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RESEARCH PAPER

NP-313, 2-acetylamino-3-chloro-1,4-naphthoquinone, a novel antithrombotic agent with dual inhibition of thromboxane A_2 synthesis and calcium entry

Heng-Lan Kuo[1], Jin-Cherng Lien[2], Chien-Hsin Chang[1], Ching-Hu Chung[3], Sheng-Chu Kuo[2], Chun-Chieh Hsu[1], Hui-Chin Peng[1] and Tur-Fu Huang[1]

[1]*Graduate Institute of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan,* [2]*Graduate Institute of Pharmaceutical Chemistry, China Medical University, Taichung, Taiwan, and* [3]*Institute of Pharmacology and Toxicology, Tzu Chi University, Hualien, Taiwan*

Correspondence
Tur-Fu Huang, Graduate Institute of Pharmacology, College of Medicine, National Taiwan University, No.1, Sec. 1, Jen-Ai Road, Taipei, Taiwan. E-mail: turfu@ntu.edu.tw; Dr Jin-Cherng Lien, Graduate Institute of Pharmaceutical Chemistry China Medical University, No.91 Hsueh-Shih Road, Taichung, Taiwan. E-mail: jclien@mail.cmu.edu.tw

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BACKGROUND AND PURPOSE

1,4-Naphthoquinones exhibit antiplatelet activity both *in vivo* and *in vitro*. In the present study, we investigated the antiplatelet 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 and P-selectin expression and examined several enzymatic activities. Furthermore, we used the irradiated mesenteric venules in fluorescein sodium–treated mice to monitor the antithrombotic effect of NP-313 *in vivo*.

KEY RESULTS

NP-313 concentration-dependently inhibited human platelet aggregation induced by collagen, arachidonic acid, thapsigargin, thrombin and A23187. 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 10 μM inhibited cyclooxygenase, thromboxane A_2 synthase, and protein kinase Cα, whereas it did not affect phospholipase A_2 or phospholipase C activity. In the presence of indomethacin and an adenosine 5-diphosphate 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-operated calcium channel but had no effect on Ca^{2+} influx through store-independent calcium channel evoked by the diacylglycerol analogue 1-oleoyl-2-acetyl-sn-glycerol. Nevertheless, it had little effect on cyclic AMP and cyclic GMP levels. Also, intravenously administered NP-313 dose-dependently inhibited the thrombus occlusion of the irradiated mesenteric vessels of fluorescein-pretreated mice.

CONCLUSIONS AND IMPLICATIONS

Taken together, these results indicate that NP-313 exerts its antithrombotic activity through dual inhibition of thromboxane A_2 synthesis and Ca^{2+} influx through SOCC.

Abbreviations

AA, arachidonic acid; CP, creatine phosphate; CPK, creatine phosphokinase; $cPLA_2$, cytosolic phospholipase A_2; DAG, diacylglycerol; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetra

acetic acid; Fura 2-AM, Fura-2 acetoxymethyl ester; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-triphosphate receptors; LDH, lactate dehydrogenase; NP-313, 2-acetylamino-3-chloro-1,4-naphthoquinone; NTG, nitroglycerin; OAG, 1-oleoyl-2-acetyl-sn-glycerol; PIP_2, phospholipid phosphatidylinositol 4,5- bisphosphate; PLA_2, phospholipase A_2; PLC, phospholipase C; PMA, phorbol-12-myristate-13-acetate; PRP, platelet-rich plasma; SOCC, store-operated Ca^{2+} channels; SOCE, store-operated Ca^{2+} entry; TXA_2, thromboxane A_2

Introduction

Myocardial infarction and ischaemic 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 and Topol, 2003; Jackson and Schoenwaelder, 2003). 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 adenosine 5-diphosphate (ADP) and thromboxane A_2 (TXA_2), reinforcing platelet adhesion and aggregation (Walsh, 2004).

The major intracellular stimulus involved in platelet aggregation is an increase in cytosolic free calcium concentration (Rink and Sage, 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 *et al.*, 2003; Berridge, 2004). The key elements involved in Ca^{2+} signalling 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 inositol 1,4,5-triphosphate receptors (IP_3R) 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.* (2002) have suggested that another SOCE-independent mechanism also contributes to Ca^{2+} entry in platelets and is activated by DAG. It has been reported that agents with inhibitory effects on the cytosolic Ca^{2+} mobilization in platelets may suppress platelet aggregation and thrombus growth (Kang *et al.*, 2001; Jin *et al.*, 2004; 2005b; Lee *et al.*, 2005), whereas few studies have further investigated whether these agents have a differential effect on the Ca^{2+} channels involved in modulating intracellular Ca^{2+} mobilization in human platelets.

The stimulation of platelets with different stimuli results in early activation of Ca^{2+}-dependent cytosolic phospholipase A_2 (PLA_2), which hydrolyzes membrane phospholipids leading to arachidonic acid (AA) release. The liberated AA is metabolized via the cyclooxygenase (COX) pathway to form prostaglandin endoperoxide, PGH_2, which sequentially, by the action of prostacyclin synthase and TXA_2 synthase, is converted to prostaglandins (Walsh, 2004; Jin *et al.*, 2005b) and TXA_2 (Arita *et al.*, 1989) respectively.

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. Nevertheless, there is evidence indicating that there are subpopulations that do not respond to the antithrombotic action of aspirin (Szczeklik *et al.*, 2005). Thus, the interest in developing other antithrombotic drugs such as TXA_2 modulators raised (Dogne *et al.*, 2004; 2006).

Compounds with the chemical structure of a 1,4-naphthoquinone backbone have been shown to have a wide variety of pharmacological effects including anticancer and antiplatelet activities (Rodriguez *et al.*, 1995; Lien *et al.*, 2002; Verma, 2006). In the present study, 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 induced by various stimulators. We investigated its antiplatelet effects and found that in addition to having inhibitory activity on TXA_2 synthesis, it also showed an inhibitory effect on SOCC involved in modulating intracellular Ca^{2+} mobilization. Furthermore, i.v. administration of NP-313 dose-dependently protected mice against platelet plug formation with only a slight effect on haemostasis.

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), BSA, PGE_1, nitroglycerin (NTG), dimethyl sulphoxide (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), creatine phosphokinase

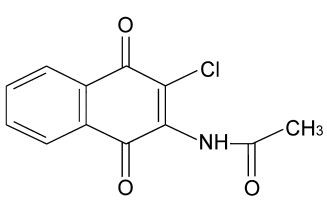

Figure 1
Chemical structure of NP-313, 2-acetylamino-3-chloro-1,4-naphthoquinone.

