Abstract

Sanguinarine is a plant alkaloid present in the root of Sanguinaria canadensis and Poppy fumaria species. Sanguinarine has been used as an antiseptic mouth rinse and a toothpaste additive to reduce dental plaque and gingival inflammation. In this study, we investigated the antiplatelet effects of sanguinarine, aiming to extend its potential pharmacological applications. Sanguinarine inhibited platelet aggregation induced by arachidonic acid (AA), collagen, U46619 and sub-threshold concentration of thrombin (0.05 U/ml) with IC50 concentrations of 8.3, 7.7, 8.6 and 4.4 μM, respectively. Sanguinarine (5–10 μM) inhibited 10–31% of platelet TXB2 production, but not platelet aggregation induced by higher concentration of thrombin (0.1 U/ml). SQ29548, a thromboxane receptor antagonist, inhibited the AA-induced platelet aggregation but not TXB2 production. Sanguinarine suppressed cyclooxygenase-1 (COX-1) activity (IC50 = 28 μM), whereas its effect on COX-2 activity was minimal. Sanguinarine (8, 10 μM) further inhibited the AA-induced Ca2+ mobilization by 27–62%. In addition, SQ22536, an adenylate cyclase inhibitor, attenuated the inhibitory effect of sanguinarine toward AA-induced platelet Ca2+ mobilization and aggregation. These results suggest that sanguinarine is a potent antiplatelet agent, which activates adenylate cyclase, inhibits platelet Ca2+ mobilization, TXB2 production as well as suppresses COX-1 enzyme activity. Sanguinarine may have therapeutic potential for treatment of cardiovascular diseases related to platelet aggregation.

Keywords: Sanguinarine; Antiplatelet; Calcium; cAMP; Cyclooxygenase; Thromboxane; Bleeding time; Platelet aggregation

1. Introduction

Sanguinarine (13-methyl-[l,3]benzodioxolo[5,6-c]-1,3-dioxolo[4,5-1] phen-anthridinium) is a benzophenanthridine alkaloid present in the root of plants Sanguinaria canadensis and other Poppy fumaria species [1]. Sanguinarine possesses antimicrobial, antioxidant as well as anti-inflammatory properties [2]. Sanguinarine also exhibits antitumor and anti-inflammatory activities in experimental animals and may inhibit neutrophil functions, including degranulation and phagocytosis in vitro [2–4]. Sanguinarine chloride is capable of lowering the levels of both ICAM-1 and VCA-1, two crucial factors in the pathogenesis of inflammatory diseases such as allergic asthma, arthritis, nephritis and
pneumonia [7]. Moreover, it is also a potent inhibitor for Na-K-dependent ATPase, cholinesterase [5,6], NF-kB and mitogen-activated protein kinase phosphatase-1 [8,9].

From human exposure studies, a number of chemicals have been evaluated for their capacities to inhibit dental plaque and promote gingival health. Sanguinarine is currently used as a mouth rinse solution or as an additive in the toothpaste with acceptable transient mouth burning sensation in some patients and could be potentially long-term used clinically. Extensive clinical trials have shown that oral rinse and toothpaste products (Viadent) containing sanguinaria extract are effective against the formation of dental plaque and gingivitis [10–12]. In addition, sanguinarine is also used as cough and cold remedies and homeopathic preparations [13], probably due to its antimicrobial and anti-inflammatory properties. Therefore, the pharmacological utilization of this plant-derived alkaloid awaits further investigation.

Platelets play central roles in control of thrombosis and bleeding via platelet adhesion, activation and aggregation [14]. Various agonists including ADP, thrombin, collagen, and prostaglandin endoperoxides may induce platelet aggregation [15]. When blood vessels are injured, platelets will adhere to the disrupted area and release some bioactive molecules from intracellular storage granules may exacerbate aggregation by recruiting additional platelets into the aggregate and lead to propagation of a gross thrombus [16]. Disorders of platelet function may contribute to many life-threatening diseases including atherosclerosis, cerebro-vascular thrombosis, coronary heart disease and tumor metastasis [17–19]. However, little is known about the effect of sanguinarine on the health of cardiovascular system. Since sanguinarine as a herbal medicine could reduce tissue inflammation and improve blood circulation, we further tested whether sanguinarine may exhibit anti-inflammatory and antplatelet effect via inhibition of cyclooxygenase (COX) enzyme activity and a decline in platelet thromboxane production.

