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Antithrombotic and Antiplatelet Activities of 2-Chloro-3-[4-(ethylcarboxy)-phenyl]-amino-1,4-naphthoquinone (NQ12), a Newly Synthesized 1,4-Naphthoquinone Derivative

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ABSTRACT. The possibility of NQ12 (2-chloro-3-[4-(ethylcarboxy)-phenyl]-amino-1,4-naphthoquinone) as a novel antithrombotic agent and its mode of action were investigated. The effects of NQ12 on platelet aggregation in human platelet-rich plasma *in vitro*, in rats *ex vivo*, and on murine pulmonary thrombosis *in vivo*, as well as the mode of antithrombotic action were examined. NQ12 potently inhibited ADP-, collagen-, epinephrine-, and calcium ionophore-induced human platelet aggregations *in vitro* concentration-dependently. NQ12 significantly inhibited rat platelet aggregation in an *ex vivo* study. NQ12 prevented murine pulmonary thrombosis in a dose-dependent manner. However, NQ12 did not affect coagulation parameters such as activated partial thromboplastin time, prothrombin time, and thrombin time. NQ12 inhibited fibrinogen binding to the platelet surface GPIIb/IIIa receptor, but failed to inhibit binding to the purified GPIIb/IIIa receptor. Thromboxane B_2 formation caused by thrombin or collagen was inhibited significantly by NQ12. The phosphoinositide breakdown induced by thrombin or collagen was inhibited concentration-dependently by NQ12. These results suggest that NQ12 may be a promising antithrombotic agent, and its antithrombotic activity may be due to antiplatelet aggregation activity, which may result from the inhibition of phosphoinositide breakdown and thromboxane A_2 formation. BIOCHEM PHARMACOL **60**;7:1001–1008, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. antithrombotic agent; 1,4-naphthoquinone; antiplatelet aggregation; phosphoinositide; thromboxane A_2; GpVb/IIIa

Considering the importance of thrombosis in cardiovascular disorders, the search for better antithrombotic strategies continues. When blood vessels are damaged, platelet aggregation occurs rapidly to form hemostatic plugs or arterial thrombi at sites of vessel injury or in regions where blood flow is disturbed. These thrombi are the source of thromboembolic complications of arteriosclerosis, heart attacks, strokes, and peripheral vascular disease [1]. Evidence that arterial thrombi are composed largely of platelet aggregates and that platelets play the major role in initiation of venous thrombi has led many investigators to postulate that platelet aggregation is a major pathogenic mechanism in thrombosis [2, 3]. As the inhibition of platelet function represents a promising approach for the prevention of thrombosis, a number of antiplatelet drugs have been developed and evaluated for their effects in preventing thrombosis or its recurrence [4–6]. Naphthoquinone vitamins (vitamin K) are recognized widely for their roles in the γ-carboxylation of specific glutamyl residues in coagulation, anti-coagulation, and extra-hepatic proteins [7]. 1,4-Naphthoquinones have been reported to possess various pharmacological effects such as antiviral, antifungal, anticancer, and antiplatelet activities [8, 9], and some synthetic naphthoquinone compounds have been shown to have very potent antiplatelet activities [10–14].

Therefore, we synthesized eighty 1,4-naphthoquinone derivatives such as 2-arylamino-3-chloro-1,4-naphthoquinones (including NQ12¶), 2-alkylamino-3-chloro-1,4-

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¶ *Abbreviations*: NQ12, 2-chloro-3-[4-(ethylcarboxy)-phenyl]-amino-1,4-naphthoquinone; TX, thromboxane; IP_3, inositol 1,4,5-triphosphate; PRP, platelet-rich plasma; PPP, platelet-poor plasma; WP, washed platelets; GP, glycoprotein; APTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time; LDH, lactate dehydrogenase; and AA, arachidonic acid.

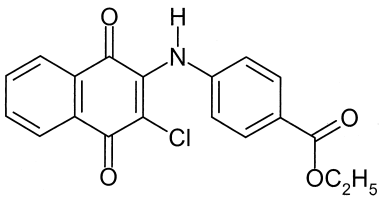

FIG. 1. Chemical structure of NQ12 (2-chloro-3-[4-(ethylcarboxy)-phenyl]-amino-1,4-naphthoquinone).

naphthoquinones, 2-arylamino-3-chloro-jugolenes, and 2-arylamino-1,4-naphazarines, and screened their antiplatelet aggregation activities. Among the tested 1,4-naphthoquinone derivatives, NQ12 (Fig. 1) showed the most potent antiplatelet aggregation activity. In the present study, the effects of NQ12 on platelet aggregation in human PRP *in vitro*, in rats *ex vivo*, and on murine pulmonary thrombosis *in vivo*, as well as the mode of antithrombotic action were examined.