(CPK) and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma Chem. (St. Louis, MO, USA). AA, PGH_2 and $cPLA_2$ assay kit was from Cayman Chemical (Ann Arbor, MI, USA). Fluorescein isothiocyanate–labelled monoclonal antibody to P-selectin (fluorescein isothiocyanate (FITC)-labelled anti-CD62P) was obtained from Becton-Dickson (Franklin Lakes, NJ, USA). Cyclic AMP (cAMP), cyclic GMP (cGMP) and TXB_2 assay kit were purchased from Amersham (Buckinghamshire, HP, UK). Lactate dehydrogenase (LDH) assay kit was purchased from Randox (Crumlin, Co. Antrim, UK). Drug and channel nomenclature conforms to the *British Journal of Pharmacology Guide to Receptors and Channels* (Alexander *et al.*, 2009).

Male ICR mice were used in animal studies. The animals were maintained on a 12 h 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 experiments 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). Blood samples, freshly obtained from healthy volunteers who had taken no medications during the preceding 2 weeks, were treated with acid citrate/dextrose in a volume ratio of 9:1. After centrifugation at 120× *g*, 25°C for 9 min, platelet-rich plasma (PRP) was transferred into another plastic tube and incubated with heparin (6.4 $U \cdot mL^{-1}$) as well as PGE_1 (1 μM). Platelets were spun down by centrifugation at 500× *g*, 25°C for 8 min and subsequently washed twice with Tyrode's solution (NaH_2PO_4, 0.4 mM; NaCl, 136.9 mM; KCl, 11.9 mM; $NaHCO_3$, 11.9 mM; $CaCl_2$, 2 mM; $MgCl_2$, 2.1 mM; glucose, 11.1 mM; BSA, 3.5 $mg \cdot mL^{-1}$; pH 7.35–7.4). The washed platelets were finally suspended in Tyrode's solution, and the platelet count was adjusted to 3.75×10^8 $platelets \cdot mL^{-1}$. For PRP 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, Buffalo, NY, USA) at 37°C while being stirred (900 rpm) (Born and Cross, 1963). Washed human platelet suspension was prewarmed to 37°C for 2 min, and NP-313 was added 3 min before the addition of platelet aggregation activator. Platelet aggregation, measured as the change in light transmission, was recorded for 8 min after the addition of aggregation agonist. The extent of inhibition of platelet aggregation is expressed as percentage of inhibition using the following equation: $X\ (\%) = (1 - B/A) \times 100\%$, where A is the maximum aggregation rate of vehicle-treated platelets, and B is the maximum aggregation of sample-treated platelets.

Flow cytometric analysis of P-selectin expression

The washed platelet suspension (3×10^8 mL^{-1}) containing tirofiban (100 $ng \cdot mL^{-1}$) 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-labelled anti-CD62P in the dark for 15 min at room temperature. The platelet suspension was immediately assayed by fluorescence-activated cell sorter (FACScan System, Becton-Dickinson, San Jose, CA, USA) 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

This was performed according to a previously described method (Chang *et al.*, 1998). The platelet suspension (3×10^8 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 (50 μM) and EDTA (2 mM) were added to terminate the reaction. The platelet suspensions were centrifuged at 14 000× *g* for 2 min, and the TXB_2 concentrations were measured using a competitive enzyme immunoassay (EIA) kit according to the instructions of the manufacturer.

TXA_2 synthase activity assay

The TXA_2 synthase activity was assayed as previously described (Son *et al.*, 2004). Briefly, indomethacin (50 μM)-pretreated platelet suspension was incubated with NP-313 imidazole or DMSO at 37°C for 3 min prior to the addition of 5 μM PGH_2. At 5 min after the addition of PGH_2, the incubation was terminated by the addition of cooling EGTA (2 mM) and centrifuged at 12 000× *g* at 4°C for 4 min. The amount of TXB_2 in the supernatant was assayed by a commercial EIA kit according to the manufacturer's instructions. TXA_2 synthase activity is reflected by the production of TXB_2.

Biochemical assays of COX-1, PLA_2, PLC and PKCα activities

These enzymatic assays were performed by MDS Pharma Services utilizing standard protocols. Briefly, COX-1 (human platelet) activity was EIA quantified by measuring the PGE_2 level converted from AA. PLA_2 activity (pig pancreas) was measured by quantifying [^{14}C]-oleate release from 1-palmitoyl-2-[1-^{14}C] oleoyl-l-3-phosphatidylcholine. PLC activity was determined by measuring the 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 phosphatidylserine, Mg^{2+} and Ca^{2+}.

Estimation of cAMP and cGMP formation

Platelet suspension (3×10^8 mL^{-1}) was warmed to 37°C for 1 min, then NP-313, PGE_1 or NTG was added and incubated for 2 min at 37°C. Incubation was stopped by the addition of 10 mM EDTA and followed by boiling the mixture for 5 min. After cooling to 4°C, the precipitated protein was collected as sediment after centrifugation. The supernatant was used to determine the cAMP and cGMP contents by EIA kits following acetylation of the samples as described by the manufacturer.

Measurement of intracellular Ca^{2+}mobilization

After centrifuging platelet-rich plasma at 500× *g*, 25°C for 8 min, isolated platelets were resuspended in Ca^{2+}-free Tyrode's solution. Platelet suspension was protected from light and incubated with Fura 2-AM (5 μM) at 37°C for 30 min. Human platelets were then prepared as previously described. The platelet count was adjusted to 3×10^8 mL^{-1} platelets. Just before $[Ca^{2+}]i$ measurements were performed, Ca^{2+} was added back to platelets to a final concentration of 1.0 mM, then NP-313, collagen, thrombin, AA, 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 10 mM 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.

LDH assay

The LDH activity released was measured spectrophotometrically by recording the decrease in the optical density of beta-nicotinamide adenine dinucleotide at 340 nm, as previously described (Jin *et al.*, 2004). NP-313 (80 μM) was incubated with platelet suspension for 60 min and then centrifuged for 4 min at 1312× *g*. The LDH activity in the platelet suspension and cellular LDH activity from platelets, which was lysed with 1% Triton-X 100, were determined. Total LDH activity was the summation of both released and cellular LDH activities. The released LDH activity was expressed as a percentage of total LDH activity.

