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# NP- 313, 2-Acetylamino-3-chloro- 1,4-naphthoquinone, a novel antithrombotic agent with dual Inhibition on thromboxane A2 synthase and calcium entry

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**Running Title:** Dual inhibitor on TXA2 synthase and calcium entry

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## Summary

Background and purpose: 1,4- Naphthoquinones were reported to exhibit antiplatelet activity both *in vivo* and *in vitro*. In the present study, we investigated the anti-platelet effect of a novel naphthoquinone derivative NP-313, 2-acetylamino-3-chloro-1,4-naphthoquinone, and its mechanism of action.

Experimental approach: We measured platelet aggregation, $Ca^{2+}$ mobilization, thromboxane B2 formation, P-selectin expression, and examined several enzymatic activities. Furthermore, we used irradiated mesenteric venules in fluorescein sodium-treated mice to observe the anti-thrombotic effect of NP-313 *in vivo*.

Key results: NP-313 concentration-dependently inhibited human platelet aggregation induced by collagen, thapsigargin, thrombin, and A23187 with IC50, 1.7±0.1, 3.8±0.2, 7.7±0.2, and 20.0±0.3μM, respectively. NP- 313 also inhibited P-selectin expression, thromboxane $B_2$ formation, and $[Ca^{2+}]_i$ elevation in platelets stimulated by thrombin and collagen. NP-313 at 10μM inhibited cyclooxygenase COX-1, thromboxane $A_2$ synthase, and protein kinase Cα by 75%, 100% and 90%, respectively, whereas it did not affect phospholipase $PLA_2$ or PLC activity. In the presence of indomethacine and ADP scavenger, NP-313 concentration-dependently inhibited thrombin and A23187-induced $[Ca^{2+}]_i$ increase through its inhibitory effects on $Ca^{2+}$-influx, rather than blocking $Ca^{2+}$ release from intracellular stores. NP-313 also inhibited

thapsigargin-mediated $Ca^{2+}$-influx through store-operating calcium channel (SOCC), but had no effect on the DAG analog, 1-oleoyl-2-acetyl-sn-glycerol evoked-$Ca^{2+}$ influx through store-independent calcium channel. Nevertheless, it has little effect on cAMP or cGMP levels. As intravenously administered, NP-313 dose-dependently inhibited the thrombus occlusion of the irradiated mesenteric vessels of fluorescein-pretreated mice.

Conclustions and Implications: Taken together, NP-313 exerts its antithrombotic activity through dual inhibition on thromboxane $A_2$ synthesis and $Ca^{2+}$ influx through SOCC.

Keywords: anti-platelet, naphthoquinone, store-operating calcium channel (SOCC), thromboxane $A_2$ synthesis

## Introduction

Myocardial infarction and ischemic stroke are the leading causes of morbidity and mortality. The crucial role of platelet activation in thrombogenesis is well known. Antiplatelet agents have been developed as potential therapies for both the treatment and prevention of cardiovascular diseases (Bhatt *et al.*, 2003; Jackson *et al.*, 2003b). Upon vessel injury, platelets rapidly adhere to the newly exposed subendothelial matrix, such as collagen and von Willebrand factor, and undergo shape change, spreading, and release of both ADP and thromboxane A2, reinforcing platelet adhesion and aggregation (Walsh, 2004).

The major intracellular stimulus involved in platelet aggregation is an increase in cytosolic free calcium concentration (Rink *et al.*, 1990). As in most non-excitable cells, the increase in platelet $[Ca^{2+}]i$ induced by various agonists involves both influx of extracellular calcium through plasma membrane calcium channels and mobilization of intracellular calcium from the dense tubular system (Berridge, 2004; Berridge *et al.*, 2003). The key elements involved in $Ca^{2+}$signaling include the activation of surface receptors that lead to the stimulation of phospholipase C (PLC), resulting in the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5- bisphosphate ($PIP_2$) to release inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG). $IP_3$ binds to the $IP_3$ receptor on intracellular stores, releasing $Ca^{2+}$, and the consequential depletion of $Ca^{2+}$ stored within the dense tubular system serves as the primary trigger for activation of store-operated $Ca^{2+}$ channels (SOCC), which mediate $Ca^{2+}$ entry

(Putney *et al.*, 2001). Besides store-operated $Ca^{2+}$ entry (SOCE), Hassock et al. have suggested that another SOCE-independent mechanism also contributed to $Ca^{2+}$ entry in platelets and is activated by DAG (Hassock *et al.*, 2002). It has been reported that agents with inhibitory effects on the cytosolic $Ca^{2+}$ mobilization in platelets may suppress platelet aggregation and thrombus growth (Jin *et al.*, 2005; Jin *et al.*, 2004; Kang *et al.*, 2001; Lee *et al.*, 2005), whereas fewer studies indicated whether these agents have differential effect on $Ca^{2+}$ channels involved in modulating intracellular $Ca^{2+}$ mobilization.