MATERIALS AND METHODS

Materials

NQ12 was synthesized and characterized as described previously [15]. NQ12: m.p. 227° (crystalline), Anal. Calc. for $C_{19}H_{14}ClNO_4$ (355.77): C, 64.14; H, 3.97; N, 3.94%. Found: C, 64.11; H, 4.00; N, 3.92%. IR (KBr, cm^{-1}): 3320 (s, NH), 1670 (s, C═O). ^1H NMR (DMSO-d_6, δ ppm) 1.21 (3H, t, CH_3, J = 7.4 Hz), 4.21 (2H, q, CH_2, J = 7.4 Hz), 8.96 (1H, s, NH), 6.54–7.76 (4H, m, aromatic ring), 7.7–8.1 (4H, m, naphthoquinone ring). Thrombin was purchased from the Chrono-Log Co. ADP, epinephrine, collagen, A23187, and BSA were purchased from the Sigma Chemical Co. Cephalin, thromboplastin, and bovine thrombin were purchased from the Instrumentation Laboratory Co. A TXB_2 enzyme immunoassay kit was obtained from the Cayman Chemical Co. An IP_3 radioimmunoassay kit was purchased from Amersham. The other chemicals were of analytical grade.

Animals

Male Sprague–Dawley rats and ICR mice were purchased from the Sam-Yook Animal Co. and acclimatized for 1 week at 24 ± 1° and 55 ± 5% humidity with free access to a commercial pellet diet obtained from the Sam-Yang Co. and drinking water before experiments. The animal studies have been carried out in accordance with the Declaration of Helsinki (Publication No. 85–23, revised 1985) and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Preparation of Platelets

Blood from healthy volunteers who had not taken any drugs for at least 15 days was collected by venipuncture into a plastic flask containing 3.15% sodium citrate (1:9, v/v). PRP was prepared by centrifugation of the blood at 120 *g* for 15 min and further centrifugation at 850 *g* for 15 min to prepare PPP. The supernatants were pooled and centrifuged at 600 *g* for 15 min at room temperature. The platelet pellets were washed with modified Tyrode–HEPES buffer (129 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, 2 mM EGTA, 5.6 mM glucose, 10 mM HEPES, 0.35% BSA, pH 7.4) and centrifuged at 600 *g* for 15 min. Then platelet pellets were resuspended gently in Tyrode–HEPES buffer (129 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, 1 mM $CaCl_2$, 5.6 mM glucose, 10 mM HEPES, 0.35% BSA, pH 7.4). The platelets in PRP or WP were counted by a Coulter Counter (Coulter Electronics) and adjusted to a concentration of 3×10^8 platelets/mL.

In Vitro *Antiplatelet Aggregation Activity*

The antiplatelet aggregation activity of NQ12 or the reference drug was determined by the turbidimetric method of Born and Cross [16] using a dual channel Whole Blood Lumi-ionized Calcium Aggregometer (Chrono-Log Co., Ltd.). A 300-μL sample of human PRP was incubated at 37° for 2 min in the aggregometer with stirring at 1000 rpm. The agent solutions were prepared in DMSO to give the final DMSO concentration of 0.3%. Then each agonist, either ADP (20 μM), collagen (200 μg/mL), epinephrine (10 μM), or A23187 (1 μM), was added to the platelets. Changes in light transmission were recorded for 5 min after stimulation. The inhibition of platelet aggregation is expressed as % inhibition (X) using the following equation: $X = [(A - B)/A] \times 100$, where A = maximal aggregation of the control, and B = maximal aggregation of the PRP-treated sample. The IC_{50} value was calculated by the least-squares method.