Fluorescein sodium–induced platelet thrombus formation in mesenteric venules of mice

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

Tail bleeding time in mice

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

Ex vivo *mouse platelet aggregation*

Male ICR mice (20–30 g) were treated i.v. with various doses of NP-313 or vehicle before being anaesthetizied with sodium pentobarbital (50 $mg \cdot kg^{-1}$ i.p.). Blood samples were collected at 5 min by intracardiac puncture after drug treatment. PRP was obtained by centrifuging the blood sample at 180× *g* for 4 min. PPP was obtained by centrifugation of remaining blood at 2000× *g* for 4 min. PRP was adjusted to 3.75×10^8 platelets·mL^{-1} with PPP. Platelet aggregation was measured as described above.

Statistical analysis

The experimental results are expressed as the means ± SEM. 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 (10 $\mu g \cdot mL^{-1}$), AA (200 μM), thapsigargin (0.1 μM), thrombin(0.1 $U \cdot mL^{-1}$) and A23187 (8 μM) induced platelet aggregation of washed human platelets in a concentration-dependent manner, with the following IC_{50} values: 1.7 ± 0.1, 2.4 ± 0.2, 3.8 ± 0.2, 7.7 ± 0.2 and 20.0 ± 0.3 μM 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-, thrombin- and AA-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

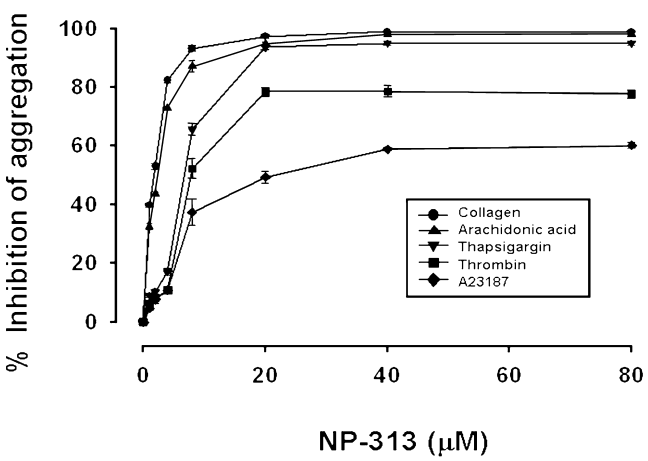

Figure 2

Effect of NP- 313 on aggregation of washed human platelets. Washed human platelets were incubated with DMSO (vehicle control) or NP-313 at 37°C for 3 min, and then collagen (10 $\mu g \cdot mL^{-1}$), arachidonic acid (AA; 200 μM), thapsigargin (0.1 μM), thrombin (0.1 $U \cdot mL^{-1}$) or A23187 (8 μM) was added to trigger platelet aggregation. Data are presented as the mean ± SEM (n = 5).

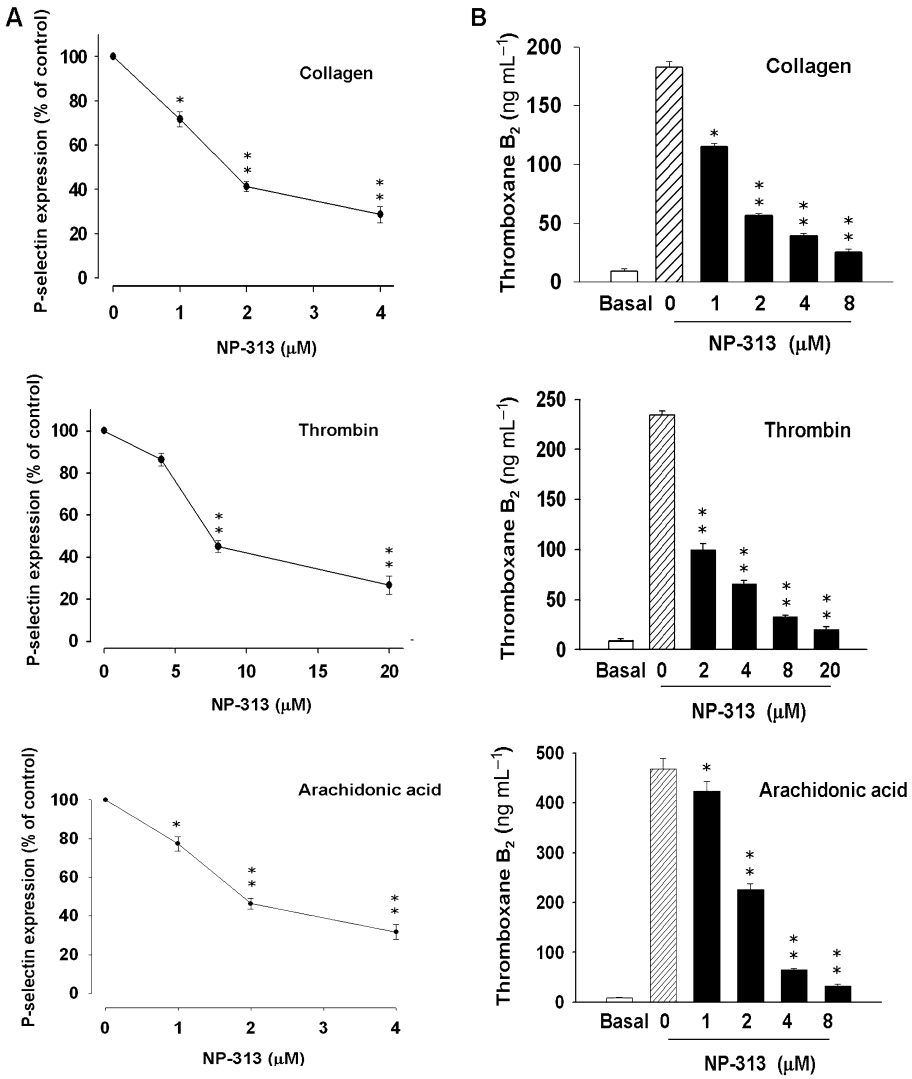

Figure 3

Effect of NP-313 on collagen-, thrombin- and arachidonic acid (AA)-induced P-selectin expression and thromboxane B_2 (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 (10 μg·mL^{-1}), thrombin (0.1 U·mL^{-1}) or AA (200 μM) in the presence of FITC-conjugated 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, one-way ANOVA (Dunnett's *post hoc* test). (B) Platelet suspensions were preincubated with DMSO (vehicle control) or NP-313 for 3 min at 37°C, and then collagen (10 μg·mL^{-1}), thrombin (0.1 U·mL^{-1}) or AA (200 μM) was added to trigger TXB_2 formation. TXB_2 formation was terminated by the addition of EDTA (2 mM) and indomethacin (50 μM). *$P < 0.05$ and **$P < 0.01$ as compared with the corresponding stimulus control, one-way ANOVA (Dunnett's *post hoc* test).

