK3β and VASP in platelets is through a cyclic AMP dependent manner.

Activation of p38 mitogen-activated protein kinase (p38MAPK) is thought a crucial stimulator for collagen-induced platelet aggregation by activating PLA_2/AA/TXA_2 pathway (Coulon et al., 2003). Of note, the PLA_2/AA/TXA_2 cascade is also suppressed by cyclic AMP (Xing et al., 1999). A marked increase of p38MAPK phosphorylation in collagen-stimulated platelets was inhibited by MP407, while the effect was attenuated by 2'5'ddAdo. Interestingly, we found that only the TXB_2 production induced by collagen not by AA was inhibited by MP407 without affecting the COX activity evidenced by no significant change on AA-induced PGE_2 formation in imidazole-treated platelets. These results imply that the inhibition of TXB_2 production by MP407 may mainly attribute to reduction of AA release and not due to directly suppressing COX activity. The PLC is a key enzyme responsible for the generation of IP_3 and DAG by hydrolyzing the phosphatidylinositol 4,5-bisphosphate (PIP_2). Then, DAG activates PKC, causing substrate p47 phosphorylation and platelet activation. Importantly, cyclic AMP is able to inhibit the functions of PLC by reducing PIP_2 resynthesis (Ryningen, et al., 1998). In this study, the increased p47 phosphorylation in collagen-stimulated platelets was inhibited by MP407, and it was attenuated by 2'5'ddAdo. Therefore, the antiplatelet activity of MP407 may be, at least partly, mediated by cyclic AMP-dependent suppression of p38MAPK and PKC activity.

A rise of $[Ca^{2+}]_i$ caused by Ca^{2+} release from intracellular stores and/or Ca^{2+} entry through plasma membrane channels is required for agonist-induced platelet granule secretion and aggregation (Rink et al., 1990). Activation of cyclic AMP/PKA is known to reduce agonist-evoked platelet $[Ca^{2+}]_i$ by phosphorylating ATP-dependent calcium pump, leading to Ca^{2+} uptake into the dense tubular system, and inhibition of calcium release from the dense tubular system via attenuating PLC-induced IP_3 generation (Kim et al., 2014; Lazarowski et al., 1989). Accordingly, the inhibition of the rise of Ca^{2+} mobilization in activated platelets by MP407 may be related to elevation of cyclic AMP level. Moreover, MP407-mediated reduction

of Ca^{2+} mobilization was similar in the presence or absence of external Ca^{2+} (1 mM), implying that the effect is mainly due to suppressing Ca^{2+} release from intracellular stores.

An exposure of platelet surface GPIIb/IIIa is considered as a common final step of platelet aggregation and clot retraction through binding to soluble fibrinogen and bridging platelets (Shattil, et al., 1998; Coller, 2001). Importantly, cyclic AMP also plays a crucial role on the inhibition of platelet surface GPIIb/IIIa expression and activation by suppressing Akt activity and GSK3β phosphorylation(Ser9), but elevating VASP phosphorylation (Ser157) (Chen et al., 2004; Horstrup et al., 1994). As expected, MP407 dose-dependent inhibited platelet surface GPIIb/IIIa expression via a cyclic AMP dependent manner. In summary, cyclic AMP-dependent inhibition of Akt, GSK3β, p38, and p47 phosphorylation, and enhancement of VASP phosphorylation(Ser157), thereby attenuating TXA_2 production, Ca^{2+} mobilization, and GPIIb/IIIa expression may all account for the antiplatelet activity of MP407 (Fig. 5).

