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# Anti-platelet Aggregation and Anti-thrombotic Effects of Marine Natural Products Sargahydroquinoic Acid and Sargaquinoic Acid

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Platelets circulate in blood and their activity is regulated by nitric oxide (NO) and prostaglandin $I_2$ ($PGI_2$) released from endothelial cells in a quiescent state under physiological conditions.[1,2] However, when platelets are activated at a site of vascular injury, they form a platelet plug and release various molecules that assist in vessel repair, which involves the stages of platelet adhesion, activation, and aggregation. These elements play a key role in preventing blood loss.[3] Platelets are also responsible for the formation of pathological thrombi through excessive activation after atherosclerotic plaque rupture and endothelial cell erosion.[4] The pathogenic thrombi cause acute symptoms in patients with atherothrombotic disease, such as acute coronary syndrome (ACS), including non-ST-elevation (NSTE) and ST-elevation (STE) myocardial infarction (MI) with unstable angina, ischemic stroke, and peripheral arterial disease (PAD).[5-7] In both type I and II diabetes, platelets exhibit increased aggregation activity and adhesiveness, which is referred to as diabetic thrombocytopathy, due to increased thromboxane $A_2$ ($TXA_2$) synthesis, the expression of adhesion molecules (*e.g.*, glycoprotein [GP] IIb/IIIa and P-selectin), and decreasing of nitric oxide and prostacyclin production. These changes finally result in the development of cardiovascular disease.[8,9] Platelets also interact with endothelial cells and leukocytes to promote inflammation, which contributes to atherosclerosis.[10]

Multiple pathways are involved in platelet activation, including those activated by collagen, thrombin, $TXA_2$, serotonin (5HT), and adenosine diphosphate (ADP).[1,11] In the initiation phase of primary hemostasis or thrombosis, platelet activation is mediated by interactions between the GP Ib/V/IX receptor complex on the platelet surface with the von Willebrand factor (vWF), and GP VI/Ia with the sub-endothelial collagen matrix at sites of vascular injury.[3,12] When activated platelets adhere to sites of vascular injury, local platelet activating factors, including ADP, $TXA_2$, 5HT, and thrombin, help to recruit additional circulating platelets to extend and stabilize the hemostatic plug.[12] Interactions between ADP and the ADP receptor ($P2Y_{12}$) result in platelet aggregation and thrombus growth.[13] $TXA_2$ is released from adherent platelets, and amplifies the platelet adhesion response by binding to thromboxane receptor $\alpha$ ($TP\alpha$) and $TP\beta$ on the platelet surface.[11] Thrombin activates platelets by binding to the protease-activated receptor (PAR-1) on the platelet surface.[14] Therefore, receptors or pathways for platelet-activating factors located on the platelet surface are significant targets for anti-platelet therapy for the treatment of cardiovascular diseases, and constitute rational approaches for the development of novel anti-platelet drugs (APD).[1,12]

Recently, several APDs have been applied as anti-platelet therapies for the treatment of atherothrombotic diseases.[15] Aspirin is an irreversible cyclooxygenase-1 (COX-1) inhibitor that blocks $TXA_2$ production, with clinical benefits in ACS and percutaneous coronary intervention (PCI), as well as the prevention of acute ischemic events.[1,16] ADP receptor antagonists, such as ticlopidine, clopidogrel, prasugrel, and cangrelor, inhibit the activation of $P2Y_{12}$-mediated platelet activation and these agents are used clinically in single or dual anti-platelet therapy.[12,15] GP IIb/IIIa antagonists interfere with platelet cross-linking and clot formation and are also used in the treatment of ACS.[3,12] Although APDs, including aspirin, ADP receptor antagonists, and GP IIb/IIIa antagonists have been widely used in the treatment of atherothrombotic diseases and recommended for the management of patients who have experienced either MI or ischemic stroke,[9,12] these agents are associated with significantly high risks including fatal or nonfatal bleeding, neutropenia, agranulocytosis, recurrent ischemic events, and thrombocytopenia.[17-19] The risk of recurrent thrombotic events and bleeding with currently available APD therapies underscores