P-selectin expression (Figure 3A), and also TXB_2 generation induced by collagen or thrombin (Figure 3B).

Effect of NP-313 on COX-1, TXA_2 synthase, phospholipase PLA_2 and PLC activity

It has been reported that some 1,4-naphthoquinone derivatives inhibit TXA_2 synthase (Jin *et al.*, 2005a,b). NP-313 inhibited COX-1 and TXA_2 synthase concentration-dependently with IC_{50} values of 1.5 ± 0.4 and 3.9 ± 0.3 μM respectively (Figure 4A,B). Consistent with these data, NP-313 concentration-dependently inhibited TXB_2 formation in platelets stimulated by collagen, thrombin and AA (Figure 3B). In contrast, NP-313 at 10 μM had little inhibitory effect on $cPLA_2$ and PLC activities (data not shown).

Effect of NP-313 on cyclic nucleotide levels in human platelets

1,4-Naphthoquinone derivatives also have been shown to inhibit platelet aggregation by suppression of intracellular calcium mobilization; this is mediated by the inhibition of IP_3

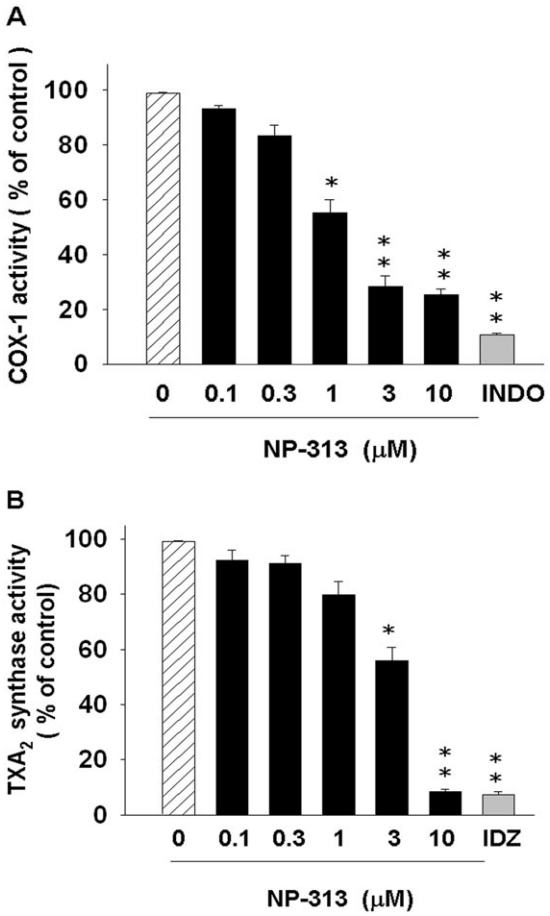

Figure 4

Effect of NP-313 on cyclooxygenase-1 (COX-1) and thromboxane A_2 (TXA_2) synthase activities. (A) The human platelet COX-1 in a reaction buffer was treated with NP-313, indomethacin (INDO; 0.3 μM) or DMSO (vehicle control) at 37°C for 15 min, and then 100 μM AA was added for 15 min. COX enzyme activities were reflected by the amount of PGE_2 produced. Data are expressed as % of PGE_2 formed in the presence of assay buffer, and data represent the mean ± SEM (n = 3). $^{**}P < 0.01$ as compared with the vehicle control, one-way ANOVA (Dunnett's *post hoc* test). (B) After preincubation with indomethacin (50 μM) at 37°C for 2 min, platelet suspensions containing NP-313, imidazole (IDZ; 50 mM) or DMSO (vehicle control) were further incubated for 3 min, and then 5 μM PGH_2 was added for 5 min. Data are expressed as % of the TXB_2 formed in the presence of assay buffer, and data represent the mean ± SEM (n = 3) $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with the vehicle control, one-way ANOVA (Dunnett's *post hoc* test).

production or elevation of cAMP level (Jin *et al.*, 2004; 2005b). 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 NTG and PGE_1 significantly increased cGMP formation and cAMP respectively; however, NP-313 affected neither cAMP levels nor cGMP levels as compared with that of resting platelets (Figure 5).

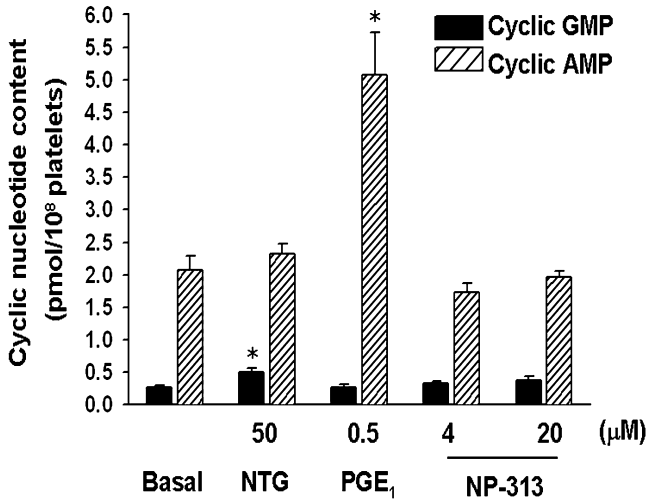

Figure 5

Effects of NP- 313 on platelet cGMP and cAMP levels. Washed platelets were incubated at 37°C for 2 min 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 (EIA). Values are presented as mean ± SEM (n = 4). $^{*}P < 0.05$ as compared with the basal control, one-way ANOVA (Dunnett's *post hoc* test).

Effect of NP-313 on collagen-, thrombin-, AA- and A23187 –induced intracellular Ca^{2+} mobilization in human platelets

Treatment of the Fura-2-loaded platelet suspension with NP-313 inhibited collagen-, thrombin-, AA- and A23187-evoked increase in $[Ca^{2+}]i$ in a concentration-dependent manner (Figure 6). NP-313 almost completely inhibited the elevation of the $[Ca^{2+}]i$ in response to collagen and AA. 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 80 μM.

