

Anticoagulant and antiplatelet activities of scolymoside

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***Cyclopia subternata* is a medicinal plant commonly used in traditional medicine to relieve pain. Here, the anticoagulant effects of scolymoside, an active compound in *C. subternata*, were examined by monitoring activated partial thromboplastin time (aPTT), prothrombin time (PT), and the activities of thrombin and activated factor X (FXa). The effects of scolymoside on plasminogen activator inhibitor type 1 (PAI-1) and tissue-type plasminogen activator (t-PA) expression were evaluated in tumor necrosis factor (TNF)- α -activated human endothelial cells. Treatment with scolymoside resulted in prolonged aPTT and PT and the inhibition of thrombin and FXa activities and production. In addition, scolymoside inhibited thrombin-catalyzed fibrin polymerization and platelet aggregation. Scolymoside also elicited anticoagulant effects in mice, including a significant reduction in the PAI-1 to t-PA ratio. Collectively, these findings indicate that scolymoside possesses anticoagulant activities and could be developed as a novel anticoagulant. [BMB Reports 2015; 48(10): 577-582]**

INTRODUCTION

Herbal teas are natural beverages containing biological active compounds that can promote health owing to their potential in vivo biological properties (1, 2). As an endemic South-African fynbos plant, *Cyclopia subternata* Vogel (Family: Fabaceae; Tribe: Podalrieae) has been used as an herbal tea called honeybush after "fermentation," a high-temperature oxidative process required to produce its characteristic sweet aroma and flavor (3). The health-promoting properties of the active compounds in *C. subternata* that have been documented include antioxidant and anti-inflammatory activity and the enhancement of recognition memory (4-6). It is known that abundant

flavonoids are contained within *C. subternata*, particularly scolymoside (SCL) (7).

There exists a well-known relationship between diet and health; food contributes significantly to the maintenance of good health (8) and is regarded as functional health foods if they benefit several functions in nutritional effects (9). When consumed in adequate amounts on a consistent basis, functional health food could decrease the vascular inflammatory diseases such as cardiovascular diseases (CVDs) by reducing lipid levels, oxidation of low-density lipoprotein, formation of plaque, and platelet aggregation, or improving arterial compliance (9, 10). This fact has garnered great interest in the search for natural bioactive compounds that contribute to platelet inhibition. Interestingly, many natural bioactive compounds from functional foods have been reported to exhibit antiplatelet activity.

In addition to previous reports demonstrating the potential antioxidant effects of SCL, we hypothesized that SCL may have anticoagulant activities. To date, it was not reported the effects of SCL on coagulation. Therefore, we investigated the anticoagulant and antiplatelet activities of SCL in terms of activated factor X (FXa) and thrombin production and its effects on prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinolytic activity.

RESULTS AND DISCUSSION

SCL (SCL, luteolin-7-O-rutinoside) is a compound found in *C. subternata* plants, which are traditionally used for the production of the South African herbal tea honeybush, and recently their aqueous extracts have been used in the food industry (3). Here, we determined the anticoagulant and antiplatelet effects of SCL (Fig. 1A) and attempted to identify the mechanisms responsible for these effects.

Effects of SCL on clotting and bleeding times

The incubation of human plasma with SCL altered coagulation properties. The anticoagulant activities of SCL were tested with human plasma using aPTT and PT assays (Table 1). Although the anticoagulant activities of SCL were weaker than those of heparin and warfarin, aPTT and PT were significantly prolonged by SCL ($\geq 5 \mu\text{M}$). Prolongation of aPTT suggests the inhibition of intrinsic and/or common pathway, and PT pro-

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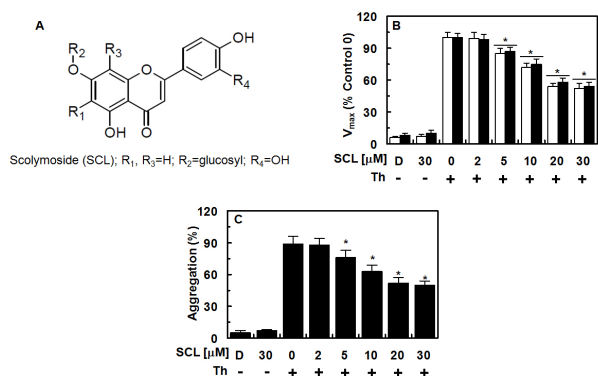