2. Materials and methods

2.1. Materials

Sanguinarine, Achilles tendon type I collagen, thrombin, arachidonic acid (AA), bovine serum albumin (BSA), indomethacin, EDTA (disodium), sodium citrate and U46619 were purchased from Sigma. Collagen (type I) was homogenized in 25 mM acetic acid and then stored at −70 °C prior to use. TXB2 ELISA kits and COX enzyme inhibitor screening assay kits were obtained from Cayman Chemical Company. The animal experiments using rabbits and mice were reviewed and approved by the Ethical Committee of Animal Experiments, Chang Gung Institute of Technology and was conducted according to the Guidelines for Animal Experiments in National Taiwan University.

2.2. Platelet aggregation assay

Washed rabbit platelets (3 × 10⁸ platelets/ml) were prepared as mentioned previously [20–22] and suspended in Tyrodes solution comprising 1 mM of CaCl₂ and 0.35% BSA. Sanguinarine dissolved in DMSO (same volume of DMSO as control) was then added into platelets, preincubated for 3 min and platelet aggregation was measured by addition of AA (100 µM), collagen (10 µg/ml), thrombin (0.05 and 0.1 U/ml) or U46619 (1 µM) by the turbidimetric method of Born and Cross [23]. The extent of platelet aggregation was recorded by an aggregometer (Model 600B, Payton Associates, Ont., Canada) and the percentage of platelet aggregation was calculated as described by Teng and Ko [24]. In some experiments, platelets were pretreated with SQ29548 (a thromboxane receptor antagonist) or aspirin (200 µM) prior to the addition of AA to clarify the role of thromboxane receptor activation in AA-induced platelet aggregation for comparison.

2.3. Thromboxane B₂ assay

Platelets were incubated with sanguinarine (or placebo) for 3 min and then treated with AA, collagen, thrombin (0.1 U/ml) or U46619 as described above [20,21]. Thereafter, EDTA (2 mM) and indomethacin (50 µM) were sequentially added into platelet suspension. After brief centrifugation in an eppendorf centrifuge (Model 5414) at 14,000 rpm for 2 min, thromboxane B₂ (TXB₂) levels in the supernatant were measured with ELISA kits (Cayman) according to manufacturer’s instruction.

2.4. Effects of sanguinarine on cyclooxygenase activity

The inhibitory potential of sanguinarine on enzyme activities of COX-1 and COX-2 was determined by using the COX inhibitor screening assay kits according to the manufacturer’s instruction. In short, sanguinarine or DMSO (as the control) was directly incubated with COX-1 or COX-2 enzyme in a reaction buffer for 10 min. Thereafter, AA was added and the reaction continued for another 2 min. Then, 0.1 N HCl and saturated stannous fluoride solution were added immediately to stop the enzymatic reaction. The amount of PGE₂ generation by COX was measured using the Cayman ELISA kits. The effect of sanguinarine on COX activity was evaluated by comparing the amounts of PGE₂ production between reactions without and with sanguinarine.

2.5. Effect of sanguinarine on Ca²⁺ mobilization in platelets

To further clarify the mechanisms responsible for antplatelet effect of sanguinarine, intraplatelet-free calcium [Ca²⁺]i was measured using the method described previously with slight modification [25]. Briefly, platelets were incubated in 4 µM Fura-2 AM at 37 °C for 30 min. Thereafter,
platelets were washed in HEPES buffer to remove unloaded dye and then re-suspended in HEPES containing 1 mM CaCl$_2$ at a cell density of $3 \times 10^8$/ml. Fura-2 loaded platelets were treated with sanguinarine alone or with AA. Fura-2 fluorescence was determined by a F4500 Fluorometer (Hitachi, Japan) equipped with thermo-stated cell holder and stirrer device. Two excitation wavelengths, 340 and 380 nm, were used with an emission at 510 nm and the ratio was analyzed automatically by computer software. Fluorescence was calibrated with lysed platelets (0.2% Triton X-100) in the absence and presence of 10 mM EGTA in each run to obtain maximum and minimum fluorescence values.