The stimulation of platelets with different stimuli results in early activation of $Ca^{2+}$-dependent cytosolic phospholipase $A_2$, which hydrolyzes membrane phospholipids leading to arachidonic acid (AA) release. The liberated AA is metabolized via cyclooxygenase (COX) pathway to form endoperoxides, prostaglandins (Jin *et al.*, 2005; Walsh, 2004), and $TXA_2$ (Arita *et al.*, 1989). The important role of $TXA_2$ synthesis in activation of platelets is underlined by the well-known clinical efficacy of aspirin related to its COX enzyme inhibition (2002). Nevertheless, there are evidences indicating that there are subpopulations that do not respond to antithrombotic action of aspirin (Szczeklik *et al.*, 2005). Thus, interests for developing other antithrombotic drugs such as $TXA_2$ modulators are in progress (Dogne *et al.*, 2004; Dogne *et al.*, 2006).

Compounds with the chemical structure of a 1,4-naphthoquinone backbone have shown a wide variety of pharmacological effects including anticancer and antiplatelet activities (Lien *et al.*, 2002; Rodriguez *et al.*, 1995; Verma, 2006). In this report, a series of 2, 3-disubstituted 1,4- naphthoquinones were synthesized and tested for their

inhibitory activities on platelet aggregation. Among them, 2-acetylamino-3-chloro-1,4-naphthoquinone (NP-313), a newly synthesized naphthoquinone derivative, was found to possess differential inhibitory effect on platelet aggregation caused by various stimulators. We investigated its antiplatelet effects and found that it possesses unique dual inhibitory activity on calcium-signaling cascade and thromboxane A2 synthesis.

## Materials and Methods

### Reagents and animals

NP-313 (2-Acetylamino-3-chloro-1,4-naphthoquinone, molecular weight, 249 Da, Figure 1) was synthesized and provided by Dr. Jin-Cherng Lien and Sheng-Chu Kuo (China Medical University, Taichung, Taiwan and its purity (>95%) was confirmed by $^{1}$H-NMR analysis. Collagen (bovine tendon type I), thrombin, 4-bromo-A23187, thapsigargin, or 1-oleoyl-2-acetyl-sn-glycerol (OAG), indomethacin, fluorescein sodium, Fura-2 acetoxymethyl ester (Fura 2-AM) , bovine serum albumin (BSA), prostaglandin $E_1$( $PGE_1$), nitroglycerin (NTG), dimethylsulfoxide (DMSO), heparin, ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetra acetic acid (EGTA), and ethylene diamine tetraacetic acid (EDTA), creatine phosphate (CP), and creatine phosphokinase (CPK) were purchased from Sigma Chem. (St. Louis, MO, USA). Cytosolic phospholipase $A_2$ ($cPLA_2$) assay kit was from Cayman Chemical (Ann Arbor, MI, USA).Fluorescein isothiocyanate labeled monoclonal antibody to P-selectin (FITC-labeled anti-CD62P) was obtained from Becton Dickson (Franklin Lakes, NJ, USA). Thromboxane $B_2$ ($TxB_2$) assay kit was purchased from Amersham (Buckinghamshire, HP, UK). Lactate dehydrogenase (LDH) assay kit was purchased from Randox (Crumlin, Co. Antrim, UK).

Male ICR mice were used in animal studies. The animals were maintained on a

12-hour light/dark cycle under controlled temperature (20±1°C) and humidity (55±5%). Animals were given continuous access to food and water. All procedures involving animal experiment were approved by the Institutional Animal Care and Use Committee at College of Medicine, National Taiwan University.

**Preparation of washed human platelets and platelet-rich plasma**

Human platelet suspension was prepared according to the method described previously (Mustard *et al.*, 1972). The washed platelets were finally suspended in Tyrode's solution and the platelet count was adjusted to $3.75 \times 10^8$ platelets $ml^{-1}$. For platelet-rich plasma preparation, whole blood was anticoagulated with sodium citrate (3.8%, w/v) in a volume ratio of 9:1 and centrifuged at 150*g*, 25°C for 9 min.