Previous studies have confirmed that dihydropyridazinone derivatives such as indolidan exhibit positive inotropic and vasodilatory activities, and ameliorate cardiovascular diseases, in particular congestive heart failure (Corder et al., 1992; Amsallem et al., 2005). However, some adverse effects, such as tachycardia were found after treatment with indolidan and its analogues. In this study, we designed and synthesized various non-steroidal and non-catecholamine cardiotonics that have a core structure deviated from the classical dihydropyridazinone-containing compounds. Among these synthetic compounds, we have demonstrated that MP407 has an inotropic activity in isolated ventricular tissues of Mongrel dogs (<10% change in contractile force at 10 μM) and an antiplatelet activity mainly through elevation of cyclic AMP formation. Notably, administration of MP407 markedly attenuated the numbers of paralysis and death due to severe pulmonary thrombosis induced by ADP injection. In addition, treatment with MP407 did not prolong the bleeding time, suggesting that MP407 has a low hemorrhagic risk. Taken together, MP407 may be a potential candidate or lead

compound for developing new inotropic, antiplatelet or antithrombotic agents for platelet hyperactivity-related disease therapy.

Conflict of interest

The authors declare that there is no conflict of interests.

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Figure 1. Synthesis of MP407.

Figure 2. Effects of MP407 on collagen or AA-induced platelet aggregation. Washed platelets were preincubated with Tyrode solution, MP407 (1-25 μM) or MP407 combination with 2'5'ddAdo (200 μM) at 37℃ for 3 min, then collagen (10 μg/ml) or AA (100 μM) was added to induce the platelet aggregation. The inhibition of platelet aggregation was expressed as the percentage of maximal aggregation. Data were expressed as mean ± S.E.M. (n=5). $^{**}P<0.01$, $^{***}P<0.001$ compared to respective MP407+collagen-treated platelets.

Figure 3. Effects of cyclic AMP on MP407-mediated platelet signaling. Washed platelets were preincubated with Tyrode solution, MP407 or MP407 (25 μM) combination with 2'5'ddAdo at 37℃ for 3 min, followed by addition of collagen for 6 min. The protein expression of target genes in various groups were examined (A, B). The blots were representative examples of five experiments.

Figure 4. Effects of MP407 on intracellular Ca^{2+} mobilization and platelet membrance GPIIa/IIIb expression. Fluo2-AM-loaded platelets or washed platelets were pretreated with MP407 in the presence or absence of 2'5'ddAdo, followed by addition of collagen. The intracellular Ca^{2+} mobilization and the expression of GPIIa/IIIb in platelets were analyzed by a flow cytometer. Data were expressed as mean ± S.E.M. (n=5). $^{**}P<0.01$, $^{***}P<0.001$ compared to the collagen-stimulated alone platelets. $^{\#}P<0.01$ compared to respective MP407 (25 μM)+collagen-treated platelets.

Figure 5. A schematic diagram represents the antiplatelet activity of MP407. MP407 increases cyclic AMP formation through inhibition of cyclic AMP phosphodiesterase (PDE) activity, resulting in suppressing Akt, GSK3β, p38, and p47 phosphorylation, and increasing VASP phosphorylation(Ser157), which in turn attenuates TXA_2 production, Ca^{2+} mobilization, and platelet surface GPIIb/IIIa expression. Taken together, the antiplatelet activity of MP407 may be mediated by cyclic AMP-dependent responses.