Fig. 1. Chemical structure of SCL and effects of SCL on cytotoxicity and fibrin polymerization in human plasma. (A) Structure of scolymoside (SCL). (B) Thrombin (Th, white box)- or reptilase (Rep, black box)-catalyzed fibrin polymerization at the indicated concentrations of SCL was monitored using a catalytic assay, as described in the “Materials and Methods” section. The results are V_{max} values expressed as percentages versus controls. (C) Effect of SCL on mouse platelet aggregation induced by 3 U/mL thrombin. D = 0.2% DMSO is the vehicle control. Data represent the mean \pm SEM of three independent experiments performed in triplicate. * $P < 0.05$ vs. Th alone (B, C) or reptilase (B).

longation suggests the inhibition of extrinsic and/or common pathway. SCL treatment at 27.7 μ M and 24.8 μ M were required to double clotting time for aPTT and PT, respectively. Therefore, the results obtained in this study indicate that SCL could also inhibit the common coagulation pathway. To confirm these *in vitro* results, *in vivo* tail bleeding times were assessed. Because the average mouse body weight is 20 g and average blood volume is 2 mL, administration of 11.9, 23.8, or 35.7 μ g SCL/mouse produced concentrations of approximately 10, 20, or 30 μ M in peripheral blood, respectively. Data showed that *in vivo* bleeding times were prolonged significantly by SCL in comparison to the controls (Table 1). We confirmed this data *ex vivo* aPTT and PT analysis dose dependent manner with SCL (Table 1).

Effects of SCL on fibrin polymerization and cellular viability

Then, we tested the effects of SCL on thrombin-catalyzed fibrin polymerization in purified human plasma, as described in the Materials and Methods section. Fig. 1B shows that SCL significantly decreases the maximal rate of fibrin polymerization. 50 mM TBS (pH 7.4) and same volume of DMSO were used to exclude the possibility that pH and volume could affect the

Table 1. Anticoagulant activity of scolymoside^a

<i>In vitro</i> coagulant assay				
Sample	Dose	aPTT (s)	PT (s)	PT (INR)
Control Scolymoside	Saline	22.4 \pm 0.4	12.4 \pm 0.2	1.00
	1 μ M	22.6 \pm 0.2	12.0 \pm 0.4	0.92
	2 μ M	22.2 \pm 0.2	12.6 \pm 0.6	1.04
	5 μ M	26.4 \pm 0.4*	14.8 \pm 0.4*	1.53*
	10 μ M	34.2 \pm 0.62*	16.8 \pm 0.4*	2.07*
	20 μ M	42.6 \pm 0.2*	21.6 \pm 0.4*	3.79*
	30 μ M	43.0 \pm 0.4*	22.2 \pm 0.2*	4.05*
Heparin	30 μ M	60.2 \pm 0.8*	26.4 \pm 0.4*	6.13*
Warfarin	30 μ M	53.2 \pm 1.2*	24.6 \pm 0.4*	5.18*
<i>In vivo</i> bleeding time				
Sample	Dose	Tail Bleeding time (s)	n	
Control Scolymoside	Saline	31.2 \pm 0.8	10	
	11.9 μ g/mouse	40.4 \pm 1.26*	10	
	23.8 μ g/mouse	52.4 \pm 1.0*	10	
	35.7 μ g/mouse	53.6 \pm 1.2*	10	
Heparin	210.0 μ g/mouse	70.2 \pm 1.4*	10	
Warfarin	18.0 μ g/mouse	62.2 \pm 1.2*	10	
<i>Ex vivo</i> bleeding time				
Sample	Dose	aPTT (s)	PT (s)	PT (INR)
Control Scolymoside	Saline	31.8 \pm 0.4	13.2 \pm 0.6	1.00
	1.2 μ g/mouse	32.0 \pm 0.6	13.4 \pm 0.4*	1.04
	5.9 μ g/mouse	36.4 \pm 1.2*	15.0 \pm 0.8*	1.36*
	11.9 μ g/mouse	42.2 \pm 0.6*	17.6 \pm 0.6*	1.99*
	23.8 μ g/mouse	48.6 \pm 1.2*	20.4 \pm 1.2*	2.84*
	35.7 μ g/mouse	50.4 \pm 1.0*	21.4 \pm 1.0*	3.19*

^aEach value represents the means \pm SEM (n=5).