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 (10 mM), CPK (1 U·mL^{-1}) and indomethacin (100 μM), to exclude the effects of ADP and TXA_2 on $[Ca^{2+}]i$ 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 1 mM EGTA, and followed by the addition of thrombin, the increase in $[Ca^{2+}]i$ was substantially smaller compared with Ca^{2+} influx (Figure 6, left). NP-313, even used at 80 μM, failed to inhibit thrombin-induced release of intracellular stored Ca^{2+}. Next, after agonist-mediated Ca^{2+} release from intracellular store, the effect of

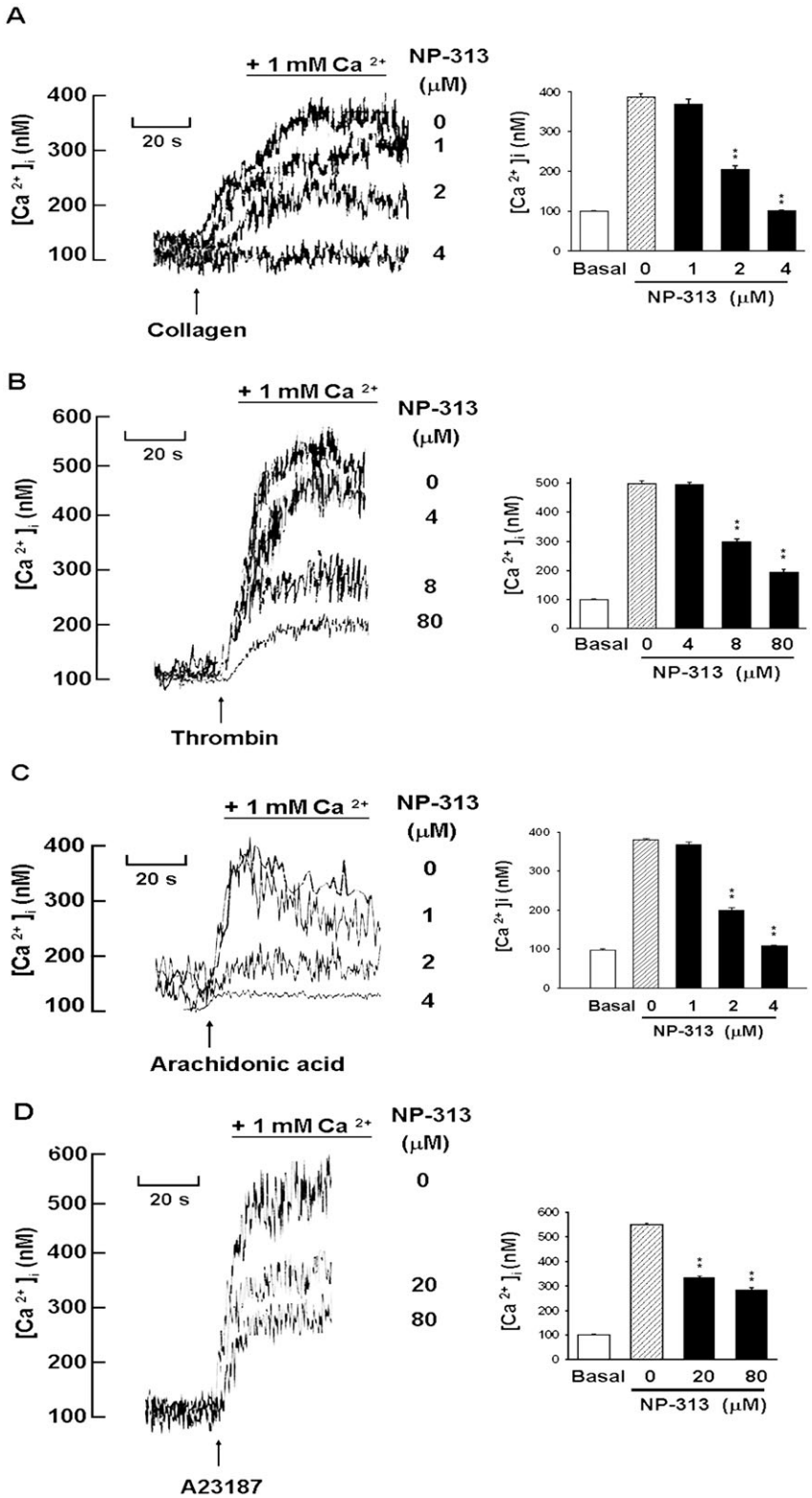

Figure 6

Effect of NP-313 on collagen-, thrombin- and A23187–induced intracellular $[Ca^{2+}]i$ mobilization in human platelets. Calcium (1 mM) was added to the platelet suspension 30 s before data collection started (zero time). Various concentrations of NP-313 were added to the platelets at 10 s, and collagen (10 $\mu g \cdot mL^{-1}$), thrombin (0.1 $U \cdot mL^{-1}$) or A23187 (8 μM) was added at 30 s. 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, one-way ANOVA (Dunnett's *post hoc* test).