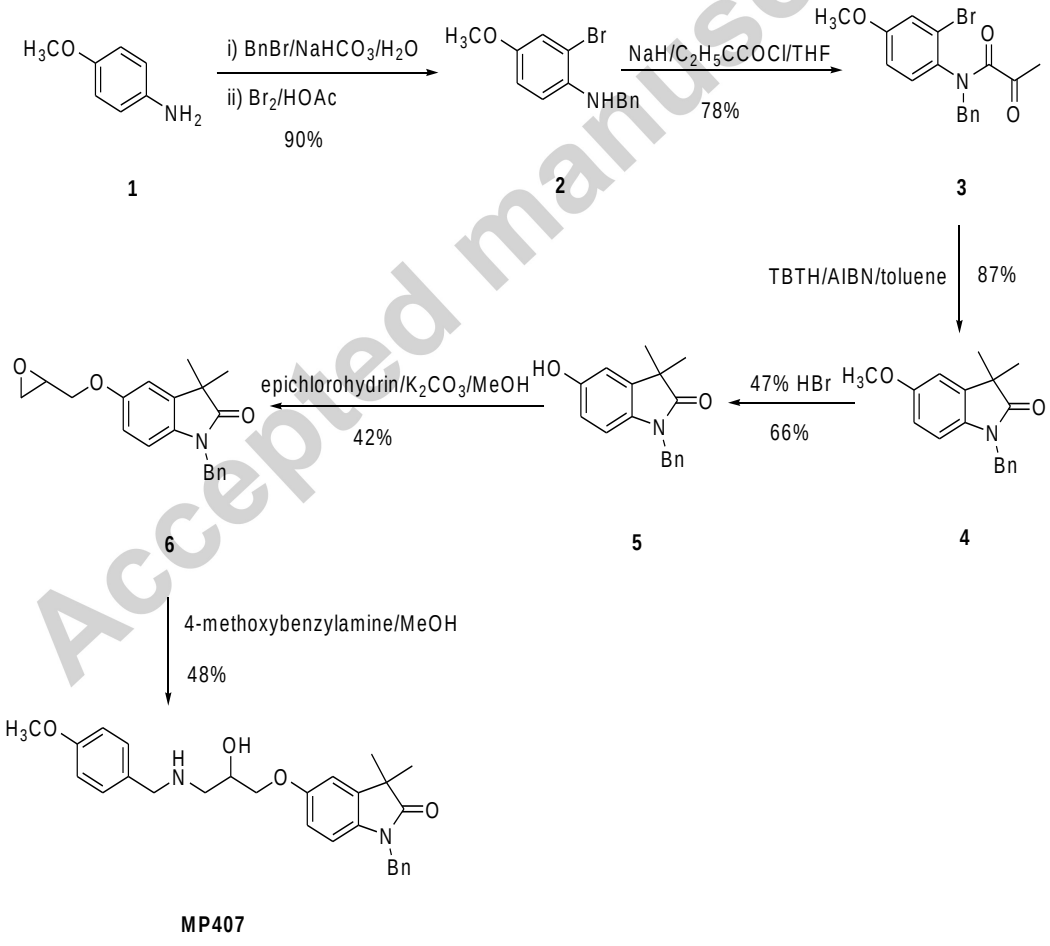


Figure 1

