Antiplatelet and Antithrombotic Activities of NQ301, 2-Chloro-3-(4-acetophenyl)-amino-1,4-naphthoquinone

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The antiplatelet and antithrombotic activities of a newly synthesized NQ301, 2-chloro-3-(4-acetophenyl)-amino-1,4-naphthoquinone, were investigated on human platelet aggregation in vitro and rats ex vivo, and murine pulmonary thrombosis in vivo. NQ301 potently inhibited ADP-, collagen-, epinephrine- and calcium ionophore A23187-induced human platelet aggregation in a concentration-dependent manner in vitro. NQ301 significantly inhibited platelet aggregation in orally administered rats ex vivo. NQ301 prevented death due to pulmonary thrombosis in mice dose-dependently in vivo. NQ301 also showed significant prolongation of tail bleeding time in conscious mice. However, NQ301 did not alter such coagulation parameters as activated partial thromboplastin time, prothrombin time, and thrombin time in human plasma. These results suggest that NQ301 may be a promising antithrombotic agent, and the antithrombotic activity of NQ301 may be due to antiplatelet aggregation activity but not to in vitro anticoagulation.

Key words 1,4-naphthoquinone; antiplatelet; antithrombosis; anticoagulation

Platelets play an important role in the pathogenesis of thrombosis. The interactions between platelets and blood vessel walls are important in the development of thrombosis and cardiovascular diseases such as myocardial infarction, stroke, and atherosclerosis. 1-3 Once blood vessels are damaged, platelet aggregation occurs rapidly to form hemostatic plugs or arterial thrombi at the sites of vessel injury or in regions where blood flow is disturbed. These thrombi are the source of thromboembolic complications of atherosclerosis, heart attacks, stroke, and peripheral vascular disease. 4 Therefore, the inhibition of platelet function represents a promising approach for the prevention of thrombosis.

A number of antiplatelet drugs have been evaluated for their effects in preventing the development of thrombosis or its recurrence. 5-7 Naphthoquinone vitamins (vitamin K) are widely recognized for their roles in the gamma-carboxylation of specific glutamate residues in coagulation, anti-coagulation, and extra-hepatic proteins. 8 It has been reported that vitamin K analogues have various pharmacological effects such as antiviral, antifungal, anticancer, and antiplatelet activities. 9-11 It has also been reported that some synthetic naphthoquinone compounds showed antiplatelet activities. 12-20

We have newly synthesized 130 naphthoquinone derivatives and screened them for antiplatelet aggregation activities. NQ301, 2-chloro-3-(4-acetophenyl)-amino-1,4-naphthoquinone (Fig. 1), showed the most potent antiplatelet aggregation activity. In the present study, we examined the possibility of NQ301 as a novel antithrombotic agent by determining its inhibitory effect on platelet aggregation induced by various aggregating agents in vitro and ex vivo, and its antithrombotic effect in vivo.

MATERIALS AND METHODS

Materials NQ301 was newly synthesized and characterized as previously described. 21 In brief, a solution of 1,4-naphthoquinone (0.01 mol) and 4-acetoaniline (0.011 mol) in 150 ml of 95% EtOH was refluxed for 5 h. After the reaction mixture was kept overnight at 4 \(^\circ\)C, a precipitate was collected by filtration. Crystalization of the precipitate from MeOH afforded NQ301. NQ301: mp 229-230 \(^\circ\)C, Anal. Calc'd for C\(_{18}\)H\(_{16}\)Cl\(_2\)N\(_2\)O: C, 66.37; H, 3.71; N, 4.30; Found: C, 66.32; H, 3.79; N, 4.27%. IR (KBr, cm\(^{-1}\)): 3320 (s, NH), 3050, 1660 (s, C=O), 1515, 1\(^{H}\)-NMR (DMSO-d\(_6\), ppm) 2.6 (3H, s, CH\(_3\)), 6.31-6.97 (4H, m, aromatic ring), 7.7-8.2 (4H, m, naphthoquinone ring), 9.1 (1H, s, NH). 1,4-Naphthoquinone and 4-acetoaniline were purchased from Aldrich Chemical Co. (St. Louis, MO, U.S.A.). Adenosine 5\(^{\prime}\)-diphosphate (ADP), epinephrine, collagen, calcium ionophore A23187, bovine serum albumin (BSA), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Thrombin and arachidonic acid were obtained from Chrono-Log Co., Ltd. (Haverton, PA, U.S.A.). Cephalin, thromboplastin and bovine thrombin were purchased from Instrumentation Laboratory Co., (Milano, Italy).

Animals Male Sprague-Dawley rats (300-330 g) and ICR mice (25-30 g) were purchased from SamYook Animal Co. (Osan, Korea) and acclimatized for 1 week at a temperature of 24±1 \(^\circ\)C and a humidity of 55±5% with free access to a commercial pellet diet obtained from Samyang Co. (Wonju, Korea) and drinking water before the experiments. Animal experiments were carried out in accordance with international guidelines.