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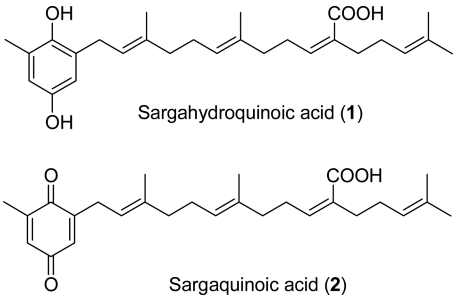


**Figure 1.** The Structures of sargahydroquinoic acid (**1**) and sargaquinoic acid (**2**).

the continued need for the development of new anti-platelet agents with no or fewer risk factors.[7,15]

In order to discover novel drugs to satisfy the unmet-medical needs of current APDs, we designed a screen for platelet aggregation inhibiting compounds by using a collagen-induced platelet aggregation test from the extract library and single natural product library derived from marine natural products collected from the South Korean East Sea. From the results of the screening, we found that two known marine natural products, sargahydroquinoic acid (SHQA, **1**) and sargaquinoic acid (SQA, **2**), strongly inhibit collagen-induced platelet aggregation. In this report, we present the results of our *in vitro* and *in vivo* studies SHQA and SQA.

SHQA and SQA, which are major constituents of *Sargassum micracanthum*[20] and *Sargassum yezoense*[21] collected from the East Sea in South Korea, have been reported to have various activities, including vasodilatation effects,[20] PPARa/g dual agonistic effects,[21] antioxidant activity,[22,23] α-glucosidase inhibitory effects,[24,25] antitumor activity,[26] and anti-malarial activity.[27] They have also been isolated from terrestrial natural source, *Roldana barba-johannis*, and showed a growth inhibition effect in insects.[28] Interestingly, SQA (2) isolated from *Iryanthera juruensis* selectively inhibited COX-1 over COX-2 in an *in vitro* study.[29] Given the findings from previous studies on the biological properties of SHQA and SQA, these compounds appear to have strong potential for diverse medical applications after *in vivo* studies, toxicological studies, and clinical trials. However, because of their scarcity in both marine and terrestrial sources, additional studies for their potential medical use are very difficult. On the basis of the yields reported in previous papers,[21-29] they are purified from their natural source extracts at yields of only 0.01-0.1%. Indeed, we also encountered this difficulty when conducting *in vivo* and toxicity studies of SHQA and SQA for clinical trials. Since then, we discovered that SHQA is extraordinarily abundant in *Sargassum yezoense* collected from a specific port of the East Sea in South Korea, with a yield of approximately 4.3% based on dry weight.[30] This finding enables additional studies, such as preclinical and clinical tests, to be carried out in an effort to develop medical applications from SHQA and SQA. In addition, we also describe a synthetic method for synthesizing SQA from SHQA, which is very useful as SQA is available only in extremely small amounts from natural sources.

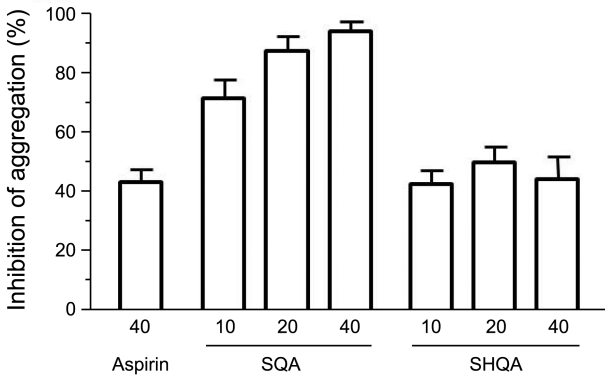


**Figure 2.** Effect of SQA and SHQA against collagen-induced platelet aggregation *in vitro*. Platelet aggregation was induced by collagen (2 μg/mL) and the resulting aggregation was recorded at 3 min. SQA and SHQA were treated with concentrations of 10, 20, 40 μg/mL. Aspirin (40 μg/mL) was used as a positive control. Data are represented mean ± SD (n = 4).

To determine the anti-platelet aggregation activity of SHQA and SAQ, freshly isolated rat platelets were pre-incubated with SQA and SHQA (10, 20, and 40 μg/mL), and aspirin (40 μg/mL), which served as a positive control. The platelets were, then exposed to collagen (2 μg/mL) to induce aggregation.[31] As shown in Figure 2, SQA and SHQA inhibited the collagen-induced platelet aggregation in a dose-dependent manner. In addition, the percentage inhibition by SQA was 94.0 ± 3.3% at 40 μg/mL, which was much stronger than the effect observed with aspirin on the collagen-induced platelet aggregation. SHQA (44 ± 7.6% inhibiion) showed a similar effect to aspirin (43.0 ± 4.5% at 40 μg/mL, n = 4). Notably, SQA at a concentration of 40 μg/mL showed a stronger inhibitory effect than both SHQA and aspirin.