**Measurement of platelet aggregation**

Platelet aggregation was measured turbidimetrically with a Lumi-aggregometer (Payton Scientific) at 37°C under stirring condition (900 rpm) (Born *et al.*, 1963). Platelet suspension was prewarmed to 37°C for 2 min and NP-313 was added 3 min before the addition of platelet aggregation activator.

**Flow cytometric analysis of P-selectin expression**

The washed platelet suspension ($3\times10^8$ $ml^{-1}$) containing tirofiban (100ng $ml^{-1}$) was preincubated with NP-313 for 3 min at 37°C and activated with the platelet activators for 10 min at 37°C. Then the sample was further incubated with FITC-labeled anti-CD62P in the dark for 15 min at room temperature. The platelet suspension was

immediately assayed by fluorescence activated cell sorter (Becton–Dickinson, FACScan Sysem, San Joe, CA) using excitation and emission wavelength of 488 and 525 nm, respectively. Data were collected from 10,000 platelets per experimental group. The level of P-selectin expression was expressed as mean fluorescence intensity.

**Measurement of $TxB_2$ formation**

Platelet suspension ($3 \times 10^8$ $ml^{-1}$) was incubated with NP-313 or DMSO for 3 min and then treated with platelet activators. At 6 min after the addition of agonists, indomethacin (50μM) and EDTA (2mM) were added to terminate the reaction. The contents of $TXB_2$ were measured using a competitive EIA kit according to the instructions of the manufacturer.

**Measurement of intracellular $Ca^{2+}$mobilization**

After centrifuging platelet-rich plasma at 500g, 25°C for 8min, isolated platelets were resuspended in $Ca^{2+}$-free Tyrode's solution. Platelet suspension was protected from light and incubated with Fura 2-AM (5μM) at 37°C for 30 min. Human platelets were then prepared as previously described. The platelet count was adjusted to $3 \times 10^8$ $ml^{-1}$ platelets. Just before $[Ca^{2+}]i$ measurements were performed, $Ca^{2+}$ was added back to platelets to a final concentration of 1.0mM, then NP-313, collagen, thrombin, A23187, thapsigargin, or OAG was added. The rise in $[Ca^{2+}]i$ was measured using a F4500 Fluorometer (Hitachi, Japan) at excitation wavelengths of 340 and 380 nm, and an emission wavelength of 500 nm. Fluorescence was calibrated with lysed platelets (0.1% Triton X-100) in the absence and presence of 10mM EGTA in each run to obtain maximum and minimum fluorescence values , and $[Ca^{2+}]i$ was calculated from

the fluorescence, using 224 nM as the $Ca^{2+}$ -Fura 2 dissociation constant.

**Biochemical assays of cyclooxygenase COX-1, -2, thromboxane A2 synthase, phospholipase PLA2 and PLC activities**

These enzymatic assays were performed by MDS pharma Services utilizing the standard protocols. Briefly speaking, COX-1 (platelet) and COX-2 (recombinant) activity was EIA quantified by measuring PGE2 level converted from arachidonic acid. Thromboxane synthase (human platelets) activity was assayed by EIA quantitation of thromboxane B2 using PGH2 as substrate. PLA2 activity (pig pancreas) was measured by quantitation of $^{14}$C-oleate release from 1-palmitoyl-2-[1-$^{14}$C] oleoyl-l-3-phosphatidylcholine. PLC activity was measured by quantitation of chromogen of phosphatidylcholine release from 1, 2-dihexanoyl sn-glycerol-3-phosphocholine. PKCα activity was measured as phosphorylation of histone ($P^{32}$-histone) stimulated by diacylglycerol in the presence of phosphatidyl-serine and $Mg^{2+}$ and $Ca^{2+}$.

**Lactate dehydrogenase (LDH) assay**

The released LDH activity was measured spectrophotometrically by recording the decrease in the optical density of h-NADH at 340nm, as previously described (Jin *et al.*, 2004).

**Fluorescein sodium-induced platelet thrombus formation in mesenteric vunules of mice**

Platelet plug formation in mesenteric microvessels was performed according to a previously described method with modification (Chang *et al.*, 1998). In brief, male ICR mice (12-14g) were anesthetized with sodium pentobarbital (50mgkg$^{-1}$) by intraperitoneal injection, and then the fluorescein sodium (12.5mgkg$^{-1}$) was IV injected into a lateral tail vein of the mouse. Venules with diameters of 30-40μm were selected to be irradiated to produce a microthrombus. In the epi-illumination system, the area of irradiation (wavelength above 520nm) was approximately 50μm in diameter on the focal plane. After the operation, the mouse was IV injected with PBS (vehicle), aspirin (100, or 200mgkg$^{-1}$), or various doses of NP-313 through other lateral tail vein. Five min after administration of these drugs, the irradiation by filtered light was started and the occlusive time (TTO, upon cessation of blood flow) was recorded.