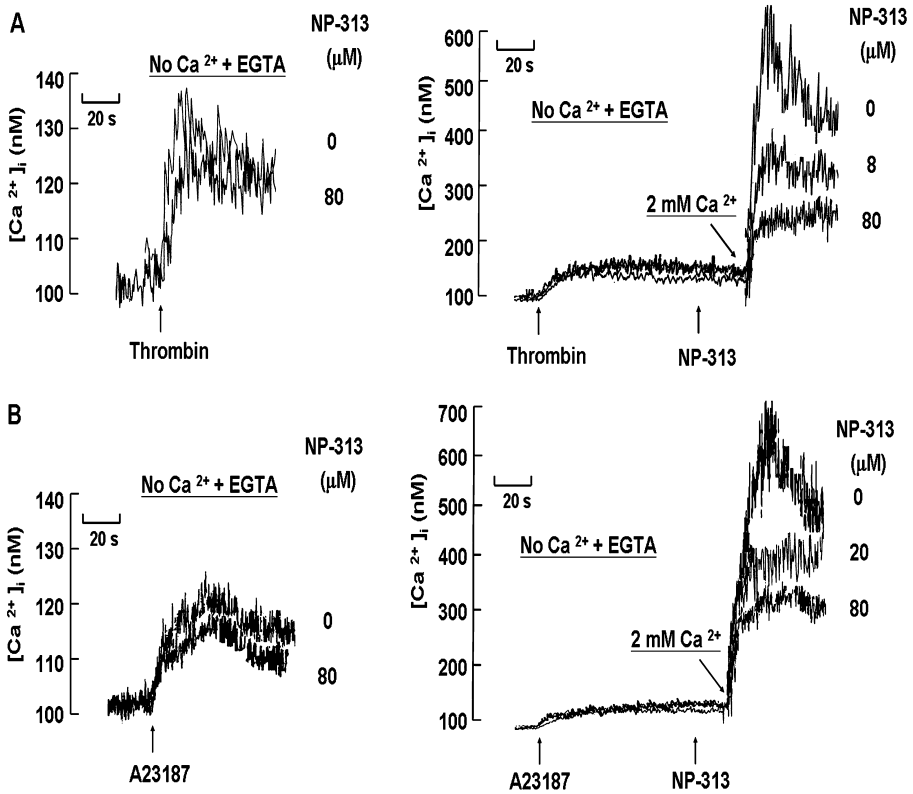

Figure 7

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 creatining phosphate (CP; 10 mM), creatining phosphatase (CPK; 1 U·mL^{-1}) and indomethacin (100 μM). Effect of NP-313 on the release of the internally stored calcium in thrombin (0.1 U·mL^{-1}; A, left) and A23187 (8 μM; B, left) stimulated platelets. External calcium was not added to the platelet suspension; 1 mM EGTA was added 30 s prior to data collection (zero time). After 10 s, various concentrations of NP-313 were added; 20 s later, thrombin or A23187 was added. Effect of NP-313 on calcium influx initiated after mobilization of intracellular Ca^{2+} by thrombin (0.1 U·mL^{-1}; A, right) and A23187 (8 μM; B, right). Platelets were preincubated in the absence of extracellular Ca^{2+} and in the presence of 1 mM EGTA. Thrombin or A23187 was added at 10 s, and various concentrations of NP-313 were added to the platelets at 90 s. At 110 s, when $[Ca^{2+}]$i was declining because of depletion of intracellular stores, 2 mM Ca^{2+} was added to the platelets. A representative of three experiments is shown. EGTA, ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetra acetic acid.

NP-313 on Ca^{2+} influx was assessed by the addition of 2 mM Ca^{2+}. In contrast, the increase in $[Ca^{2+}]$i induced 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 80 μM.

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 (10 mM), CPK (1 U·mL^{-1}) and indomethacin (100 μM), to exclude the effects of ADP and TXA_2 on $[Ca^{2+}]$i elevation during agonist-induced stimulation. The data in Figure 7A show 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 1 mM EGTA, and followed by the addition of thrombin, the increase of $[Ca^{2+}]$i was substantially smaller compared with Ca^{2+} influx (Figure 7A, left). NP-313, even used at 80 μM, 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 2 mM Ca^{2+}. In contrast, the increase in $[Ca^{2+}]$i by thrombin in the presence of extracellular Ca^{2+} was concentration-dependently inhibited by NP-313 (Figure 7A, 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. Furthermore, the influence of NP-313 on A23187-evoked Ca^{2+} mobilization was similar to that

observed with thrombin (Figure 7B). Nevertheless, A23187 can diffuse through membranes and directly increase $[Ca^{2+}]i$ from intracellular stores without increasing IP_3 and through extracellular Ca^{2+}-influx, which is also partly due to SOCE activation after Ca^{2+} depletion of ER (Fuse *et al.*, 2001; Kunzelmann-Marche *et al.*, 2001).

Effect of NP-313 on thapsigargin- and OAG-induced Ca^{2+} entry

There are mainly two Ca^{2+} entry channels on platelet plasma membrane, that is 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. It is well known that thapsigargin mediates Ca^{2+} influx through SOCC by inhibiting the sarcoplasmic/ endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump without increasing the level of IP_3, thus promoting a loss of Ca^{2+} via a 'leak' process in the ER (Pozzan *et al.*, 1994; Sage, 1997). This Ca^{2+}-depleted condition of the ER then causes an increase in Ca^{2+} influx through SOCC. Figure 8A shows that NP-313 markedly elicited a concentration-dependent inhibition of thapsigargin-mediated Ca^{2+} mobilization. However, because NP-313 had no effect on the thapsigargin-evoked release of Ca^{2+} from the intracellular stores in the absence of extracellular Ca^{2+} (data not shown), the effect of NP-313 to inhibit the Ca^{2+} influx was most probably caused by a more direct inhibitory effect on SOCC itself and not indirectly by preventing the loss of Ca^{2+} from the ER. In contrast, NP-313, at concentrations up to 80 μM, had no effect on the moderate and slow $[Ca^{2+}]i$ increase evoked by a DAG analogue, OAG (Figure 8B), 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 (80 μM) 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 of mesenteric venules induces 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 TTO. In the vehicle-treated group, i.v. application of 12.5 $\mu g \cdot g^{-1}$ fluorescein sodium induced an average TTO of 95.0 ± 7.5 s ($n = 6$). However, after i.v. administration at 4, 8 and 16 $\mu g \cdot g^{-1}$, NP-313 significantly prolonged the average TTO to 127.0 ± 12.1, 188.3 ± 25.7 and 242.5 ± 16.4 s respectively ($n = 6$; $P < 0.01$ for each, one-way ANOVA, Dunnett's *post hoc* test).

As shown in Figure 9B, NP-313 did not affect the bleeding time in mice compared with vehicle-treated mice after i.v. administration at effective antithrombotic dose of 4 and