As seen in the results shown in Figure 2, SQA had a higher potency than SHQA, suggesting that SQA may be a possible effective and novel anti-platelet aggregation drug candidate. Therefore, prior to starting *in vivo* studies, we needed to obtain sufficient quantities of SQA. Although the yield of SQA from local *S. yezoense* was relatively higher than other sources, the natural quantity of SQA available was still not sufficient to conduct *in vivo* experiments. To address this challenge, we synthesized SQA from SHQA that was purified from *S. yezoense* extracts *via* a hydroquinone-based oxidation reaction, as shown in Table 1.

Synthetic SQA was obtained at a yield of 51% from the auto-oxidation with 35% hydrogen peroxide as an oxygen source in the catalytic amount of sodium nitrite ($NaNO_2$)/hydrogen chloride. These reaction conditions were very mild and inexpensive, without the presence of a harmful metal oxidation agent; however, the yield was not satisfactory (entry 1).[32] To improve the yield, the oxidation reaction of SHQA was conducted with chromium trioxide ($CrO_3$) and acetic acid as a strong oxidant, and the resulting yield was relatively high at 86% (entry 2).[33] The scaled-up reaction with 10 g starting material using the chromium(VI) condition was satisfactory for obtaining sufficient quantities of

**Table 1.** Oxidation reaction conditions for the synthesis sargaquinoic acid (**2**) from sargahydroquinoic acid (**1**).

Sargahydroquinoic acid (**1**) —Oxidative conditions→ Sargaquinoic acid (**2**)

| Entry | Reagents | Solvent | Temperature | Times | Yield (%)[a] |
|---|---|---|---|---|---|
| 1 | $NaNO_2$, $H_2O_2$ (35%), HCl (cat.) | Dichloromethane | RT | 2.5 h | 51 |
| 2[b] | $CrO_3$ | Acetic acid | 0 °C | 2 h | 86 |
| 3 | $Ce(SO_4)_2$, $SiO_2$ | Dichloromethane | RT | 24 h | 50 |
| 4 | Ammonium cerium(IV) nitrate, $SiO_2$ | Dichloromethane | RT | 24 h | 66 |
| 5[c] | $CrO_3$ | Acetic acid | 0 °C | 5 h | 74 |

[a]Isolated yield. [b]Starting material 1 g scale. [c]Starting material 10 g scale

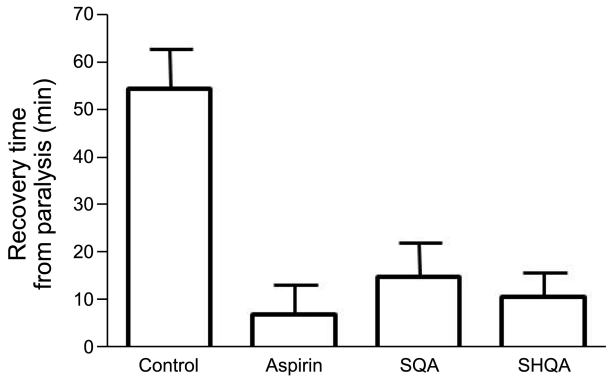


**Figure 3.** Effects of SQA and SHQA on pulmonary thrombosis-mediated paralysis in mouse. Control means the mouse was injected with collagen (8 mg/kg) and epinephrine (160 μg/mL) without any drugs such as aspirin, SQA, or SHQA. SQA and SHQA (5 mg/kg) were intravenously injected for 10 min before the intravenous injection of collagen (8 mg/kg) and epinephrine (160 μg/mL) into the tail vein of ICR mouse. Aspirin (5 mg/kg) was used as a positive control. Antithrombotic efficacy was evaluated by recovery time from paralysis. Data are represented mean ± SD (n = 4).