**Tail bleeding time in mice**

The bleeding time was measured with transection of the tail in a mouse model (Dejana *et al.*, 1982). Various doses of NP-313 or vehicle were given IV 5 min prior to tail cutting.

**Statistical analysis**

The experimental results are expressed as the means ± S.E.M. The statistical comparisons were made by ANOVA (following a paired Students'*t*-test), and differences were considered to be significant at $P$ value $< 0.05$.

## Results

### Effect of NP-313 on Platelet Aggregation

As shown in Figure 2, NP-313 inhibited collagen (10μg/ml), thapsigargin (0.1μM), thrombin(0.1U/ml), and A23187 (8μM) induced platelet aggregation of washed human platelets in a concentration-dependent manner, with $IC_{50}$ values, 1.7 ± 0.1, 3.8 ± 0.2, 7.7 ± 0.2, and 20.0 ± 0.3μM, respectively. However, in response to thrombin and A23187-induced platelet aggregation, the maximal degree of inhibition with NP-313 reached around 80 and 60%, respectively.

### Effect of NP-313 on Collagen- and Thrombin-Induced Granule Secretion and $TxB_2$ Formation in Platelets

We examined whether NP-313 could inhibit the processes of platelet activation, such as granule secretion, and $TxB_2$ formation. NP-313 concentration-dependently inhibited P-selectin expression (Figure 3A), and also $TxB_2$ generation induced by collagen or thrombin (Figure 3B).

### Effect of NP-313 on COX-1, -2, TxA2 synthase, phospholipase PLA2, PLC, and PKCα activity

It has been reported that some 1, 4-naphthoquinone derivatives inhibit $TxA_2$ synthase (Jin *et al.*, 2005; Jin *et al.*, 2004; Pozzan *et al.*, 1994). Similarly, NP-313 at 10 μM inhibited COX-1 (75% inhibition), PKCα (90% inhibition) and thromboxane synthase (100% inhibition), whereas it had little inhibitory effect on COX-2, PLA2, and PLC activities. Consistent to these data, NP-313 concentration-dependently inhibited TxB2

formation in platelets stimulated by collagen and thrombin (Figure 3B). NP-313 also inhibited PMA (300nM)-induced platelet aggregation in a concentration-dependent manner. At 15 μM, a complete inhibition was observed (data not shown).

**Effect of NP-313 on Cyclic Nucleotide Levels in Human Platelets**

To assess whether the action of NP-313 was due to elevation of intracellular levels of cAMP and/or cGMP, two major inhibitory messengers in regulating platelet aggregation (Schwarz *et al.*, 2001), the effect of NP-313 on cyclic nucleotide levels in platelets was examined. In washed human platelets, the addition of nitroglycerin (NTG) and PGE1 significantly increased cyclic GMP formation and cyclic AMP, respectively, however NP-313 affected neither cAMP levels nor cGMP levels as compared with that of resting platelets (Figure 4).

**Effect of NP-313 on Collagen-, Thrombin- , and A23187 –Induced Intracellular $Ca^{2+}$ Mobilization in Human Platelets**

. Treatment of the Fura-2-loaded platelet suspension with NP-313 inhibited collagen, thrombin and A23187-evoked increase of $[Ca^{2+}]i$ in a concentration-dependent manner (Figure 5). NP-313 almost completely inhibited the elevation of the $[Ca^{2+}]i$ in response to collagen. Nevertheless, in response to thrombin and A23187-evoked increase of $[Ca^{2+}]i$, the residual 20% and 40% of $[Ca^{2+}]i$ mobilization, respectively, were unaffected by NP-313, even used at 80μM. Notably, these results were

correlated with the residual platelet aggregation caused by thrombin and A23187 (Figure 2).