2.6. Effect of SQ22536 on AA-induced platelet aggregation and Ca$^{2+}$ mobilization

Platelets were prepared for aggregation and Ca$^{2+}$ mobilization as described above. They were pretreated with SQ22536 (125, 250 and 500 nM) for 3 min and followed by the addition of sanguinarine (final 10 μM). Platelet aggregation and Ca$^{2+}$ mobilization were measured by addition of AA to elucidate whether antiplatelet effect of sanguinarine was mediated by stimulation of cAMP production. Platelets exposed to SQ22536 and then AA was used for control.

2.7. Statistical analysis

Three or more independent experiments were performed. The data were analyzed and expressed as mean ±S.E.M.
Results were expressed as either percent inhibition or percent of control (as 100%). Fifty-percent inhibitory concentration (IC$_{50}$) of sanguinarine against agonist-induced platelet function was calculated with a regression analysis. Whenever appropriate, paired Student’s *t*-test was used to demonstrate statistical significance ($p<0.05$) between different groups.

3. Results

3.1. Effect of sanguinarine on platelet aggregation

Sanguinarine showed inhibitory effects on AA-, collagen- and thrombin-induced platelet aggregation. As shown in one representative aggregation assay, sanguinarine showed dose-dependent inhibition on AA-induced platelet aggregation, whereas thrombin (0.1 U/ml, Thr)-induced platelet aggregation was only slightly inhibited by 10 [$\mu$]M of sanguinarine ($p>0.05$) (Fig. 1). Quantitatively, sanguinarine effectively inhibited the AA-induced platelet aggregation with a 50% inhibitory concentration (IC$_{50}$) of 8.3 [$\mu$]M. Sanguinarine also suppressed the collagen-induced platelet aggregation with an IC$_{50}$ of about 7.7 [$\mu$]M (Fig. 2B). Sanguinarine showed only mild inhibitory effect (10%) on thrombin (0.1 U/ml)-induced platelet aggregation at a concentration of 10 [$\mu$]M (Fig. 2C). However, sanguinarine could inhibit platelet aggregation induced by a sub-threshold concentration of thrombin (0.05 U/ml) with an IC$_{50}$ concentrations of 4.4 [$\mu$]M.

3.2. Effect of sanguinarine on platelet TXB$_2$ production

Sanguinarine inhibited the AA-induced TXB$_2$ production, with a calculated IC$_{50}$ concentration of 4.5 [$\mu$]M (Fig. 3A). Similarly, the IC$_{50}$ concentration for sanguinarine or DMSO (solvent, as a control) was incubated with COX-1 or COX-2 for 10 min and then AA was added. COX enzyme activities were reflected by the amount of PGE$_2$ produced. PGE$_2$ production was measured by ELISA. Enzyme activity in the sample without sanguinarine treatment (DMSO alone) was served as control and results were expressed as % of COX-1 and COX-2 enzyme activity in control (as 100%) (mean ± S.E.M.). Asterisk (*) denotes statistically significant difference when compared with solvent control ($p<0.05$).

Fig. 4. Direct effect of different concentrations of sanguinarine on the enzyme activities of COX-1 (panel A) and COX-2 (panel B). Sanguinarine or DMSO (solvent, as a control) was incubated with COX-1 or COX-2 for 10 min and then AA was added. COX enzyme activities were reflected by the amount of PGE$_2$ produced. PGE$_2$ production was measured by ELISA. Enzyme activity in the sample without sanguinarine treatment (DMSO alone) was served as control and results were expressed as % of COX-1 and COX-2 enzyme activity in control (as 100%) (mean ± S.E.M.). Asterisk (*) denotes statistically significant difference when compared with solvent control ($p<0.05$).
ine on collagen-induced platelet TXB2 production was about 5.7 μM (Fig. 3B). On the other hand, sanguinarine inhibited the thrombin (0.1 U/ml)-induced platelet TXB2 production by 10–31% at concentrations of 5–10 μM (Fig. 3C).