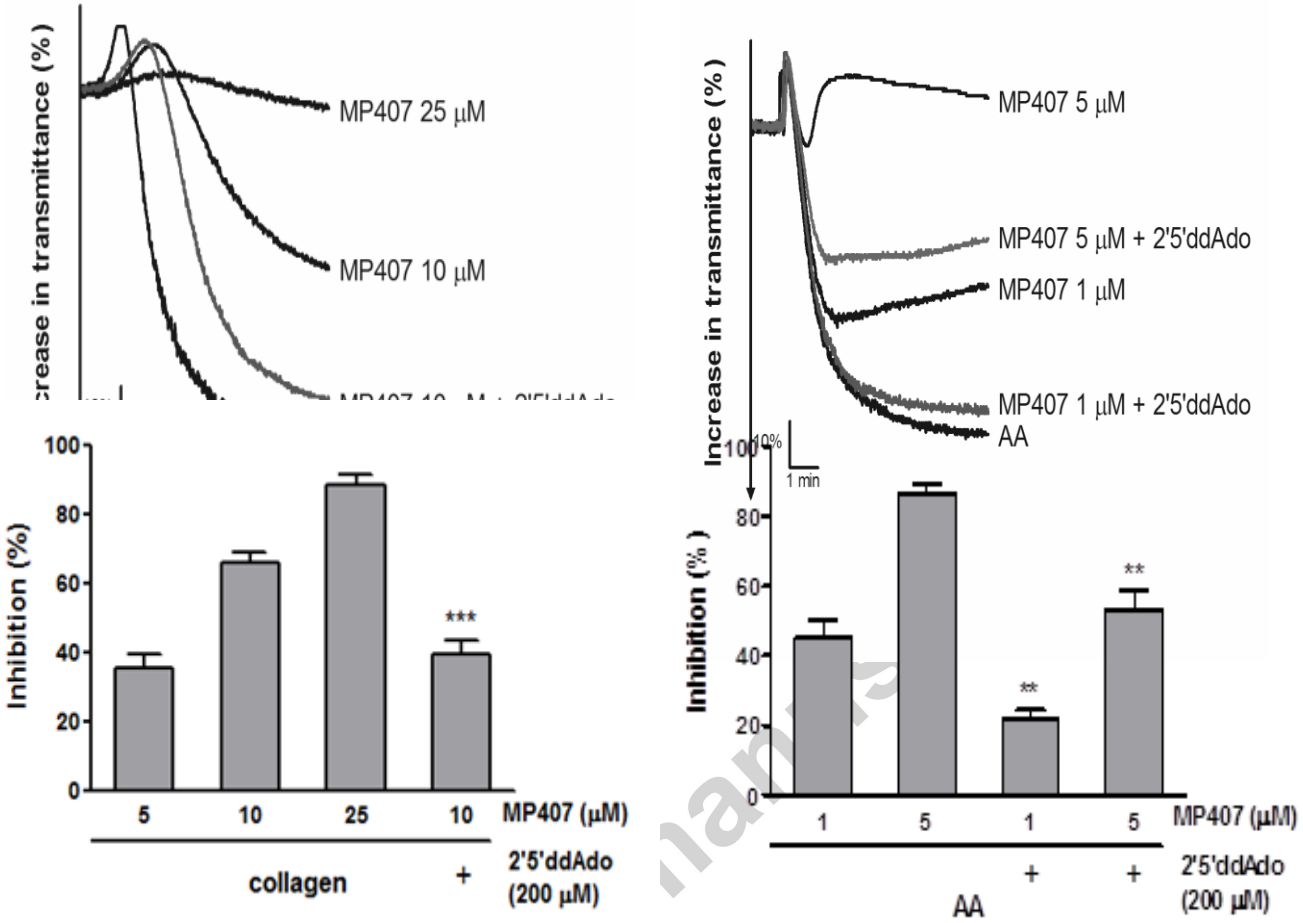
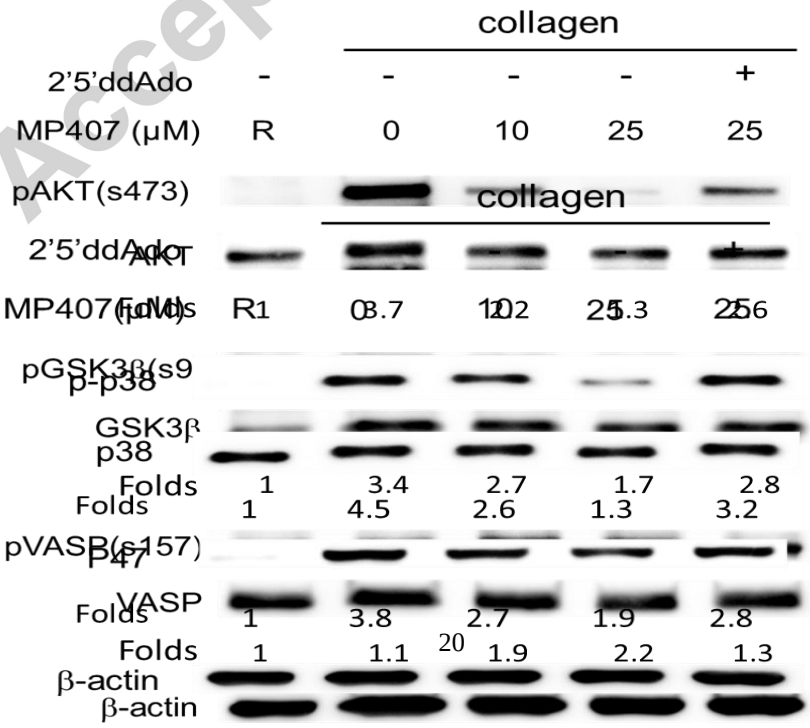