**Effect of NP-313 on Thrombin- and A23187 –Induced $Ca^{2+}$ Release from Internal Stores and Influx of Extracellular $Ca^{2+}$ in Human Platelets**

Cytosolic $Ca^{2+}$ elevation occurs as a consequence of release of $Ca^{2+}$ from intracellular stores and influx from the extracellular medium. In the following experiments, Fura-2-loaded platelets were preincubated in the buffer containing CP (10mM), CPK (1U $ml^{-1}$), and indomethacin (100μM), to exclude the effects of ADP and $TXA_2$ on [Ca2+]i elevation during agonist-induced stimulation. The data in Figure 6A showed the effect of NP-313 on intracellular $Ca^{2+}$ mobilization and $Ca^{2+}$influx mediated by thrombin. As platelets were incubated in $Ca^{2+}$-free medium containing 1mM EGTA, and followed by the addition of thrombin , the increase of $[Ca^{2+}]i$ was substantially smaller compared to $Ca^{2+}$ influx (Figure 6, left). NP-313, even used at 80μM, failed to inhibit thrombin-induced release of intracellular stored $Ca^{2+}$. Next, after agonist-mediated $Ca^{2+}$ release from intracellular store, the effect of NP-313 on $Ca^{2+}$ influx was assessed by the addition of 2mM $Ca^{2+}$. In contrast, the increase of $[Ca^{2+}]i$ by thrombin in the presence of extracellular $Ca^{2+}$ was concentration-dependently inhibited by NP-313 (Figure 6A, right).These results indicate NP-313 mainly prevents the entry of $Ca^{2+}$ into the cytoplasm but has little influence on $Ca^{2+}$ mobilization from the dense tubular system. In addition, the influence of NP-313 on A23187-evoked $Ca^{2+}$ mobilization was similar to that observed with thrombin (Figure 6B). It is known that A23187, a $Ca^{2+}$ ionophore, mobilizes $Ca^{2+}$ across membranes and directly increases $[Ca^{2+}]_i$. Furthermore, the residual $[Ca^{2+}]i$ mobilization observed both in thrombin and A23187-induced $Ca^{2+}$ influx were unaffected by NP-313 even at 80μM.

**Effect of NP-313 on Thapsigargin and OAG- Induced $Ca^{2+}$ Entry**

There are two $Ca^{2+}$-entry channels on platelet plasma membrane, i.e. store-operated $Ca^{2+}$ channel (SOCC) and SOCC-independent channel. Studies were carried out to determine if NP-313 had an impact on $Ca^{2+}$ entry mediated by these two channels. Figure 7A showed that NP-313 markedly elicited a concentration-dependent inhibition of thapsigargin-mediated $Ca^{2+}$ influx through the activation of SOCC by inhibiting intracellular $Ca^{2+}$-ATPase. However, NP-313 did not inhibit thapsigargin-mediated mobilization of intracellular $Ca^{2+}$ in the absence of extracellular $Ca^{2+}$ (data not shown). It is well-known that thapsigargin mediated-$Ca^{2+}$ efflux from dense tubular system in platelets is independent of IP3 receptors and occurs via inhibiting SERCA, and therefore causes intracellular $Ca^{2+}$ store depletion by uncovering a possive leak conductance from the intracellular stores (Pozzan *et al.*, 1994). On the contrary, NP-313, at the concentration up to 80μM, has no effect on the moderate and slow $[Ca^{2+}]i$ increase evoked by a DAG analog, OAG (Figure 7B), which was reported to activate store-independent $Ca^{2+}$ entry (Hassock *et al.*, 2002). Thus, NP-313 is considered to selectively inhibit $Ca^{2+}$ influx mediated by SOCC.

**Lack of Cytotoxic Effect of NP-313 on Human Platelets**

No significant increase in LDH release was observed with NP-313 (80μM) and vehicle-treated platelets, even when the incubation time of NP-313 with platelets was prolonged to 30 min (about 2.7% vs 2.6% release), suggesting that it neither affects platelet permeabilization nor induces platelet cytolysis at the concentration used.

**Effect of NP-313 on *in vivo* thrombosis and bleeding time in a mouse model**

It has been previously demonstrated that administration of fluorescein sodium and the subsequent irradiation on mesenteric venules induced the formation of marked mixed thrombi, composed of activated platelets and fibrin clots (Chang *et al.*, 1998). Figure 8A shows that the effects of NP-313 on average time to occlusion (TTO). In the vehicle-treated group, intravenous application of the dose of 12.5μg $g^{-1}$ fluorescein sodium induced an average TTO of 95.0 ± 7.5s (n = 6). However, after intravenous administration at 4, 8 and 16μg $g^{-1}$, NP-313 significantly prolonged the average TTO to 127.0 ± 12.1, 188.3 ± 25.7 and 242.5 ± 16.4s, respectively (n = 6; $P$ <0.01 for each, one way ANOVA, Dunnett's post hoc test).