Ex Vivo *Antiplatelet Aggregation Activity*

Ex vivo antiplatelet activity was examined as described previously [17]. Male Sprague–Dawley rats weighing 320–350 g were used after overnight fasting. Rats were administered orally 100 mg/kg of NQ12 solution or 50 mg/kg of aspirin suspended in 0.5% carboxymethylcellulose solution. Blood was collected 90 min after sample administration, and PRP was prepared as described above. Platelet aggregation was induced by 32.8 μg/mL of collagen or 1.3 μM ADP in 300 μL PRP. Antiplatelet aggregation activity was tested as described above.

In Vivo *Antithrombotic Activity*

Antithrombotic activity *in vivo* was investigated by the mouse thromboembolism test according to the method of DiMinno and Silver [18]. Male ICR mice weighing 25–30 g were used after overnight fasting. Collagen (114 μg) plus epinephrine (13.2 μg) solution showing about 90% thromboembolism was injected into the tail vein 90 min after oral

administration of NQ12 (50 mg/kg, 100 mg/kg). Aspirin (50 mg/kg) as a positive control or 0.5% carboxymethylcellulose solution as vehicle was administered orally. The number of dead or paralyzed mice was counted for 15 min, and the percent (%) of protection was calculated as follows: [1 − (dead or paralyzed)/total] × 100.

In Vitro *Anticoagulation Activity*

Anticoagulation activity was evaluated by measuring plasma clotting times. Plasma clotting times (APTT, PT, and TT) were measured by a modification of the method of Hara *et al.* [19] using an Automated Coagulation Laboratory 100 Instrument (Instrumentation Laboratory Co.) with PPP. The plasma was incubated with NQ12 or heparin as a reference drug for 7 min at 37°. One hundred microliters of the incubated plasma was mixed with 50 μL of cephalin in the process plate, and coagulation was started with the addition of $CaCl_2$, 100 μL of thromboplastin, and 100 μL of bovine thrombin to 100 μL of incubated plasma for the APTT, PT, and TT assays, respectively.

FITC-Labelled Fibrinogen Binding to Human Platelet GPIIb/IIIa

Fibrinogen was conjugated with fluorescein isothiocyanate by incubating the protein (1 mg/mL) at 4° overnight with 50 mM carbonate buffer (pH 9.5) containing 0.1 mg/mL of FITC. Free FITC molecules were eliminated by overnight dialysis against carbonate buffer. The preparation of conjugate compounds, 3.2 mg/mL, was designated as FITC–fibrinogen. The inhibition of NQ12 in fibrinogen binding to GPIIb/IIIa receptor of ADP-stimulated platelets was examined by flow cytometric analysis. Histograms were generated by quantitating the fluorescence intensity of 10,000 platelets, with the peak height being indicative of the number of platelets having a given intensity. WP was prepared and adjusted to 2×10^8 platelets/mL with modified Tyrode's buffer (pH 7.4). NQ12 was dissolved in DMSO and then diluted 10 times with 100% ethanol to a final concentration of 50 μM. To determine the inhibitory effect of NQ12 on fibrinogen binding to platelets, FITC-labelled fibrinogen (10 μg/mL) and ADP (20 μM) were added. The fluorescence signal of ADP-stimulated platelets was analyzed by Flow Cytometry (FACSCalibur, Becton Dickinson) after 5 min of incubation.

Binding of Purified Human Platelet GPIIb/IIIa Receptor to Fibrinogen

The peptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) was synthesized using f-Moc chemistry and purified on a reverse-phase C_{18} column. Fibrinogen in carbonate/bicarbonate buffer (pH 9.0) was coated onto microtiter wells overnight at 4°. Purified GPIIb/IIIa was co-incubated in the wells with either NQ12 or GRGDSP, a selective inhibitor of the GPIIb/IIIa receptor. The amount of receptor bound was measured by ELISA.

Assay of TXB_2 Formation

TXA_2 was measured as its stable hydrolysis product TXB_2 with an enzyme immunoassay kit. After incubation of human WP with the inducer for 6 min, EDTA (2 mmol/L) and indomethacin (50 μmol/L) were added to stop the formation of TXB_2. The mixture was centrifuged using an Eppendorf microcentrifuge (model Mega17R, Hanil) for 2 min, and the amount of TXB_2 in the supernatant was measured with an enzyme immunoassay kit (Cayman Chemical Co.).