Increase in transmittance (%)
MP407 25 µM
MP407 10 µM
Inhibition (%)
100
80
60
40
20
0
***
5
10
25
10
MP407 (µM)
collagen
+
2'5'ddAdo
(200 µM)
Increase in transmittance (%)
MP407 5 µM
MP407 5 µM + 2'5'ddAdo
MP407 1 µM
MP407 1 µM + 2'5'ddAdo
AA
10%
1 min
Inhibition (%)
**
**
1
5
1
5
MP407 (µM)
+
+
2'5'ddAdo
(200 µM)
AA

Figure 2

A

B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2'5'ddAdo - - - - +
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pAKT(s473)
collagen
2'5'ddAdo
AKT
MP407 (µM)
Folds R1 0.7 1.2 1.3 1.6
pGSK3β(s9
p-p38
GSK3β
p38
Folds 1 3.4 2.7 1.7 2.8
Folds 1 4.5 2.6 1.3 3.2
pVASP(s157)
p47
VASP
Folds 1 3.8 2.7 1.9 2.8
Folds 1 1.1 1.9 2.2 1.3
β-actin
β-actin

Figure 3

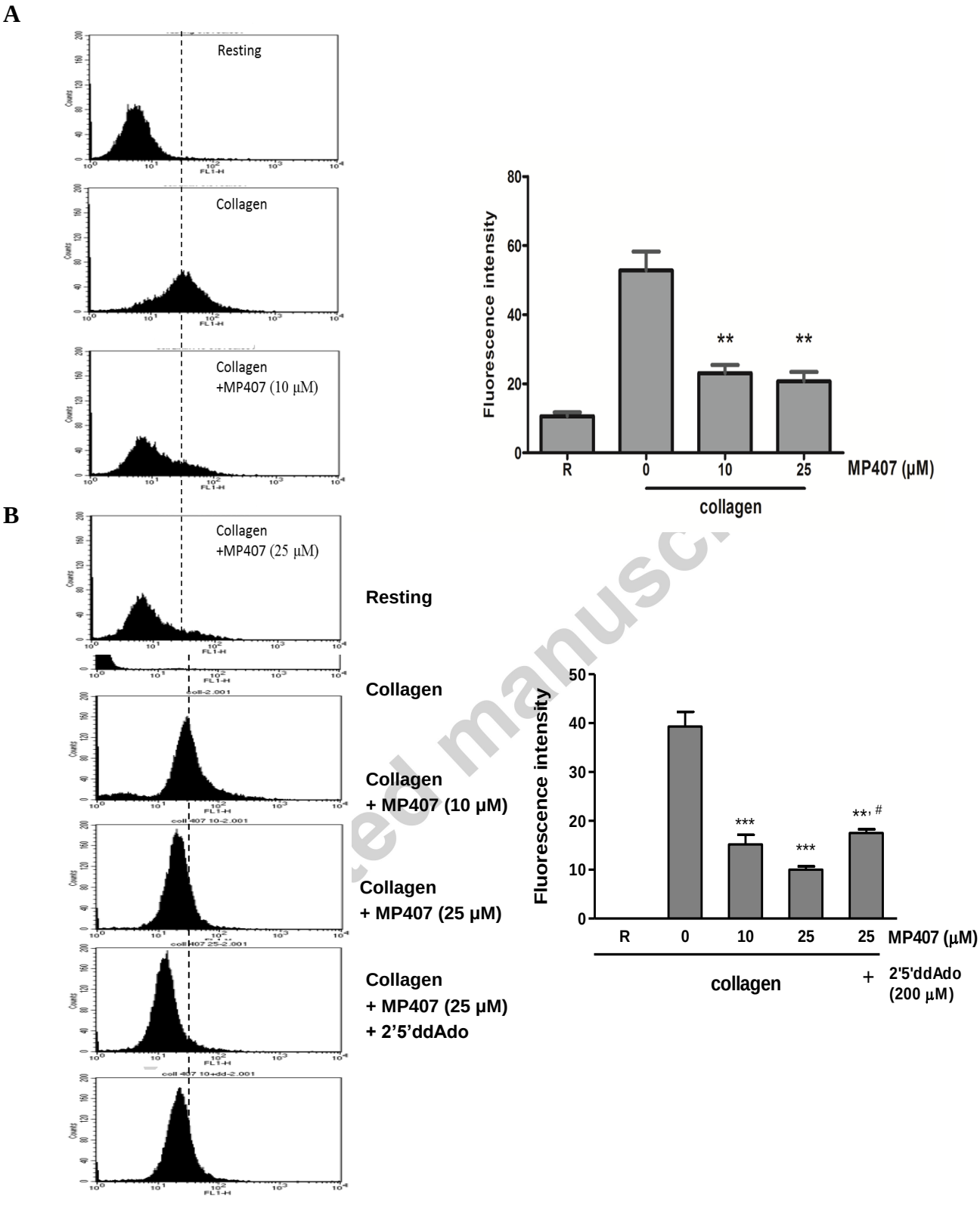

Figure 4

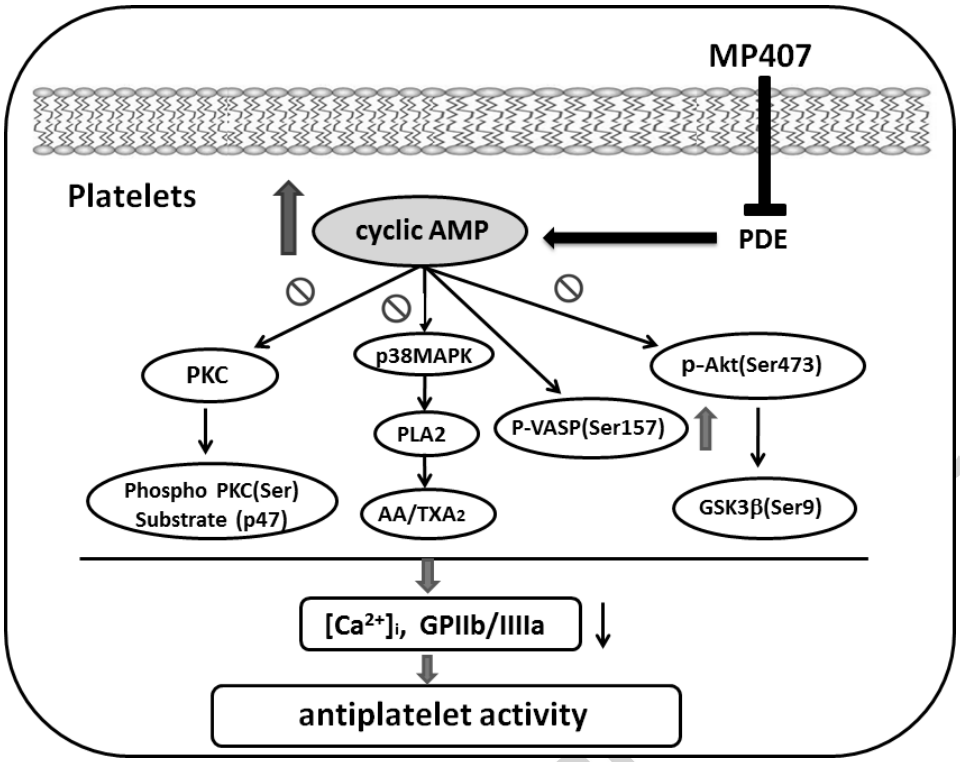

Figure 5

Table 1. Effects of MP407 on cyclic AMP formation

Treatment	Cyclic AMP (pmol/10^8 platelets)
Resting	11.5 ± 1.2
MP407 (25 μM)	15.1 ± 1.2[a]
collagen (10 μg/ml)	9.8 ± 0.4
+ MP407 (10 μM)	13.8 ± 0.7[b]
+ MP407 (25 μM)	18.2 ± 0.6[c]
+ forskolin (10 μM)	42.1 ± 0.4[d]
+ forskolin + MP407 (25 μM)	98.7 ± 5.6[d,e]