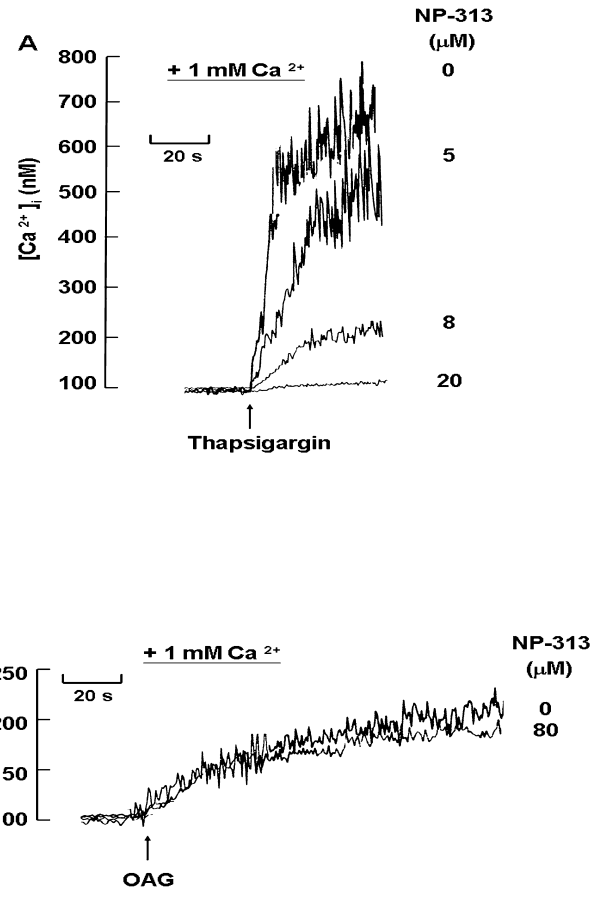

Figure 8

Effect of NP-313 on thapsigargin- and OAG- induced Ca^{2+} entry. Fura-2-loaded platelets were suspended in Tyrode's buffer containing creatine phosphate (CP; 10 mM), creatine phosphokinase (CPK; 1 $U \cdot mL^{-1}$) and indomethacin (100 μM). Calcium (1 mM) was added to the platelets 30 s before data collection was started plus various concentrations of NP-313 were added at 10 s, and 0.1 μM thapsigargin (A) or 60 μM OAG (B) was added at 30 s. A representative of three experiments is shown. OAG, 1-oleoyl-2-acetyl-sn-glycerol.

8 $\mu g \cdot g^{-1}$. In contrast, aspirin caused a marked prolongation of tail bleeding time when an effective antithrombotic dose of 200 $\mu g \cdot g^{-1}$ was administered i.v. However, NP-313 slightly prolonged bleeding time when administered at 16 $\mu g \cdot g^{-1}$. Furthermore, we evaluated the *ex vivo* antiplatelet action of NP-313 on PRP. As shown in Figure 9C, upon i.v. administration of NP-313 at 4, 8 and 16 $\mu g \cdot g^{-1}$ for mice, NP-313 inhibited *ex vivo* platelet aggregation of PRP caused by collagen (10 $\mu g \cdot mL^{-1}$) 5 min after drug administration.

Discussion

The present study has shown that NP-313 inhibits collagen- and thrombin-induced platelet aggregation and platelet activation, such as α-granule secretion, TXA_2 formation and intracellular Ca^{2+} mobilization in a concentration-dependent manner. However, NP-313 preferentially inhibited

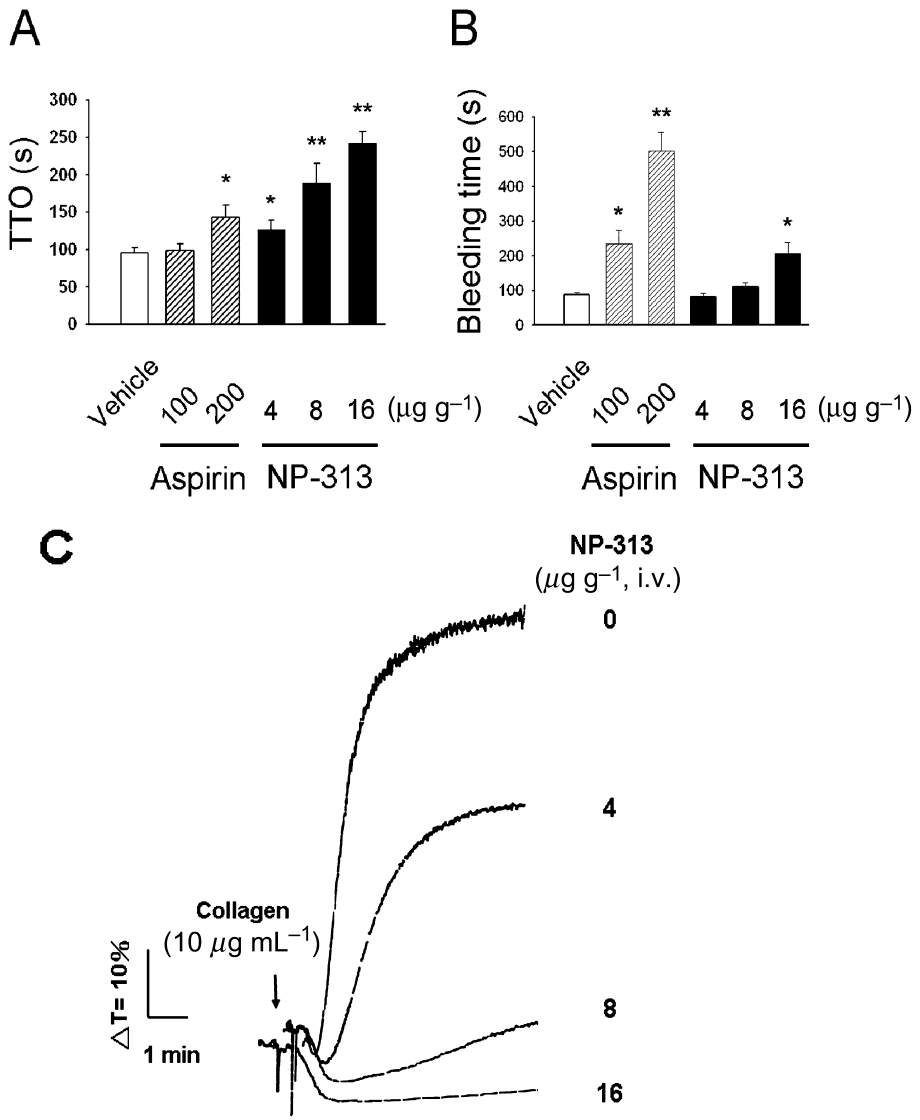

Figure 9

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 5 min after its i.v. administration upon light irradiation of mesenteric venules of mice pretreated with fluorescein sodium. Data are presented as the mean ± SEM ($n = 6$). (B) Effect of NP-313 on tail bleeding time of mice measured 5 min after i.v. administration. Data are presented as the mean ± SEM ($n = 6$). *$P < 0.05$ and **$P < 0.01$ as compared with the vehicle control, one-way ANOVA (Dunnett's *post hoc* test). (C) Effect of NP-313 on *ex vivo* mouse platelet aggregation of PRP induced by collagen (10 μg·mL^{-1}). Aggregation traces shown are representative of three independent experiments.