IP_3 Content Assay

The level of IP_3 in human WP was measured by the modified method of Ko *et al.* [11]. WP (3×10^8 platelets/mL) was incubated with 0.4% DMSO, or NQ12 at 37° for 3 min and then added with thrombin or collagen. The reaction was terminated by the addition of 100 μL of ice-cold perchloric acid (20%) followed by a 20-min incubation in an ice bath. The mixture was centrifuged at 2000 *g* for 15 min at 4°, the supernatant was collected, and its pH was adjusted to 7.5 with a 10 N KOH solution.

The precipitate of $KClO_4$ formed for 30 min at 4° was sedimented by centrifugation at 2000 *g* for 15 min at 4°. The radioactivity in the supernatant indicative of IP_3 was measured, and the intracellular amount of IP_3 was determined.

LDH Assay

The released LDH activity was measured spectrophotometrically by recording the decrease in the optical density of β-NADH at 340 nm as described previously [20]. Samples were incubated with human PRP for 120 min, and then centrifuged for 4 min at 1312 *g*. The cellular LDH activity from platelets was determined in the platelet suspension, which was lysed with 1% Triton-X 100. Total LDH activity was the summation of both released and cellular activities. The released LDH activity was expressed as a percentage of the total LDH activity.

Statistics

The difference between the treated groups and the control group was analyzed by an unpaired Student's *t*-test for the *ex vivo* antiplatelet aggregation and the χ^2 test for the *in vivo* antithrombotic activity.

RESULTS

In Vitro *Antiplatelet Aggregation*

NQ12 potently inhibited ADP-, collagen-, epinephrine-, and A23187-induced human platelet aggregations *in vitro* in a concentration-dependent manner, and the IC_{50} values were 7.2, 12.6, 4.3, and 28.3 μM, respectively. On the other hand, aspirin inhibited the platelet aggregations induced by

TABLE 1. Inhibitory effect of NQ12 on human platelet aggregation

Aggregating agent	IC_{50} (μM)	
	NQ12	Aspirin
ADP (20 μM)	7.2 ± 3.3	> 1000*
Collagen (200 μg/mL)	12.6 ± 9.4	160.0 ± 49.3
Epinephrine (10 μM)	4.3 ± 1.1	70.4 ± 0.9
A23187 (1 μM)	28.3 ± 6.1	> 1000*

IC_{50} values were calculated from at least three separate experiments. Means ± SD.
* Less than 50% inhibition at 1000 μM.

collagen and epinephrine, but failed to inhibit those induced by ADP and A23187 in this experimental condition (Table 1).

Ex Vivo *Antiplatelet Aggregation*

In the *ex vivo* antiplatelet aggregation study, NQ12 significantly ($P < 0.05$) inhibited ADP- or collagen-induced rat platelet aggregation, and the inhibitory effects were 72.1 ± 11.1 and 33.7 ± 5.3%, respectively (Fig. 2).

In Vivo *Antithrombosis*

Oral administration of NQ12 to mice showed significant protection from death due to pulmonary thrombosis. Protection from pulmonary thrombosis was observed in a dose-dependent manner in mice. The protection of NQ12 was 54.5 and 73.7% at doses of 50 and 100 mg/kg, respectively, whereas aspirin (50 mg/kg) showed 68.4% protection (Table 2).

In Vitro *Anticoagulation*

The effects of NQ12 on coagulation times were evaluated by APTT, PT, and TT assays using human PPP. APTT, PT, and TT were not changed by NQ12 treatment at concentrations up to 100 μM (Table 3).

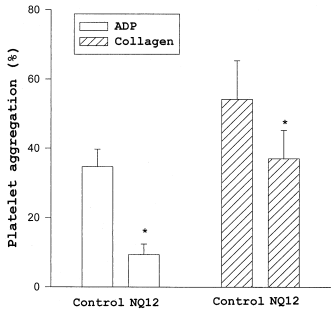

FIG. 2. Inhibitory effect of NQ12 on platelet aggregation in rats. PRP was obtained from the blood 90 min after NQ12 (100 mg/kg) was administered orally to Sprague–Dawley rats, and platelet aggregation was induced by ADP (1.3 μM) or collagen (32.8 μg/mL). Key: (*) Significantly different from control at $P < 0.05$. Values are means ± SD (N = 5).