3.3. Effect of sanguinarine on COX-1 and COX-2 enzyme activity

As shown in Fig. 4A, COX-1 enzyme activity was reduced by 40, 100 and 200 μM of sanguinarine, with a calculated IC50 concentration of about 28 μM. However, sanguinarine showed little inhibitory effect on COX-2 enzyme activity even at a concentration of 200 μM (Fig. 4B).

3.4. Roles of thromboxane receptor activation in platelet aggregation

SQ29548 (2–20 μM), a TXA2 receptor antagonist, suppressed AA-induced platelet aggregation, with complete inhibition at concentrations higher than 10 μM (IC50 = 3.5 μM) (Fig. 5A). Aspirin (200 μM), as a positive control, also inhibited AA-induced platelet aggregation (data not shown). Interestingly, SQ29548 (2–20 μM) could not suppress the AA-induced platelet TXB2 production (Fig. 5B). For further evaluation the antiplatelet effect of sanguinarine, we found that it effectively suppressed the U46619-induced platelet aggregation (IC50 about 8.6 μM) (Fig. 5C). Unlike the induction by AA, platelet aggregation induced by U46619 showed little stimulatory effect on platelet TXB2 production, indicating that TXA2 production is possibly not the major factor for induction of platelet aggregation by U46619 (data not shown).

3.5. Effect of sanguinarine on AA-induced platelet calcium mobilization

Since Ca2+ mobilization is a crucial factor for TXB2 production and platelet aggregation [26], we therefore tested whether sanguinarine may suppress AA-induced Ca2+ mobilization. As shown in Fig. 6A, AA evidently stimulated the Ca2+ mobilization in platelets as indicated by an increase in the ratio of fluorescence intensities between excitation wavelengths of 340 and 380 nm. Quantitatively, sanguinarine inhibited the AA-induced Ca2+ mobilization by 27 and 62%, respectively, at concentrations of 8 and 10 μM (Fig. 6B).

3.6. Effect of SQ22536 on inhibitory effect of sanguinarine toward AA-induced platelet Ca2+ mobilization and aggregation

Activation of adenylyl cyclase and cAMP production has been shown to inhibit platelet aggregation [27]. We therefore tested whether SQ22536, an adenylyl cyclase inhibitor, could reverse the inhibitory effect of sanguinarine toward AA-induced platelet aggregation and Ca2+ mobilization.
Fig. 6. Effect of sanguinarine on Ca\textsuperscript{2+} mobilization in platelets. Ca\textsuperscript{2+} mobilization was measured by the ratio of the fluorescence intensities of Fura-2 AM-labeled platelets at excitation wavelengths of 340 nm:380 nm. Platelets were loaded with fluorescence dye Fura-2 AM, treated with different concentrations of sanguinarine and then activated with AA. Fura-2 fluorescence was determined by a F4500 Fluorometer (Hitachi, Japan). (A) Fluorescence 340/380 ratio for Fura-2 AM-labeled platelets from a representative experiment. The 340/380 ratio decreased with an increase of sanguinarine. (B) Quantification of the percentage inhibition (mean ± S.E.M.) by sanguinarine toward AA-induced platelet Ca\textsuperscript{2+} mobilization was shown by the histogram (n = 7).

itself showed little effect on AA (100 μM)-induced platelet aggregation (data not shown).