* $P < 0.05$ as compared to control.

obtained results, respectively. Data was not affected by two factors (data not shown). To eliminate the effects of thrombin on the reduction in fibrin production rather than the polymerization of formed fibrin, a reptilase was used to induce fibrin polymerization. Data show that reptilase-catalyzed fibrin polymerization was significantly decreased by SCL (Fig. 1B). To determine the effect of SCL on cellular viability, a cellular viability assay (MTT assay) was performed in human endothelial cells treated with SCL for 24 h. SCL did not affect cell viability at concentrations up to 50 μM (data not shown).

Effects of SCL on platelet aggregation catalyzed by thrombin

To confirm the anticoagulant activities of SCL, a thrombin was used to catalyze the platelet aggregation. In washed murine platelets, SCL treatment (0-30 μM) for 5 min inhibited the platelet aggregation induced by thrombin (3 U/mL) with an IC_{50} value of 29.4 μM in a concentration-dependent manner. The 5-min incubation period was chosen because an optimal inhibitory effect of SCL was observed at 5 min.

Effects of SCL on the activities of thrombin and FXa

In order to determine the underlying mechanism of SCL-mediated inhibition of coagulation, we tested the effects of SCL on

the activities of thrombin and FXa. Data showed that the activity of thrombin was inhibited by SCL treatment (Fig. 2A), suggesting the direct inhibition of thrombin activity by SCL. The direct thrombin inhibitor argatroban was used as a positive control. We also tested the effects of SCL on the activity of FXa. SCL inhibited the activity of FXa (Fig. 2B). The direct FXa inhibitor rivaroxaban was used as a positive control. Therefore, Fig. 2 A and B indicated that SCL exerts its anticoagulant effect by inhibiting fibrin polymerization and/or the intrinsic/extrinsic pathway.

Effects of SCL on thrombin and FXa production

Next, we determined the effects of SCL on the production of thrombin and FXa, which are the most important enzymes in the blood coagulation cascade, to define the anticoagulant mechanism of SCL. Incubation of HUVECs with SCL inhibited the production of thrombin by FVa and FXa in the presence of CaCl_2 (Fig. 2C). Next, we also investigated the effects of SCL on the activation of FX by FVIIa. Cells were stimulated with $\text{TNF-}\alpha$ to induce TF expression, and as shown in Fig. 2D, the rate of FX activation by FVIIa was 17-fold higher in stimulated HUVECs (95.2 ± 7.2 nm) than that in non-stimulated HUVECs (5.3 ± 1.0 nm). This increase in activation was abrogated by anti-TF IgG (17.2 ± 2.4 nm). In addition, pre-incubation with SCL resulted in a dose-dependent inhibition of FX activation by FVIIa (Fig. 2D). Therefore, these results suggest that SCL can inhibit the production of thrombin and FXa.

Effects of SCL on the secretion of PAI-1 and t-PA protein

$\text{TNF-}\alpha$ inhibited the fibrinolytic system in human endothelial cells by mediating the PAI-1 production and disturbing the harmony between PAI-1 and t-PA, which is known to modulate coagulation and fibrinolysis (11, 12). In order to test the direct effects of SCL on $\text{TNF-}\alpha$ -stimulated PAI-1 secretion, cells were cultured in media with or without SCL in the absence or presence of $\text{TNF-}\alpha$ for 18 h. Data showed that SCL treatment inhibited $\text{TNF-}\alpha$ -induced PAI-1 secretion from HUVECs in a dose-dependent manner, with a significant decrease observed at concentrations of 5 μM and above (Fig. 3A).

Because $\text{TNF-}\alpha$ has no significant effect on the production of t-PA (13) and the balance between plasminogen inhibitors and their activators reflects net plasminogen-activating capacity (14-16), we tested the effect of $\text{TNF-}\alpha$ with SCL on t-PA secretion from human endothelial cells. Data were consistent with those of a previous study, showing a modest decrease in the production of t-PA by $\text{TNF-}\alpha$ in HUVECs (17). This decrease was not significantly altered by treatment with SCL (Fig. 3B). Therefore, these results suggest that the PAI-1/t-PA ratio increased after $\text{TNF-}\alpha$ treatment and that SCL prevented this increase (Fig. 3C).