TABLE 2. Effect of NQ12 on pulmonary thrombosis in mice

Sample	Dose (mg/kg)	No. killed/ No. tested	% Protection
Control	Vehicle	18/21	14.3
NQ12	50	10/22*	54.5
	100	5/19†	73.7
Aspirin	50	6/19†	68.4

NQ12 was administered orally 90 min before i.v. injection of epinephrine (13.2 μg/mouse) plus collagen (114 μg/mouse).
χ^2-test was used to examine the difference between vehicle- and sample-treated groups.
*$P < 0.01$.
†$P < 0.001$.

Platelet Surface GPIIb/IIIa Binding

The binding of FITC-labelled fibrinogen to human platelet GPIIb/IIIa is shown in Fig. 3. The mean fluorescence intensity value was below 5 in the untreated platelets (data not shown), whereas it was increased to about 90 after stimulation of human platelets with ADP. NQ12 inhibited binding by 58% compared with control (at a concentration of 5 μM). The increased expression of the GPIIb/IIIa complex on the platelet surface was confirmed by flow cytometric analysis using a FITC-labelled mouse anti-human GPIIb/IIIa antibody. To determine whether NQ12 inhibits platelet surface GPIIb/IIIa directly, a binding assay was performed using purified GPIIb/IIIa. A synthetic peptide containing the RGD sequence, GRGDSP, was used as a reference, because the RGD peptide is a potent inhibitor of the interaction between the GPIIb/IIIa receptor and fibrinogen. The peptide GRGDSP exhibited inhibition with an IC_{50} of 300 nM, whereas NQ12 did not inhibit GPIIb/IIIa binding to fibrinogen (Fig. 4).

Platelet TXB_2 Formation

The amount of TXB_2 in resting WP was 24 ± 6 pg/3 × 10^8 platelets, whereas TXB_2 formation was increased greatly to 15.8 ± 0.1, 17.0 ± 0.4, and 17.4 ± 0.1 ng/3 × 10^8 platelets by thrombin, collagen, and AA stimulation, respectively. NQ12 potently inhibited TXB_2 formation stimulated by thrombin and collagen, whereas a weak inhibition of TXB_2

TABLE 3. Effect of NQ12 on human plasma coagulation times

Sample	Dose (μM)	APTT (sec)	PT (sec)	TT (sec)
Control	Vehicle	42.6 ± 0.4	13.7 ± 0.2	6.4 ± 0.1
NQ12	1	41.7 ± 0.3	13.9 ± 0.0	6.5 ± 0.2
	10	40.7 ± 0.5	13.7 ± 0.1	6.6 ± 0.0
	100	41.5 ± 0.3	13.7 ± 0.1	6.5 ± 0.1
Heparin	1	54.4 ± 0.3*	14.1 ± 0.0	13.3 ± 1.1†
	10	NC	32.3 ± 0.7*	NC

NC means no coagulation.
APTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time. Results are expressed as means ± SD (N = 5).
*Significantly different from control at $P < 0.05$.
†$P < 0.01$.

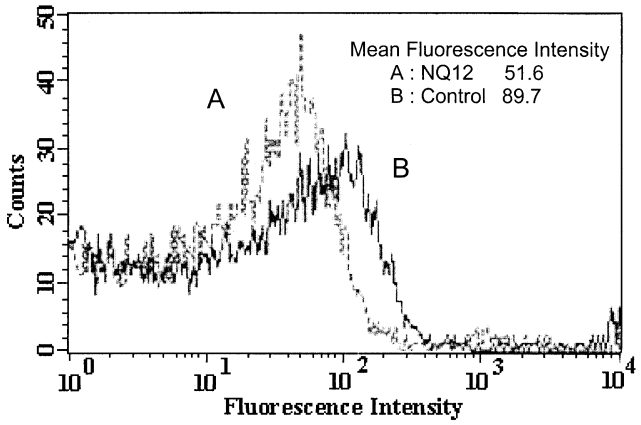

FIG. 3. Effect of NQ12 on the binding of FITC-labelled fibrinogen to human platelet GPIIb/IIIa. The inhibitory effect of NQ12 on fibrinogen binding to the GPIIb/IIIa receptor on ADP-stimulated platelets was examined by flow cytometric analysis. Washed platelets were prepared and adjusted to 2 × 10^8 platelets/mL with modified Tyrode's buffer (pH 7.4). To determine the inhibitory effect of NQ12 on fibrinogen binding to platelets, fluorescence signal was measured 5 min after FITC-labelled fibrinogen (10 μg/mL) and ADP (20 μM) were added. The curves show the fluorescence signal of ADP-activated platelets in the presence or absence of 50 μM NQ12 and are representative of three experiments.

production was shown in the case of stimulation by AA (Table 4).