As shown in Figure 8B, NP-313 did not affect the bleeding time in mice compared with vehicle-treated mice after IV administration at effective antithrombotic dose of 4, and 8μg $g^{-1}$. In contrast, aspirin caused a marked prolongation of tail bleeding time as it was intravenously given at an effective antithrombotic dose of 200 μg $g^{-1}$. However, NP-313 slightly prolonged bleeding time when administered at 16 μg $g^{-1}$.

## Discussion

The present study has shown that NP-313 inhibits collagen and thrombin-induced platelet aggregation and platelet activation, such as α-granule secretion, thromboxane formation, and intracellular $Ca^{2+}$ mobilization in a concentration-dependent manner. However, NP-313 preferentially inhibited collagen-induced platelet aggregation as compared with thrombin-induced one ($IC_{50}$, 1.7 vs 7.7μM, respectively). Upon being stimulated by collagen or thrombin, platelets synthesize and release $TxA_2$, which amplifies platelet aggregation (Jackson *et al.*, 2003a). Nevertheless, unlike the $TxA_2$-dependency of aggregation with collagen (Nieswandt *et al.*, 2003), thrombin induces intracellular $Ca^{2+}$ mobilization and platelet aggregation independent of the $TXA_2$ pathway (Freedman, 2005), in line with our results that majority of platelet responses remain unaffected even in the presence of ADP scavenger and cyclooxygenase inhibitors (Figure 6A). Enzymatic assays showed that NP-313 exhibits a potent inhibitory effect on COX-1, and thromboxane $A_2$ synthase and PKCα activity while it has little effect on phospholipase $A_2$ or PLC activity. In our experiments, we observed NP-313, at 4μM, abolished thrombin mediated-$TxB_2$ formation, without inhibiting thrombin-induced intracellular $Ca^{2+}$ mobilization in the presence of EGTA (Figure 3B, right panel and Figure 6A). On the other hand, collagen activates platelets through a tyrosine kinase-based signaling pathway and activation of $PLC\gamma_2$, which produces DAG and $IP_3$, leading to the release of $Ca^{2+}$ from intracellular $Ca^{2+}$ stores (Jackson *et al.*, 2003a). These processes are exclusively dependent on GPVI activation through collagen. However, GPVI alone mediates only a part of platelet aggregation and full-cell response and aggregation secondary mediators (like TxA2) are needed (Atkinson *et al.*, 2003).

NP-313 was also found to inhibit platelet aggregation caused by thapsigargin and

A23187 that bypass receptor-mediated processes. The mechanism of calcium release from the IP3-sensitive internal stores is well characterized. On the other hand, $Ca^{2+}$ entry is thought to occur predominantly as a consequence of stored $Ca^{2+}$ depletion and has been referred to as SOCC or capacitative $Ca^{2+}$entry (Putney *et al.*, 2001). However, another $Ca^{2+}$channel has also been reported to exist in human platelet membrane, and mediates $Ca^{2+}$entry in a SOCE-independent mechanism (Hassock *et al.*, 2002). It is noteworthy that we found that NP-313, even used at high concentration (80 μM), did not inhibit the residual 30% of $[Ca^{2+}]$i mobilization upon thrombin stimulation, and a residual aggregation was also observed (Figure 5B and Figure 2). These results suggest that NP-313 exerts differential effect on the platelet $Ca^{2+}$ channels or key elements involved in activation of $Ca^{2+}$ channels. NP-313 has no effect on intracellular $Ca^{2+}$ mobilization by thrombin in the absence of extracellular $Ca^{2+}$, indicating that it does not interfere with signaling elements implicated in the regulation of the phosphoinositide breakdown mediated by thrombin. On the contrary, after depletion of the intracellular $Ca^{2+}$stores, NP-313 concentration-dependently inhibits thrombin-induced $Ca^{2+}$ influx. Thapsigargin, a tool used to study SOCC, effectively elevated intracellular $[Ca^{2+}]$i by inhibiting the calcium ATPase pump of the dense tubular system without increasing the level of $IP_3$ (Pozzan *et al.*, 1994). On the other hand, DAG analog OAG has been used to investigate SOCC-independent $Ca^{2+}$ entry channel, attributable to directly activating this channel independent of PKC (Hofmann *et al.*, 1999). In this report, thapsigargin-induced cytosolic $Ca^{2+}$mobilization was completely inhibited by NP-313 at a concentration of 20μM (Figure 4), which has no influence on OAG-mediated intracellular $Ca^{2+}$ mobilization in platelets (Figure 7A and B), suggesting that NP-313 selectively inhibits SOCC rather than blocking SOCC-independent or intracellular $IP_3R$-$Ca^{2+}$ channels, in