There are several beneficial characteristics associated with SCL use as opposed to standard pharmaceutical products, if SCL is developed as an anticoagulant drug. First, SCL may exhibit less side effects or toxicities. Most herbal medicines are

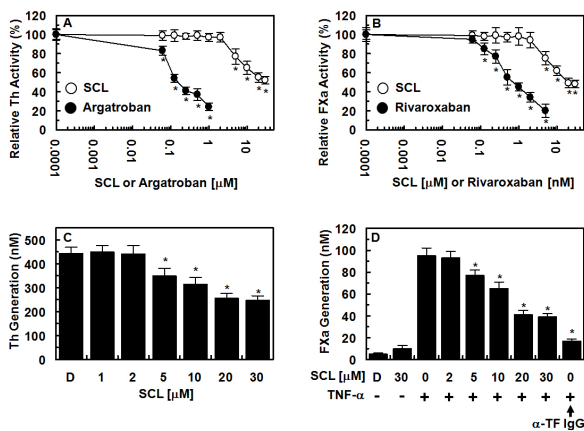


Fig. 2. Effects of SCL on the inactivation and production of thrombin and factor Xa. (A) Inhibition of thrombin (Th) by SCL was measured using a chromogenic assay, as described in the "Materials and Methods" section. (B) Inhibition of factor Xa (FXa) by SCL was monitored using a chromogenic assay, as described in the "Materials and Methods" section. Argatroban (A) or rivaroxaban (B) was used as positive control. (C) HUVECs monolayers were pre-incubated with FVa (100 pM) and FXa (1 nM) for 10 min with the indicated concentrations of SCL. Prothrombin was added to a final concentration of 1 μM , and prothrombin activation was determined 30 min later, as described in the "Materials and Methods" section. (D) HUVECs were pre-incubated with the indicated concentrations of SCL for 10 min. $\text{TNF-}\alpha$ (10 ng/mL for 6 h)-stimulated HUVECs were incubated with FVIIa (10 nM) and FX (175 nM) in the absence or presence of anti-TF IgG (25 $\mu\text{g}/\text{mL}$), and FXa production was determined as described in the "Materials and Methods" section. * $P < 0.05$ vs. 0.0001 (A, B), D (C) or $\text{TNF-}\alpha$ alone (D).

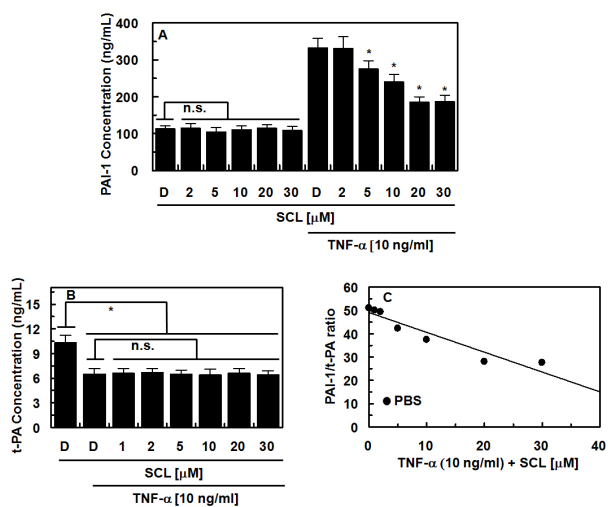


Fig. 3. Effects of SCL on the secretion of PAI-1 and tPA. (A) HUVECs were cultured with SCL in the absence or presence of TNF- α (10 ng/mL) for 18 h, and PAI-1 concentrations in culture media were determined as described in the "Materials and Methods" section. (B) HUVECs were cultured with SCL in the absence or presence of TNF- α (10 ng/mL) for 18 h, and t-PA concentrations in culture media were determined as described in the "Materials and Methods" section. (C) The PAI-1/t-PA ratio in TNF- α -activated HUVECs from (A) and (B). D = 0.2% DMSO is the vehicle control. *P < 0.05 vs. TNF- α alone or D; n.s., not significant.

well tolerated by patients and display fewer side effects than pharmaceutical drugs (18). The second advantage of SCL use is its low cost. Herbs are inexpensive compared to prescription medications. Research, testing, and marketing considerably add to the cost of prescription medicines. Finally, the pre-clinical evaluation of the antithrombotic potential of novel molecules requires the use of reliable and reproducible experimental models. PT, aPTT, fibrin polymerization, and platelet aggregation are the most established and commonly used preparations to determine the efficacy of novel anticoagulant drugs (19, 20).