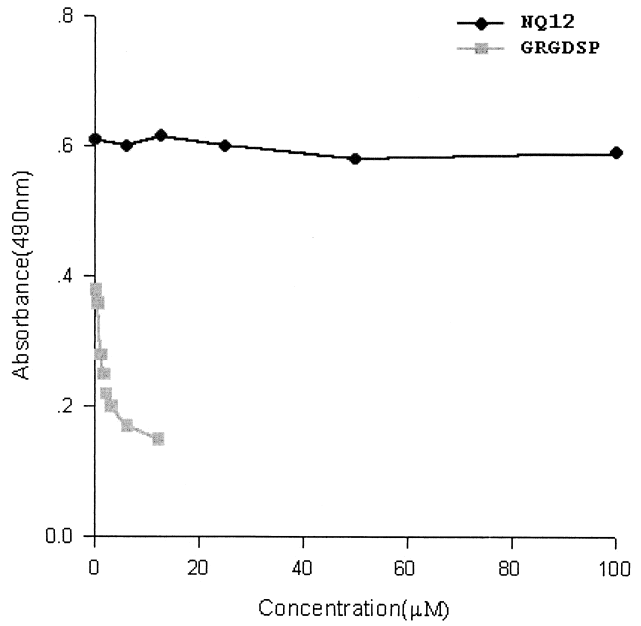

FIG. 4. Effect of NQ12 on the binding of purified human platelet GPIIb/IIIa receptor to fibrinogen. Purified GPIIb/IIIa was co-incubated in the wells with NQ12 at concentrations of 0.01, 1.5, 3.1, 6.25, 12.5, 25, 50, and 100 μM or GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro) at concentrations of 0.01, 0.19, 0.38, 0.75, 1.5, 3.1, 6.25, and 12.5 μM. The amount of receptor bound was determined by ELISA. Data are expressed as the mean absorbance of two experiments performed in duplicate.

TABLE 4. Effect of NQ12 on thromboxane B_2 formation in human washed platelets

Sample	Dose (μM)	TXB_2 (ng/3 × 10^8 platelets) Thrombin	Collagen	AA
Control	Vehicle	15.8 ± 0.1	17.0 ± 0.4	17.4 ± 0.1
NQ12	10	11.8 ± 0.0	6.5 ± 0.1*	17.3 ± 0.1
	30	5.0 ± 0.1†	3.1 ± 0.3*	16.2 ± 0.2
	50	4.8 ± 0.1†	3.1 ± 0.2*	13.9 ± 0.5†
Aspirin	50	4.8 ± 0.1†	2.4 ± 0.3*	14.4 ± 0.1†

Samples were preincubated with platelets (3 × 10^8 platelets/mL) at 37° for 4 min, followed by the addition of thrombin (0.5 U/mL), collagen (50 μg/mL) and arachidonic acid (50 μM). The TXB_2 formation was terminated by 2 mM EDTA and 200 μM indomethacin 5 min after the addition of aggregating agents. Data are expressed as means ± SEM (N = 4).

*$P < 0.001$.

†$P < 0.01$.

Platelet IP_3 Formation

The level of IP_3 was 0.15 ± 0.07 pmol/10^9 platelets in resting platelets, whereas the formations of IP_3 were greatly increased up to 34.4 ± 3.1 and 14.9 ± 1.1 pmol/10^9 platelets by thrombin and collagen, respectively. NQ12 significantly inhibited the IP_3 formation caused by these inducers in a concentration-dependent manner (Table 5).

Platelet Cytotoxicity

The released activity of cytosolic LDH was assayed to study the cytotoxic effect of NQ12 on platelets. The LDH activities were approximately 3.9% for the vehicle and 4.1% for the samples treated for 120 min. No significant increase in LDH release was observed between NQ12- and vehicle-treated platelets (Table 6).