concert with the results that it did not inhibit $IP_3$-mediated $Ca^{2+}$ mobilization induced by thrombin or A23187. The identity of the store-operated $Ca^{2+}$ channels in platelet remains elusive. Among them, Orai 1 has been found to be expressed in human platelets, and its function has been linked to thrombus formation *in vitro* as well as *in vivo* (Braun *et al.*, 2009). Orai 1 belongs to a family of channels that have 4 putative transmembrane domains and contain the pore-forming subunits of SOCE channels. Orai 1 deficient platelets showed an almost absence of cation entry after store depletion by thapsigargin and much reduced $Ca^{2+}$ entry when stimulated by agonists. NP-313 has a preferential inhibitory activity on collagen-induced platelet aggregation and cytosolic $Ca^{2+}$-mobilization than thrombin-induced ones, consisting with the recent observation that Orai 1-mediated $Ca^{2+}$ entry is particularly essential for collagen receptor, a GPVI-ITAM-mediated cell activation (Authi, 2009). The molecular nature of the Ca2+-sensor(s) and the interplay between Ca2+-and DAG-driven signal pathways is less clearly defined. Recent work of Bergmeier et al. showed that $Ca^{2+}$ and DAG-regulated guanine nucleotide exchange factor I (Cal DAG-GEFI), played a role in regulating $Ca^{2+}$-dependent activation of integrin in platelets (Cifuni *et al.*, 2008; Stefanini *et al.*, 2009). However, the question remains open regarding whether NP-313 acts primarily on this SOCC or indirectly through PKC inhibition deserves further investigation.

Intravenous administration of NP-313 (4-8μg $g^{-1}$) dose-dependently prevented thrombus formation caused by the irradiation of mesenteric vessel of the fluorescein sodium-pretreated mice, however, it did not significantly prolong bleeding time, implying that NP-313 preferentially inhibits thrombus formation with slight effect on haemostasis.

In conclusion, this study reported NP-313, a 1,4-naphthoquinone derivative, in

possessing the dual inhibition of thromboxane $A_2$ synthase and SOCC. This compound does not affect cyclic nucleotide level, and causes no LDH release. The unique inhibitory effects of NP-313 on platelet aggregation may provide a new strategy for treatment of platelet-dependent thrombosis. NP-313 may become a new pharmacological tool for investigating the signal transduction pathways in regulating $[Ca^{2+}]_i$ through platelet SOCC.

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## Legends for Figures

**Figure 1. Chemical structure of NP-313**

**2-acetylamino-3-chloro-1,4-naphthoquinone**

**Figure 2 Effect of NP- 313 on washed human platelet aggregation.** Washed human platelets were incubated with DMSO (vehicle control) or NP- 313 at 37°C for 3min, and then collagen(10μg/ml), thapsigargin (0.1μM), thrombin (0.1U/ml), or A23187 (8μM) was added to trigger platelet aggregation. Data are presented as the means ± SEM (mean ± SEM, n=5).

**Figure 3. Effect of NP- 313 on collagen- and thrombin -induced P-selectin expression and $TXB_2$ formation in human platelets.** (A) Washed human platelets were preincubated with DMSO (vehicle control) or NP-313 for 3 min and then treated with collagen (10μg $ml^{-1}$) or thrombin (0.1U $ml^{-1}$) in the presence of FITCconjugated anti-CD62P for 15 min at room temperature. Data are presented as the means ± SEM (*n*=3). * *P*<0.05 and ***P*<0.01 as compared with the corresponding stimulus control. (B) Platelet suspensions were preincubated with DMSO (vehicle control) or NP-313 for 3min at 37°C, and then collagen (10μg $mL^{-1}$) (left) or thrombin (right) was added to trigger $TXB_2$ formation. $TXB_2$ formation was terminated by the addition of EDTA (2mM) and indomethacin (50μM).

**Figure 4 Effects of NP- 313 on platelet cGMP and cAMP levels.** Washed platelets

were incubated at 37 °C for 2min with NP-313, $PGE_1$, or NTG. The reaction was stopped, platelets were then pelleted and the supernatants were assayed for cGMP and cAMP by enzyme immunoassay. Values are presented as mean±S.E.M. (n=4). *P<0.05 as compared with the basal control.