+ IBMX (50 μM)	16.9 ± 0.5[b]
+ IBMX + MP407 (25 μM)	20.1 ± 1.9[c]

Tyrode solution, MP407, forskolin, IBMX or the combination of forskolin + MP407 or IBMX

+ MP407 were preincubated with platelets at 37℃ for 3 min. Then, collagen was added for 6 min followed by boiling for 3 min. The amounts of cyclic AMP in supernatant were measured. Values are presented as the mean ± S.E.M. (n=5). [a]*P*<0.05 vs resting platelets, [b]*P*<0.05, [c]*P*<0.01, [d]*P*<0.001 vs collagen-stimulated alone platelets, [e]*P*<0.001 vs collagen and MP407 (25 μM)-treated platelets.

Table 2. Effects of MP407 on TXB_2 formation induced by collagen or AA

Treatment	TXB_2 (ng/ml) formation induced by collagen (10 μg/ml)	AA (100 μM)
Resting	1.2 ± 0.0	1.4 ± 0.0
Control	168.0 ± 9.8	671.7 ± 32.7
MP407 (1 μM)		651.3 ± 45.5
MP407 (5 μM)		613.7 ± 28.5
MP407 (10 μM)	29.0 ± 4.0[a]	
MP407 (25 μM)	6.7 ± 1.7[b]	
MP407 (25 μM) + 2'5'-ddAdo (200 μM)	38.7 ± 4.5[a,c]	

Washed platelets were preincubated with Tyrode solution or MP407 at 37℃ for 3 min. Then, collagen or AA was added for another 6 min to trigger TXB_2 formation. Data were expressed as the mean ± S.E.M. (n=5). [a]*P*<0.01, [b]*P*<0.001 vs collagen-stimulated alone platelets. [c]*P*<0.01 vs collagen + MP407 (25 μM)-treated platelets.