Preparation of Platelets Blood from healthy volunteers who had not taken any drugs for at least 15 d was collected

![Fig. 1. Chemical Structure of NQ301, 2-Chloro-3-(4-acetophenyl)-amino-1,4-naphthoquinone](https://example.com/fig1.png)

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by venopuncture into a plastic flask containing 3.15% sodium citrate (1:9 v/v). Platelet rich plasma (PRP) was prepared by centrifugation of the blood at 120×g for 15 min and further centrifuged at 850×g for 15 min to prepare platelet poor plasma (PPP).\(^{22}\) 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\(_2\)PO\(_4\), 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 gently resuspended 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\(_2\)PO\(_4\), 1 mm CaCl\(_2\), 5.6 mm glucose, 10 mm HEPES, 0.35% BSA, pH 7.4). The platelet number was adjusted with autologous PPP. The platelets in PRP or washed platelet (WP) were counted by Coulter Counter (Coulter Electronics, Hialeah, FL, U.S.A.) and adjusted to a concentration of 3×10\(^6\) platelets/ml.

**In Vitro Antiplatelet Aggregation** The platelet aggregation was measured by turbidimetry using a dual channel Whole Lumi-ionized Calcium Aggregometer (Chrono-Log Co., Ltd., Havertown, PA, U.S.A.) according to the method of Born.\(^{23}\) Briefly, human PRP (300 μl) was incubated at 37°C for 4 min in the aggregometer with stirring at 1000 rpm and then stimulated with ADP, collagen, epinephrine and calcium ionophore A23187. NQ301 or aspirin, a reference agent, was incubated with PRP for 3 min, followed by addition of the aggregation agents. Changes in light transmission were recorded for 10 min after stimulation with these agents. Each inhibition rate was obtained from the maximal aggregation induced by respective agonists at the concentration using Eq. 1, and then the values of IC\(_{50}\) were calculated from the data using a probit method.

\[ \text{Eq. 1: inhibition rate} = (1 - \text{MAR of sample-treated PRP/MAR of vehicle-treated PRP}) \times 100 \]

where MAR is the maximal aggregation rate.

**Ex Vivo Antiplatelet Aggregation** Male Sprague-Dawley rats were used after overnight fasting. Rats were orally administered 90 mg/kg of NQ301 and 0.5% carboxymethyl cellulose (CMC) as a vehicle. Blood was collected 90 min after sample treatment and PRP was prepared as previously described. Platelet aggregation was induced by 32.8 μg/ml of collagen or 1.3 μM of ADP. Antiplatelet activities of the sample were investigated according to the method of Kimura et al.\(^{24}\)

**In Vivo Antithrombotic Activity** The antithrombotic effects of NQ301 were investigated by the mouse thromboembolism test according to the method of DiMinno et al.\(^ {25} \) Male ICR mice were used after overnight fasting. NQ301 (50, 100 mg/kg), aspirin (50 mg/kg) as a positive control and 0.5% CMC solution as a vehicle were administered orally. A mixture solution of collagen (114 μg) and epinephrine (13.2 μg) was injected into the mouse tail vein and pulmonary thrombosis was induced 90 min after oral administration of the samples. The number of dead or paralyzed mice was recorded up to 15 min and the percentage of protection was calculated as follows:

\[ \left( \frac{1 - \text{dead paralyzed}}{\text{total}} \right) \times 100 \]

**Tail Bleeding Time in Conscious Mice** The bleeding time was measured as described by Hornstra et al.\(^ {26}\) The bleeding time is designed to determine the blood's ability to form a hemostatic plug, in which platelet, plasma factor, and blood vessel wall are involved. In short, ninety minutes after the oral administration of samples, the tail of male ICR mouse was transected at 2 mm from the tip and 1.5 cm of the distal portion was vertically immersed in saline at 37°C.

**In Vitro Coagulation Parameters** The plasma clotting times, activated partial thromboplastin (APTT), prothrombin time (PT) and thrombin time (TT) were automatically measured by the modification of Hara's method\(^ {27} \) using an Automated Coagulation Laboratory 100 Instrument (Instrumentation Laboratory Company, Milano, Italy). The PPP was incubated with samples for 7 min at 37°C. One hundred microliters of incubated plasma was mixed with 50 μl of cephalin in the process plate and the coagulation was started with the addition of CaCl\(_2\), 100 μl of thromboplastin, and 100 μl of bovine thrombin into the 100 μl of incubated plasma for APTT, PT and TT assay, respectively.