SQA for our *in vivo* study, although the yield was slightly decreased (entry 5). In an attempt to identify mild oxidation conditions, we tried cerium sulfate/$SiO_2$ (entry 3) and ammonium cerium (IV) nitrate (CAN)/$SiO_2$ (entry 4), but the yield was not satisfactory.[34] The spectral data of synthetic SQA coincided with that of natural SQA previously published by our group.[20] The synthetically derived SQA and natural SHQA were used in the subsequent *in vivo* anti-thrombotic effect experiments.

To evaluate the *in vivo* anti-thrombotic effects of SQA and SHQA, we used the pulmonary thromboembolism model induced by the intravenous injection of collagen and epinephrine. The death or paralysis effect of collagen-induced pulmonary thrombosis was shown to be caused by massive occlusion of the pulmonary microcirculation *via* platelet thromboembolism.[35] SQA/SHQA (5 mg/kg) and aspirin (5 mg/kg), which served as a positive control, were intravenously administered into the tail veins of ICR mice. After 10 min, a mixed solution of collagen (8 mg/kg) and epinephrine (160 μg/kg) was injected into the tail vein to induce pulmonary thrombosis, which resulted in mouse paralysis for a period of 40 min or death. In the present study, the anti-thrombotic effect was evaluated by the recovery time (RT) from paralysis. As shown in Figure 3, the RTs of SQA, SHQA, and aspirin were 6.8 ± 6.2, 14.7 ± 7.2, and 10.3 ± 5.2 min (n = 4), respectively, and showed a fast recovery compared with the positive control, aspirin (54.3 ± 8.3 min, n = 4). However, negative control groups (n = 4) in Figure 3 untreated with any positive control drug (aspirin) or drug candidates (SQA or SHQA) were dead not recovering from paralysis after treatment collagen (8 mg/kg) and epinephrine (160 μg/kg). These results suggested that SQA and SHQA may be useful in the treatment or prevention of atherothrombotic diseases caused by platelet-mediated pathogenic thrombi. Moreover, SQA and SHQA are promising lead structures or anti-platelet agents.

In conclusion, on the basis of the results of the *in vitro* platelet aggregation inhibition test, we discovered that of SHQA (**1**) and SQA (**2**) show a strong inhibitory effect on platelet aggregation. To enable preclinical and clinical studies to be conducted, we synthesized SQA from natural SHQA in high yield. In addition, we confirmed that SHQA (**1**) and SQA (**2**) show a fast recovery time from paralysis in the mouse pulmonary thromboembolism model, indicating that they are strong, novel anti-platelet drug candidates. As bleeding is a main side effect of the APDs used clinically, we also plan to conduct a bleeding test with SHQA and SQA and will report these results in a future proper paper.

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30. The brown alga, *Sargassum yezoense*, was collected at the Jangho Harbor, Gangwon, South Korea, in March 2008, at a depth of 2-3 m. A voucher specimen (sample No. MNP-O-78) has been deposited at the Marine Biomedical Research Center, Kwandong University College of Medicine, Gangwon, South Korea, under the supervision of Professor Gab-Man Park. After purification of the ethyl acetate extract from 10 g of dried *Sargassum yezoense* powder using flash column chromatography with hexane/ethyl acetate (5:2), 433 mg and 87 mg of SHQA (isolated yield, 4.3%) and SQA (isolated yield, 0.9%) were obtained, respectively.
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35. Diminno, G.; Silver, M. J. J. *Pharmacol. Exp. Therap*. **1983**, *225*, 57. Male ICR mice (Orient BIO Inc.) were used in the *in vivo* anti-thrombotic assay. The study adhered to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by the Committee for the Care and Use of Laboratory Animals of Kwandong University. The anti-thrombotic effects of SQA/SHQA were evaluated using the mouse pulmonary thrombosis assay, as described previously. In brief, SQA/SHQA (5 mg/kg) and aspirin (5 mg/kg), which served as a positive control, were intravenously administered into the tail veins of ICR mice weighing 25-28 g. After 10 min, a mixed solution of collagen (8 mg/kg) and epinephrine (160 μg/kg) was injected into the tail veins to induce pulmonary thrombosis, which resulted in mouse paralysis for a period of 40 min or death. In this study, the anti-thrombotic efficacy of SQA/SHQA and aspirin were evaluated based on the recovery time from pulmonary thrombosis-induced paralysis compared with the control, aspirin.