4. Discussion

Many life-threatening diseases including atherosclerosis, cerebrovascular thrombosis, coronary artery disease, stroke, tumor metastasis are correlated to platelet dysfunctions [17–19,28]. Antiplatelet agents have been used for treatment of these diseases [28]. However, little is known about the effect of sanguinarine on platelet functions. Sanguinarine has been used as a mouth rinse solution against dental plaque formation and gingival inflammation as well as for treatment of cough/common cold [10–13,29]. In this study, we further demonstrated that sanguinarine exhibited antiplatelet effect, suggesting its potential pharmacological use in prevention and treatment of cardiovascular diseases. Interaction of platelets with collagen at sites of vascular injury may activate platelet adhesion, activation, secretion of platelet granular contents and final aggregation [30], leading to atherosclerosis and thromboembolism. AA-metabolites may contribute to the pathogenesis of these cardiovascular, pulmonary, inflammatory and thromboembolic diseases [31]. Agonists other than collagen, such as thrombin, fibrinogen, von Willebrand Factor and soluble agonists released from activated platelets or other cells (TXA\textsubscript{2} and ADP) are also involved in platelet aggregation [30]. Sanguinarine inhibited
the platelet aggregation as well as TXB2 production triggered by AA and collagen. This suggests that TXB2 production is crucial for AA- and collagen-induced platelet aggregation and sanguinarine attenuated aggregation of platelets by inhibition of TXB2 production. We further found that sanguinarine effectively inhibited platelet aggregation induced by 0.05 U/ml (sub-threshold concentration) thrombin but not 0.1 U/ml thrombin. These differential inhibitory effects of sanguinarine on platelet aggregation induced by different concentrations of thrombin may be due to the presence of different thrombin receptors in platelets. The complex signaling transduction pathways may thus lead the antiplatelet effect on thrombin difficult [32–34]. This notion is supported by the observation that other antiplatelet agents such as betel leaf extract, cresol, and *piper betle* inflorescence extract also have different effects on AA-, collagen- and thrombin-induced platelet TXB2 production and aggregation [20–22].

COX-1 and COX-2 play crucial role in a number of physiological processes such as homeostasis of gastrointestinal tract, blood clotting, pain, inflammation and fever [35]. However, little is known about the effect of sanguinarine on COX activity. Since most of the platelet TXB2 production by AA was associated with activation of COX-1 and thromboxane synthase, which cannot be inhibited by SQ29548 (Fig. 5B), inhibition of COX-1 in platelets by sanguinarine in our study may partly explain why sanguinarine may suppress platelet TXB2 production and therefore attenuate platelet aggregation. The concentrations of sanguinarine needed to inhibit COX-1 are higher than that to inhibit platelet aggregation and TXB2 production in this study, suggesting the presence of discrepancy between the test tube

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**Fig. 7.** Effect of SQ22536 on AA-induced Ca\(^{2+}\) mobilization and aggregation in platelets. (A) Fura-2 loaded platelets were exposed to AA-, AA plus sanguinarine or pretreated with SQ22536 for 3 min, followed by addition of sanguinarine (final 10 \(\mu\)M) and AA. Ca\(^{2+}\) mobilization (340/380 ratio changes, as mean ± S.E.M.) in platelets was measured for comparison (\(n = 7\)). (B) Platelet suspension was incubated with AA-, AA plus sanguinarine or pretreated with SQ22536 (250, 500 \(\mu\)M) for 3 min, followed by addition of sanguinarine (final 10 \(\mu\)M) and AA. One representative platelet aggregation histogram was shown. (C) Platelet aggregation (mean ± S.E.M.) by AA-, AA plus sanguinarine and its attenuation by SQ22536 (125, 250 and 500 \(\mu\)M) (\(n = 4\)). \(P < 0.05\) indicates marked difference between groups.
COX activity reaction and in vitro platelet thromboxane production or other inhibitory mechanisms by sanguinarine. This should be further addressed in the future. A number of studies have found that mouth rinse solution and toothpaste containing sanguinarine are effective in reducing dental plaque formation and gingivitis [10–12,29]. Sanguinarine-containing toothpaste and mouth rinse solution are also able to inhibit the redevelopment of gingivitis. Patients in the study group have 26% fewer gingival bleeding sites at 14 weeks than that of control group [36]. However, Grossman et al. [37] further found that sanguinarine mouth rinse reduce the plaque formation by 24%, whereas it showed little effect on control of gingival bleeding. Antimicrobial and anti-inflammatory properties of sanguinarine have been suggested to be responsible for these clinical efficacies. Sanguinarine concentrations in the mouthrinse and toothpaste contain about 0.03–0.075% sanguinaria extract in which about 33% is sanguinarine. The estimated sanguinarine concentration in the mouth rinse and toothpaste is about 300–750 μM [29] that is high enough to inhibit COX-1 activity in platelets. Although COX-2 inhibition by sanguinarine was not evident, the anti-gingivitis effect is possible due to antimicrobial effect and also partially associated with COX-1 inhibition, because recently both COX-1 and COX-2 are considered to be contributing factors of tissue inflammation [38].