DISCUSSION

Antiplatelet therapy has become a useful means of preventing acute thromboembolic artery occlusions in cardiovascular diseases, because platelet activation and thrombus formation are important in the pathophysiology of isch-

TABLE 5. Effect of NQ12 on inositol 1,4,5-triphosphate formation in human washed platelets

Sample	Dose	IP_3 (pmol/mL) Thrombin	Collagen
Control	Vehicle	34.4 ± 3.1	14.9 ± 1.1
NQ12	1 μM		4.2 ± 0.2*
	5 μM		3.8 ± 0.4*
	10 μM	32.1 ± 3.8	2.5 ± 0.2*
	30 μM	27.5 ± 6.5†	
	50 μM	15.8 ± 3.9*	
LiCl	10 μM	2.4 ± 0.1*	2.7 ± 0.4*

Washed platelets were preincubated with the samples at 37° for 3 min. Thrombin (0.5 U/mL) or collagen (50 μg/mL) then was added, and the mixture was incubated for 2 min. Data are expressed as means ± SEM (N = 5).

*$P < 0.001$.

†$P < 0.01$.

TABLE 6. Effect of NQ12 on platelet lactate dehydrogenase release

	Released LDH (%)	
Time (min)	Vehicle	NQ12
0	2.0 ± 0.9	1.2 ± 0.5
10	1.4 ± 0.3	1.4 ± 0.3
20	1.7 ± 0.6	1.5 ± 0.2
30	2.6 ± 1.6	1.8 ± 0.5
60	2.0 ± 0.5	2.1 ± 0.3
90	3.8 ± 0.9	2.8 ± 0.6
120	3.9 ± 0.5	4.1 ± 0.9

Extents of LDH release were expressed as the percentage of total enzyme activity lysed with 0.1% Triton X-100. Data are expressed as means ± SD.

aemic events in the heart, brain, and peripheral arterial territories. Antiplatelet compounds such as aspirin, ticlopidine, and dipyridamole are in clinical use. Aspirin is an antithrombotic drug whose antiplatelet activity is due to the inhibition of cyclooxygenase activity, which results in several clinical disadvantages including gastrointestinal side-effects and hemorrhage [21]. The search for more effective and safe antiplatelet agents has resulted in the development of thromboxane synthase inhibitors (ridogrel and picotamide), receptor blockers (clopidogrel), and antagonists of platelet receptor glycoproteins Ib and IIb/IIIa (GR-83895, SHP-106760, MK-852, Ro44–9883, and MK-383) [22, 23].

We synthesized eighty 1,4-naphthoquinone derivatives and screened their antiplatelet aggregation activities. Among the tested compounds, NQ12, 2-chloro-3-[4-(ethylcarboxy)-phenyl]-amino-1,4-naphthoquinone, showed the most potent antiplatelet aggregation activity. In the present study, the antithrombotic activities and mode of antithrombotic action of NQ12, a newly synthesized 1,4-naphthoquinone derivative, were investigated. NQ12 showed potent inhibition of platelet aggregation in human PRP *in vitro* and rat PRP *ex vivo*, and prevented death due to pulmonary thrombosis *in vivo*, whereas it did not affect the coagulation pathway.

NQ12 potently inhibited ADP-, collagen-, epinephrine-, and A23187-induced human platelet aggregation, whereas aspirin only inhibited platelet aggregation induced by collagen and epinephrine, but failed to inhibit ADP- and A23187-induced platelet aggregation. We chose several agonists to compare platelet aggregation among agonists with different intensities of receptor–ligand binding. The agonists used in this study were ADP, epinephrine, collagen, thrombin, and A23187, which have different platelet aggregation mechanisms. ADP and epinephrine are weak agonists, whereas collagen and thrombin are strong agonists. This study was focused on showing the comparative activity of NQ12 with the different agonists being studied. The concentrations of the agonists used in this study were optimized according to the results of our preliminary experiments.

The results of the *ex vivo* study indicate that NQ12 has a significant inhibitory effect on platelet aggregation when administered orally. The lethal effect of aggregating agonists on mice is caused by massive occlusion of the microcirculation of the lung by platelet thromboembolism or by vasoconstriction due to the increase of TXA_2 and prostaglandin $F_{2\alpha}$ in platelets [24–26]. NQ12 inhibited collagen plus epinephrine-induced thromboembolic death in mice in a dose-dependent manner. On the other hand, NQ12 did not alter the clotting parameters, whereas heparin, a reference drug, prolonged the clotting time. These results suggest that NQ12 has antithrombotic activity, and its mode of antithrombotic action may be due to antiplatelet activity but not to anticoagulation activity.