**Lactate Dehydrogenase (LDH) Assay** LDH is a cytosolic enzyme present in all eukaryotic cells, which catalyses pyruvate to lactate in the presence of β-NADH. The amount released into the buffer is proportional to the number of membrane-damaged cells. The released LDH activity was measured spectrophotometrically by recording the decrease in the optical density of β-NADH at 340 nm. Samples were incubated with human washed platelet for 0, 10, 20, 30, 60, 90 or 120 min, respectively, in a time-dependent manner, and then centrifuged for 4 min at 11000 rpm. The activity of LDH activity was calculated from the absorption change at 340 nm. The total LDH activity of platelets was determined in a platelet suspension that was lysed by incubation with 1% Triton-X 100. The data were represented as a percentage of released LDH activity from total LDH activity.

**Statistics** Differences between the sample-treated group and control group were analyzed by Student's t-test for ex vivo antiplatelet aggregation and by the \( \chi^2 \)-test for in vivo antithrombotic activity.

**RESULTS**

**In Vitro Antiplatelet Aggregation Effect** NQ301 potently inhibited ADP-, collagen-, epinephrine- and A23187-induced human platelet aggregation in vitro in a dose-dependent manner. The IC\(_{50}\) values of NQ301 were 3.21±1.10, 8.44±2.58, 15.69±3.73 and 44.20±2.37 μM, respectively. Aspirin, a reference drug which is the most widely used anti-platelet drug in clinical practice, caused only collagen- and epinephrine-induced human platelet aggregation, but failed to inhibit ADP- and A23187-induced platelet aggregation. The antiplatelet activity of NQ301 was more potent than that of aspirin, and its antiplatelet mode might be different from that of aspirin (Table 1).

**Ex Vivo Antiplatelet Aggregation Effect** The inhibition of platelet aggregation of NQ301 after a single oral administration into SD rat is shown in Fig. 2. The inhibition of ADP- and collagen-induced platelet aggregation in NQ301-treated groups was statistically significant from the control group (\( p<0.01 \)) by 34% and 31%, respectively.

**In Vivo Antithrombotic Effect** NQ301 showed significant protection from death due to pulmonary thrombosis in...
Table 1. IC50 Values of NQ301 on Human Platelet Aggregation in Vitro

<table>
<thead>
<tr>
<th>Aggregating agents</th>
<th>NQ301 (μM)</th>
<th>Aspirin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP (20 μM)</td>
<td>3.21±1.10</td>
<td>&gt;10000*</td>
</tr>
<tr>
<td>Collagen (200 μg/ml)</td>
<td>16.69±3.73</td>
<td>164.61±41.31</td>
</tr>
<tr>
<td>Epinephrine (10 μM)</td>
<td>8.44±2.58</td>
<td>70.45±0.96</td>
</tr>
<tr>
<td>A23187 (10 μM)</td>
<td>44.20±2.37</td>
<td>&gt;10000*</td>
</tr>
</tbody>
</table>

IC50 values were calculated from at least 3 separate experiments. * Less than 50% inhibition at 1000 μM. The results are expressed as mean±S.D.

Fig. 2. Effect of Oral Administration of NQ301 on Platelet Aggregation in Rats

Samples (90 mg/kg NQ301, 0.5% CMC) were orally administered and platelet aggregation was induced by ADP (1.3 μM) or collagen (32.8 μg/ml). * p<0.01. Mean±S.D. (n=5-7).

Table 2. Effect of NQ301 on Pulmonary Thrombosis in Mice

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (mg/kg)</th>
<th>No. killed or paralyzed/No. tested</th>
<th>Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Vehicle</td>
<td>18/21</td>
<td>14.3</td>
</tr>
<tr>
<td>NQ301</td>
<td>50</td>
<td>8/20**</td>
<td>60.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4/19**</td>
<td>79.0</td>
</tr>
<tr>
<td>Aspirin</td>
<td>50</td>
<td>10/19*</td>
<td>47.4</td>
</tr>
</tbody>
</table>

The samples were orally administered 90 min before tail vein injection of epinephrine (132 μg/mouse) plus collagen (124 μg/mouse). * p<0.01, ** p<0.001.

Table 3. Effect of NQ301 on the Mouse Tail Bleeding Time

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (mg/kg)</th>
<th>Tail bleeding time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Vehicle</td>
<td>59.4±3.1</td>
</tr>
<tr>
<td>NQ301</td>
<td>50</td>
<td>237.6±28.1*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>288.2±34.5*</td>
</tr>
<tr>
<td>Aspirin</td>
<td>50</td>
<td>234.4±22.8*</td>
</tr>
</tbody>
</table>

The samples were orally administered 90 min before the test. The values are expressed as mean±S.D. (n=10). * p<0.01.