SQ29548, a TXA2 receptor antagonist, suppressed AA-induced platelet aggregation but lacked inhibitory effect on AA-induced TXB2 production. This suggests that positive feedback activation of TXA2 receptors by TXA2 is essential for AA-induced platelet aggregation. However, most AA-induced platelet TXB2 production was generated via metabolism of AA by COX-1, but not mediated by positive feedback activation of TXA2 receptor. Consistently IBI-P-05006, the other thromboxane receptor antagonist, may inhibit both U46619 and AA-induced platelet aggregation but show little effect on AA metabolism [39]. In this study, we further found that sanguinarine not only inhibited TXB2 production but also inhibited a thromboxane receptor agonist (U46619)-induced platelet aggregation, suggesting that sanguinarine inhibited AA-induced aggregation through inhibition of COX-1 and thromboxane production as well as other downstream signaling following thromboxane receptor activation.

Sanguinarine may suppress the phenylephrine-induced vaso-contraction and Ca2+ mobilization in rat aorta [40]. However, stimulating mice skeletal muscle contracture by sanguinarine is shown to be associated with sarcoplasmic reticulum calcium release [41]. Since adenylate cyclase activation and Ca2+ mobilization are crucial for platelet TXB2 production and aggregation [26,27], we further tested whether sanguinarine may affect the Ca2+ mobilization by using AA as the inducer. We found that AA-induced Ca2+ mobilization was obviously inhibited by sanguinarine. This indicates that sanguinarine may block cellular Ca2+ mobilization related to platelet aggregation. Moreover, activation of adenylate cyclase may suppress platelet activations [27]. Interestingly, SQ22536 at higher concentrations was able to attenuate the AA-induced Ca2+ mobilization and platelet aggregation in our study, but failed to affect the platelet aggregation induced by AA. Similarly, PGEi and sodium nitroprusside have been elucidated to stimulate cAMP production, which interferes Ca2+ mobilization and platelet aggregation [27,42]. SQ22536 also showed little effect on U46619-induced primary platelet secretion [43] and ADP-induced platelet aggregation [44]. This reveals that attenuation of AA-induced Ca2+ mobilization and platelet aggregation by sanguinarine is possibly correlated to activation of adenylate cyclase and cAMP production. Basal levels of cAMP do not directly contribute to AA-induced platelet aggregation.

In conclusion, sanguinarine exhibits antiplatelet effect by inhibition of TXB2 production and Ca2+ mobilization, which is possibly correlated to adenylate cyclase activation. Sanguinarine was also able to suppress COX-1 enzyme activity. Sanguinarine has been shown to induce an endothelium-independent vaso-relaxation in isolated rat thoracic aorta [40]. However, sanguinarine (2.3–65 μM) may increase contractility but inhibit Na+/K+ ATPase in isolated guinea pig atria myocardium [45] and also induce the contracture in isolated cardiac muscle strip from Wistar rat [46], indicating potential cardiotoxicity. Though sanguinarine has been used as a mouth rinse solution or for cough remedy, further studies are necessary to evaluate the pharmacological and toxicological effect of sanguinarine on human health.

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References