**Figure 5 Effect of NP-313 on collagen-, thrombin-, and A23187–induced intracellular [$Ca^{2+}$]i mobilization in human platelets.** Calcium (1mM) was added to the platelet suspension 30s before data collection started (zero time). Various concentrations of NP-131 were added to the platelets at 10s, and collagen (10μg $ml^{-1}$), thrombin (0.1U $ml^{-1}$), or A23187 (8μM) was added at 30s. The traces shown in left panel are from a representative experiment; similar results were obtained from three separate experiments and average data are presented in the right panel (A, B, and C). *$P$<0.05 as compared with the corresponding stimulus control.

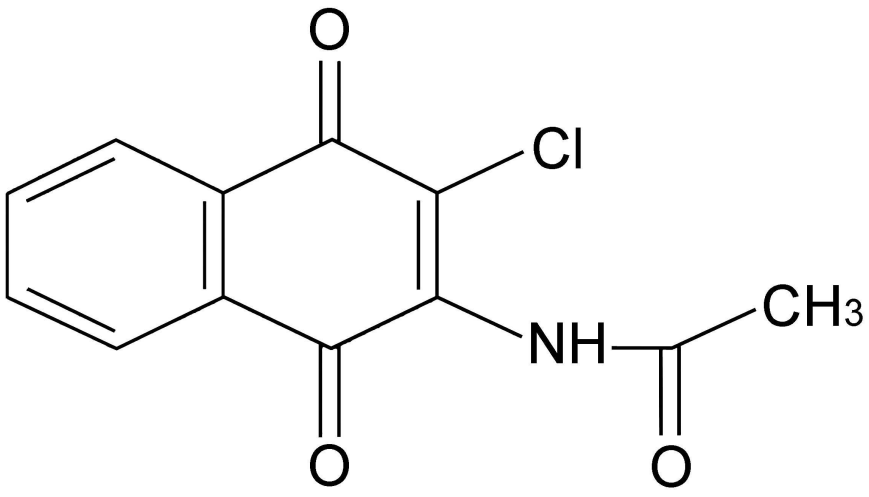
**Figure 6 Effect of NP-313 on thrombin- and A23187 –induced $Ca^{2+}$ release from internal stores and influx of extracellular $Ca^{2+}$ in human platelets.** Fura-2-loaded platelets were suspended in Tyrode's buffer containing CP (10mM), CPK (1U $ml^{-1}$), and indomethacin (100μM). Effect of NP-313 on the release of the internally stored calcium in thrombin (0.1U $ml^{-1}$; A, left) and A23187 (8 μM; B, left) stimulated platelets. External calcium was not added to the platelet suspension, 1mM EGTA was also added 30 s prior to data collection (zero time). After 10s, various concentrations of NP-131 was added; 20s later thrombin or A23187 was added.**;** effect of NP-313 on calcium influx initiated after mobilization of intracellular $Ca^2$+ by thrombin (0.1U $ml^{-1}$; A, right) and A23187 (8μM; B, right), Platelets were incubated in the absence of extracellular $Ca^{2+}$ and in the presence of 1mM EGTA. Thrombin or A23187 was

added at 10s and various concentrations of NP-131were added to the platelets at 90 s. At 110s, when $[Ca^{2+}]i$ was declining because of depletion of intracellular stores, 2.0mM $Ca^{2+}$ was added to the platelets. A representative of three experiments is shown.

**Figure 7 Effect of NP-313 on thapsigargin and OAG- induced $Ca^{2+}$ entry.** Fura-2-loaded platelets were suspended in Tyrode's buffer containing CP (10mM), CPK (1U $ml^{-1}$), and indomethacin (100µM). Calcium (1mM) was added to the platelets 30s before data collection was started.Various concentrations of NP-131 are added at 10s, and 0.1µM thapsigargin (A) or 60µM OAG (B) was added at 30s. A representative of three experiments is shown.

Figure 8 **Effect of NP-313 on *in vivo* thrombosis and bleeding time in a mouse model.** (A) Effect of NP-313 on the time to occlusion (TTO) measured 5min after IV administration upon light irradiation of mesenteric venules of mice pretreated with fluorescein sodium. Data represent the mean±S.E.M. (n= 6). (B) Effect of NP-313 on tail bleeding time of mice measured 5min after IV administration. Data represent the mean±S.E.M. (n=6). **P* <0.05 and ***P* <0.01 as compared with the vehicle control, one way ANOVA (Dunnett's post hoc test).

Statement of conflicts of interest: N/A




Chemical structure of NP-313.
2-acetylamino-3-chloro-1,4-naphthoquinone

112x67mm (600 x 600 DPI)