Table 4. Effect of NQ301 on Human Plasma Coagulation Time (s)

<table>
<thead>
<tr>
<th>Sample</th>
<th>APTT (s)</th>
<th>PT (s)</th>
<th>TT (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>50.0±0.1</td>
<td>80.0±0.1</td>
<td>120.0±0.1</td>
</tr>
<tr>
<td>NQ301</td>
<td>40.0±0.1</td>
<td>70.0±0.1</td>
<td>100.0±0.1</td>
</tr>
<tr>
<td></td>
<td>60.0±0.1</td>
<td>100.0±0.1</td>
<td>150.0±0.1</td>
</tr>
</tbody>
</table>

The activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) were assayed by adding samples to human platelet poor plasma 7 min before initiating activation and coagulation. Results are expressed as mean±S.D. (n=3). N.C. means no coagulation. * p<0.0001. ** p<0.0005.

Discussion

The results of the present study indicate that 2-chloro-3-(4-acetophenyl)-amino-1,4-naphthoquinone (NQ301), a newly synthesized 1,4-naphthoquinone derivative, significantly inhibits human platelet aggregation in vitro and ex vivo in rats, prevents death due to pulmonary thrombosis, and prolongs tail bleeding time in mice in vivo, but it does not affect the coagulation parameters such as APTT, PT and TT. These results show that the antithrombotic activity of NQ301 may be mediated by the inhibition of platelet aggregation, and it may not act directly on the release of thromboplastin and/or thrombin formation.

In the present in vitro antiplatelet study, NQ301 potently inhibited human platelet aggregation induced by ADP, collagen, epinephrine, and calcium ionophore A23187, whereas aspirin, a reference drug, only inhibited collagen- and epinephrine-induced human platelet aggregation but failed to inhibit ADP- and A23187-induced platelet aggregation. These results suggest that the mode of inhibitory action of NQ301 on platelet aggregation may be different from that of aspirin. Aspirin is an antithrombotic drug widely used for clinical antiplatelet therapy, such as prophylaxis or prevention of the recurrence of thrombosis and is assessed to be effective in some cases of stroke and ischemia. However, aspirin has several clinical disadvantages including gastrointestinal side-effects and hemorrhage. This appears to be ascribed to the fact that aspirin produces its antiplatelet effect by inhibiting cyclooxygenase activity, but it also affects blood...
vessels and decreases the production of prostaglandin (PG) I₂, a biological substance that inhibits the formation of thrombi in blood vessels at the same time. Considering the importance of thrombosis in cardiovascular diseases, the search for better antithrombotic drugs continues.

In the present antithrombotic study, NQ301 significantly prevented death due to pulmonary thrombosis induced by platelet aggregation in a dose-dependent manner. The bleeding time was also examined to investigate the effect of NQ301 on the hemostatic system in blood vessels. NQ301 showed significant prolongation of the mouse tail bleeding time compared to the control. The results of the ex vivo study in rats indicate that NQ301 has a significant inhibitory effect on platelet aggregation when administered orally. These results indicate that NQ301 may be a novel antithrombotic compound.

Liao et al. previously reported that 1,4-naphthoquinone derivatives such as 2-chloro-3-methyl-1,4-naphthoquinone (CMN), 3-methyl-5,8-dihydroxy-1,4-naphthoquinone, 5,8-dihydroxy-1,4-naphthoquinone, and 3-methyl-1,4-naphthoquinone (vitamin K₃) inhibited the aggregation of washed rabbit platelets. Ko et al. reported the antplatelet mechanism of a synthetic compound, 2-chloro-3-methoxybenzylpropionamido-1,4-naphthoquinone (PP1D-I). PP1D-I inhibited the aggregation and ATP release in washed rabbit platelets concentration-dependently. Chang et al. reported the effects of 2-[(4-cyanophenyl)amino]-3-chloro-1,4-naphthalenedione (NQ-Y15), a synthetic 1,4-naphthoquinone derivative, on platelet activity and its mechanism of action. NQ-Y15 caused a concentration-dependent inhibition of platelet aggregation. The results of the above 1,4-naphthoquinone derivatives support the findings of the antplatelet activities of NQ301.

The antplatelet mechanism of NQ301 is not yet clear. It was already 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. It was reported that 2-chloro-3-methoxybenzylpropionamido-1,4-naphthoquinone exerted antplatelet effects by inhibiting phosphoinoside turnover. It was reported that the antplatelet effect of 2-[(4-cyanophenyl)amino]-3-chloro-1,4-naphthalenedione is due to a combination of thromboxane (TX) A₂ synthase inhibition with TXA₂ receptor blockade.

The platelet LDH release was assayed to examine whether the antplatelet aggregation activity of NQ301 was due to damage to the platelet membrane. No significant increase in released LDH has been shown compared to the vehicle group. NQ301 did not affect the coagulation parameters such as APTT, PT and TT.

In conclusion, these results suggest that NQ301 has antithrombotic activity, and the mode of antithrombotic action may be due to antplatelet activity, but not to in vitro anticoagulation activity.

REFERENCES