Liao *et al.* [27] have reported previously that several 1,4-naphthoquinone derivatives inhibit the aggregation of washed rabbit platelets. Ko *et al.* [11] have reported that 2-chloro-3-methoxycarbonylpropionamido-1,4-naphthoquinone (PP1D-1) inhibits the aggregation and ATP release in washed rabbit platelets concentration-dependently. Chang *et al.* [14] have reported that 2-[(4-cyanophenyl)amino]-3-chloro-1,4-naphthalenedione (NQ-Y15) causes concentration-dependent inhibition of platelet aggregation. The results with the above 1,4-naphthoquinone derivatives support the findings of the antiplatelet activities of NQ12.

We also tried to elucidate the mode of antiplatelet action of NQ12. NQ12 inhibited fibrinogen binding to the platelet surface GPIIb/IIIa receptor, but failed to inhibit binding to the purified GPIIb/IIIa receptor. The TXB_2 formation and phosphoinositide breakdown induced by thrombin or collagen were inhibited significantly by NQ12. Platelet aggregation is a consequence of complex signal transduction cascade reactions induced by stimulants. Platelet membrane GPIIb and IIIa exist as a complex and are important in platelet plug formation after vascular injury [28]. This complex acts as a receptor for fibrinogen, fibronectin, von Willebrand factor, and vitronectin, and its binding of these adhesive proteins mediates platelet aggregation, adhesion, and spreading [29–33]. The mean fluorescence intensity value was below 5 in the untreated platelets (data not shown), whereas it was increased up to about 90 after stimulation of human platelets with ADP. This result suggests that the FITC–fibrinogen binding is increased after the GPIIb/IIIa complex is activated. The increased expression of the GPIIb/IIIa complex on the platelet surface was confirmed preliminarily by flow cytometric analysis using a FITC-labelled mouse anti-human GPIIb/IIIa antibody. This result is almost similar with the previous report [33] that the expression of the GPIIb/IIIa complex on the platelet surface was increased after the activation of platelets with agonists (including ADP). However, the results from the binding assay using purified GPIIb/IIIa indicate that NQ12 does not inhibit directly the binding of fibrinogen to GPIIb/IIIa. These results suggest that the inhibitory activity of NQ12 on platelet aggregation is due to the inhibition of the intracellular pathway preceding GPIIb/IIIa receptor exposure, but not to the direct inhibition of the receptor.

NQ12 potently inhibited TXB_2 formation stimulated by thrombin and collagen in human platelets. This result indicates that NQ12 may inhibit platelet aggregation by blocking the formation of TXA_2 or at an earlier step. NQ12 inhibited IP_3 formation in platelets induced by thrombin and collagen. These data indicate that NQ12 may inhibit platelet activation by suppressing the phosphoinositide breakdown caused by the agonists.

The antiplatelet mechanism of NQ12 is not clear. But it already has been reported that the inhibitory effect of 2-chloro-3-methyl-1,4-naphthoquinone on rabbit platelet aggregation may be due to the inhibition of phosphoinositide breakdown caused by the inducers [27]. It has been reported that 2-chloro-3-methoxycarbonylpropionamido-1,4-naphthoquinone exerts antiplatelet effects by inhibiting phosphoinositide turnover [11]. It has been reported that the antiplatelet effect of 2-[(4-cyanophenyl)amino]-3-chloro-1,4-naphthalenedione is due to a combination of TXA_2 synthase inhibition with TXA_2 receptor blockade [14]. The results of the above 1,4-naphthoquinone derivatives support the inhibitory effect of NQ12 on TXB_2 and IP_3 formation.

We also assayed the platelet membrane damage of NQ12 as indicated by cytoplasmic LDH activity and examined the toxic effects of NQ12 in *ex vivo* and *in vivo* animal studies. No significant increase in released LDH by NQ12 was shown compared with the vehicle. Also, NQ12 did not show any toxic effects when it was administered orally to rats and mice.

In conclusion, these results suggest that NQ12 may be a promising antithrombotic agent, and its antithrombotic activity may be due to antiplatelet aggregation. The inhibition of platelet aggregation may result from the inhibition of IP_3 and TXA_2 formation.

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