collagen-induced platelet aggregation as compared that induced by thrombin (IC_{50}, 1.7 vs. 7.7 μM, respectively). On being stimulated by collagen or thrombin, platelets synthesize and release TXA_2, which amplifies platelet aggregation (Jackson *et al.*, 2003). It is known that collagen and AA depend on the formation of TXA_2 in triggering platelet aggregation (Parise *et al.*, 1984; Atkinson *et al.*, 2003). On the other hand, collagen activates platelets through a tyrosine kinase-based signalling pathway and sequential activation of PLCγ_2, leading to intracellular Ca^{2+} mobilization (Jackson *et al.*, 2003), and these processes are exclusively dependent on glycoprotein VI activation. However, GPVI alone mediates only a part of platelet aggregation, and full-cell response and aggregation secondary mediators (like TXA_2) are needed (Atkinson *et al.*, 2003). Enzymatic assays showed that NP-313 exhibits a potent inhibitory effect on COX-1 and TXA_2 synthase activities, while it has little effect on PLA_2 or PLC activity. NP-313 also concentration-dependently inhibited platelet aggregation and TXB_2 generation induced by AA. These results suggest that NP-313 produces its effect on TXA_2 synthesis mainly by inhibiting both COX-1 and TXA_2 synthase activities, which may contribute to the inhibition of platelet aggregation with collagen. Nevertheless, unlike the TXA_2 dependency of aggregation with collagen (Nieswandt and Watson, 2003), thrombin induces intracellular Ca^{2+} mobilization and platelet aggregation independent of the TXA_2

pathway (Freedman, 2005), in line with our results that the majority of the platelet responses were unaffected even in the presence of ADP scavenger and COX inhibitors (Figure 7A). Furthermore, NP-313, below or at a concentration of 4 μM, abolished thrombin-mediated TXB_2 formation while not inhibiting thrombin-induced PLC-dependent intracellular Ca^{2+} mobilization (Figure 3B, right panel and Figure 6B).

The mechanism of calcium release from the IP_3-sensitive internal stores is well characterized. 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 the intracellular IP_3R–Ca^{2+} channel activity (Figure 7A, left panel). Ca^{2+} entry is thought to occur predominantly as a consequence of stored Ca^{2+} depletion and has been referred to as SOCE or capacitative Ca^{2+} entry through SOCC (Putney *et al.*, 2001). The identity of the SOCC in platelets 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 four putative transmembrane domains and contain the pore-forming subunits of SOCE channels. Orai 1–deficient platelets showed an almost absence of Ca^{2+} entry after store depletion by thapsigargin and much reduced Ca^{2+} entry when stimulated by agonists. On the other hand, activation of the SOCE-independent channel, transient receptor potential canonical (TRPC)6 with high expression in human platelets, by DAG and some of its metabolites also have been shown to contribute to the Ca^{2+} entry via G-protein–linked receptors (such as thrombin receptor) (Authi, 2009). NP-313 was found to inhibit the platelet aggregation caused by thapsigargin that bypasses receptor-mediated processes. Thapsigargin, a tool used to study SOCC, effectively elevates 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, the DAG analogue OAG has been used to investigate SOCE-independent Ca^{2+} entry (TRPC)6 channel, attributable to direct activation of this channel independent of PKC (Hofmann *et al.*, 1999). In the present study, thapsigargin-induced cytosolic Ca^{2+} mobilization was almost completely inhibited by NP-313 at a concentration of 20 μM (Figure 5), which has no influence on OAG-mediated intracellular Ca^{2+} mobilization in platelets (Figure 8A,B), suggesting that NP-313 selectively inhibits SOCE rather than blocking SOCE-independent channels. This may also explain why NP-313 inhibited thrombin-induced Ca^{2+} influx but did not affect the residual 30% of $[Ca^{2+}]i$ influx (Figure 7A, right panel). NP-313 preferentially inhibits collagen-induced platelet aggregation and cytosolic Ca^{2+}-mobilization compared with these effects induced by thrombin, consistent 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). In addition, the inhibitory action of NP-313 on TXA_2 synthesis was not only mediated by inhibiting both COX-1 and TXA_2 synthase activities but also possibly by the inhibition of cytosolic free Ca^{2+}. It is well known that upon stimulation of platelets, AA is liberated from the sn-2-position of membrane phospholipids through the action of $cPLA_2$, activated by an increase in cytosolic free Ca^{2+} (McNicol and Shibou, 1998). However, the question remains open regarding whether NP-313 acts primarily on this SOCC or indirectly on some molecule that regulates SOCC. Furthermore, we found that NP-313 has an inhibitory action on PKCα, since NP-313 at 10 μM inhibited PKCα (90% inhibition), and PMA (300 nM)-induced platelet aggregation in a concentration-dependent manner. A complete inhibition was observed at 15 μM (data not shown). The inhibitory effect on PKCα may contribute to the observation that NP-313 also could partly inhibit thrombin-induced aggregation (Konopatskaya *et al.*, 2009).

We further evaluated the antithrombotic efficacy/ side effect profile of NP-313 in a mouse model. Intravenous administration of NP-313 (4–8 $\mu g \cdot g^{-1}$) 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 little effect on haemostasis. In addition, it was found that the antithrombotic activity of NP-313 was in parallel with *ex vivo* antiplatelet activity. Notably, it was shown that NP-313 is more effective at inducing an antithrombotic action than the antiplatelet agent, aspirin, which mainly affects only the TXA_2 pathway of platelet activation. The promising antithrombotic efficacy of NP-313 may result from a dual inhibition of TXA_2 synthesis and a suppression of Ca^{2+} influx through SOCC. It has been shown that mice deficient in Orai 1 are markedly protected systemically and arterially from ischaemia-induced thrombosis but have only a slightly prolonged bleeding time (Braun *et al.*, 2009). However, further investigation is needed to address the precise mechanism of the antithrombotic effect of NP-313.

In conclusion, this study demonstrated that NP-313, a 1,4-naphthoquinone derivative, possesses the ability to inhibit both TXA_2 synthesis and SOCE. This compound does not affect cyclic nucleotide levels or induce LDH release. With its distinct action on the Ca^{2+} channels involved in modulating intracellular Ca^{2+} mobilization, NP-313 may become a new pharmacological tool for investigating the signal transduction pathways involved in regulating $[Ca^{2+}]_i$ through platelet SOCC. NP-313 also shows *in vivo* protection against thrombous formation with little effect on haemostasis, suggesting that it may be a potential candidate for development as an antithrombotic agent.

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Conflicts of interest

N/A.

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