As a commercial anticoagulant, heparin has been used for the prevention of venous thromboembolic diseases for more than 60 years (21, 22). However, many side effects of this drug have been observed, including the inability to inhibit thrombin bound to fibrin, ineffectiveness in congenital or acquired antithrombin deficiencies, development of thrombocytopenia, increased risk of thromboembolic disease when the therapeutic response is not achieved, and increased risk of bleeding when the therapeutic range is exceeded (22, 23). Furthermore, the amount of available heparin is low in bovine lung and pig intestine, where heparin is primarily extracted (23). Therefore, the necessity of discovering alternative anticoagulant sources has arisen with the increasing demand for safer anticoagulant therapy. Based on the current findings and advantages of herb-

al medicines, SCL could replace current agents if they demonstrate successful therapeutic effects.

In conclusion, the results of this study demonstrate that SCL inhibited the extrinsic and intrinsic blood coagulation pathways through the inhibition of FXa and thrombin production in HUVECs. Additionally, these results show that SCL inhibited TNF- α -induced secretion of PAI-1. These results add to previous work on the topic, and should be of interest to those designing pharmacological strategies for the treatment or prevention of vascular diseases.

MATERIALS AND METHODS

Reagents

Scolymoside was purchased from Sigma (St. Louis, MO). TNF- α was purchased from Abnova (Taiwan). Anti-tissue factor antibody was purchased from Santa Cruz Biologics (Santa Cruz, CA). Factor V, VII, VIIa, FX, FXa, antithrombin III (AT III), prothrombin, and thrombin were obtained from Haematologic Technologies (Essex Junction, VT). aPTT assay reagent and PT reagents were purchased from Fisher Diagnostics (Middletown, Virginia), and the chromogenic substrates, S-2222 and S-2238, were purchased from Chromogenix AB (Sweden). Rivaroxaban (direct FXa inhibitor) and argatroban (direct FIIa inhibitor) were purchased from Santa Cruz Inc. (Dallas, Texas). PAI-1 and t-PA ELISA kits were purchased from American Diagnostica Inc. (Stamford, CT). Other reagents were of the highest commercially available grade.

Anticoagulation assay

aPTT and PT were determined using a Thrombotimer (Behnk Elektronik, Germany), according to the manufacturer's instructions, as described previously (24-26). Citrated normal human plasma (90 μ l) was mixed with 10 μ l of scolymoside and incubated for 1 min at 37°C. PT results are expressed in seconds and as International Normalized Ratios (INR), and aPTT results are expressed in seconds. INR = (PT sample / PT control)^{ISI}. ISI = international sensitivity index.

Platelet aggregation assay

Platelet-rich plasma (PRP) was obtained from syngeneic donor mice by double centrifugation (200 g to create platelet/plasma phase, 500 g to pellet platelets). The platelet-rich plasma was adjusted to a concentration of 1×10^9 platelets/mL with use of a hemocytometer for cell counts. The platelet aggregation study was carried out according to a method previously reported (26, 27). Washed platelets were incubated with indicated scolymoside in TBS for 3 min, and then stimulated by thrombin (3 U/mL, Sigma) in 0.9% saline solution at 37°C for 5 min. Platelet aggregation was recorded using an aggregometer (Chronolog, Havertown, PA).

Thrombin or reptilase-catalyzed fibrin polymerization

Thrombin-catalyzed polymerization was determined every 6 s

for 20 min by monitoring turbidity at 360 nm using a spectrophotometer (TECAN, Switzerland) at ambient temperature. Control plasma and plasma incubated with scolymoside were diluted three times in TBS (50 mM Tris-buffered physiological saline solution pH 7.4) and clotted with thrombin or reptilase (final concentration - 0.5 U/mL). The maximum polymerization rate (V_{max} , $\Delta OD/min$) of each absorbance curve was recorded (28). All experiments were performed three times.