Table 3. Effects of MP407 on cyclooxygenase activity

Treatment	PGE_2 (ng/ml)
Resting	0.02 ± 0.00
AA (100 μM)	
+ imidazole (1 mM)	139.1 ± 10.4
+ imidazole + MP407 (1 μM)	119.1 ± 24.6
+ imidazole + MP407 (5 μM)	123.6 ± 8.3
+ imidazole + MP407 (10 μM)	124.1 ± 15.0
+ indomethacin (2 μM)	11.6 ± 4.8

Tyrode solution or MP407 was preincubated with platelets at 37℃ for 3 min in the presence of imidazole (1 mM). Then, AA was added for another 6 min followed by boiling for 3 min. The amounts of PGE_2 in the samples were measured using ELISA kit. Data were expressed as the mean ± S.E.M. (n=5).

Table 4. MP407 inhibited ADP-induced pulmonary thrombosis in mice.

groups	Numbers of paralysis and death
Vehicle	9
MP407 (2 mg/kg, i.p.)	4
MP407 (4 mg/kg, i.p.)	2

The mice were treated with solvent or MP407 for 30 min followed by injection of ADP (300 mg/kg) to induce pulmonary thrombosis. Then, recorded the numbers of paralysis and death at 24 h after ADP injection. Each group contained 10 mice.