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bio Science (Charles City, IA) and were maintained using a previously described method (25, 26, 29, 30).

Animals and husbandry

Male C57BL/6 mice (6-7 weeks old, weighing 18-20 g), purchased from Orient Bio Co. (Sungnam, Republic of Korea), were used after a 12-day acclimatization period. Animals were housed five per polycarbonate cage under controlled temperature (20-25°C) and humidity (40-45%) and a 12:12 h light:dark cycle. Animals received a normal rodent pellet diet and water ad libitum during acclimatization. All animals were treated in accordance with the 'Guidelines for the Care and Use of Laboratory Animals' issued by Kyungpook National University (IRB No. KNU2012-13).

Factor Xa production on the surfaces of HUVECs

TNF- α (10 ng/mL for 6 h in serum-free medium) stimulated confluent monolayers of HUVECs (preincubated with the indicated concentrations of scolymoside for 10 min) in a 96-well culture plate were incubated with FVIIa (10 nM) in buffer B (buffer A supplemented with 5 mg/mL bovine serum albumin [BSA] and 5 mM CaCl₂) for 5 min at 37°C in the presence or absence of anti-TF IgG (25 μ g/mL). FX (175 nM) was then added to the cells (final reaction mixture volume, 100 μ l) and incubated for 15 min. The reaction was stopped by addition of buffer A (10 mM HEPES, pH 7.45, 150 mM NaCl, 4 mM KCl, and 11 mM glucose) containing 10 mM EDTA and the amounts of FXa generated were measured using a chromogenic substrate. Changes in absorbance at 405 nm over 2 min were monitored using a microplate reader. Initial rates of color development were converted into FXa concentrations using a standard curve prepared with known dilutions of purified human FXa.

Thrombin production on the surfaces of HUVECs

Measurement of thrombin production by HUVECs was quantitated as previously described (31, 32). Briefly, HUVECs were pre-incubated in 300 μ l containing scolymoside in 50 mM Tris-HCl buffer, 100 pM FVa, and 1 nM FXa for 10 min, followed by addition of prothrombin to a final concentration of 1 μ M. After 10 min, duplicate samples (10 μ l each) were transferred to a 96-well plate containing 40 μ l of 0.5 M EDTA in

Tris-buffered saline per well in order to terminate prothrombin activation. Activated prothrombin was determined by measuring the rate of hydrolysis of S2238 at 405 nm. Standard curves were prepared using amounts of purified thrombin.

Thrombin activity assay

Scolymoside in 50 mM Tris-HCl buffer (pH 7.4) containing 7.5 mM EDTA and 150 mM NaCl was mixed in the absence or presence of 150 μ l of AT III (200 nM). Heparins with AT III (200 nM) were dissolved in physiological saline and placed in the sample wells. Following incubation at 37°C for 2 min, thrombin solution (150 μ l; 10 U/mL) was added, followed by incubation at 37°C for 1 min. S-2238 (a thrombin substrate; 150 μ l; 1.5 mM) solution was then added and absorbance at 405 nm was monitored for 120 s using a spectrophotometer (TECAN, Switzerland).

Factor Xa (FXa) activity assay

These assays were performed in the same manner as the thrombin activity assay, but using factor Xa (1 U/ mL) and S-2222 as substrates.

In vivo bleeding time

Tail bleeding times were measured using the method described previously (24-26). Briefly, C57BL/6 mice were fasted overnight before experiments. One hour after intravenous administration of scolymoside, tails of mice were transected at 2 mm from their tips. Bleeding time was defined as the time elapsed until bleeding stopped. When the bleeding time exceeded 15 min, bleeding time was recorded as 15 min for the analysis. All animals were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by Kyungpook National University (IRB No. KNU2012-13).

Ex vivo clotting time

Male C57BL/6 mice were fasted overnight and scolymoside in 0.5% DMSO was administered by intravenous injection. One hour after administration, arterial blood samples (0.1 mL) were withdrawn into 3.8% Na-citrate (1/10; v/v) for ex vivo aPTT and PT determination.

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