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Revisiting the effect of cholesteryl sulfate on clotting and fibrinolysis: Inhibition of human thrombin and other human blood proteases

Rami A. Al-Horani

Division of Basic Pharmaceutical Sciences, College of Pharmacy, Xavier University of Louisiana, New Orleans LA 70125, USA

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ABSTRACT

Cholesteryl sulfate (CS) was quantitatively synthesized by microwave-assisted sulfonation of cholesterol followed by sodium exchange chromatography. In vitro effects of CS on human thrombin and other serine proteases of the coagulation and fibrinolysis processes were investigated using a series of biochemical and biophysical techniques. CS was found to inhibit thrombin with an IC_{50} value of 140.8 ± 21.8 μM at pH 7.4 and 25 °C. Michaelis-Menten kinetics indicated that thrombin inhibition by CS is non-competitive (allosteric) in nature. Fluorescence-based binding studies indicated that CS binds to thrombin with a K_D value of 180.9 ± 18.9 μM. Given the lack of competition with heparins and a hirudin peptide in competitive inhibition assays, it appears that CS does not bind to thrombin's exosites 1 or 2 and it rather recognizes a different allosteric exosite. CS was found to partially inhibit thrombin-mediated fibrinogen activation with an IC_{50} value of 175.5 ± 17.5 μM and efficacy of ~26.0 ± 6.6%. Likewise, CS selectively doubled the activated partial thromboplastin time with EC_{2x} of 521 μM. Interestingly, CS was found to also inhibit factors Xa and XIa as well as plasmin with IC_{50} values of ~85–250 μM and efficacy of 94–100%. Nevertheless, CS most potently inhibited factor XIIa with an IC_{50} Value of ~17 μM and efficacy of 60%. Surprisingly, CS did not inhibit factor IXa. These results encourage further *in vitro* and *in vivo* investigation of CS to better understand its (patho-) physiological roles in coagulation and hemostasis.

1. Introduction

Cholesterol sulfate (CS) is the most abundant steroidal sulfo-conjugate in human plasma. The sulfate moiety of CS has a pKa of ~3.3, indicating that it is predominantly ionized under physiologic conditions. Thus, CS has also been detected in many other biological fluids such as urine, bile, and seminal plasma as well as in many tissues. Recent advances have revealed important roles for CS in cellular membranes, lipid metabolism, inflammation, immune response, and signaling pathways [1–6]. Importantly, CS regulates the activity of a variety of enzymes. For example, it was reported that CS inhibits bovine thrombin and human plasmin, two serine proteases involved in coagulation and fibrinolysis, respectively [7]. It also suppressed sterol synthesis in cultured human fibroblasts by acting as an inhibitor of HMG CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis [8]. CS was also found to modulate the specificity of phosphatidylinositol-3-kinase [9]. CS has also been reported to inhibit the pancreatic serine proteases trypsin and chymotrypsin [10,11] as well as yeast pronase [10]. It also inhibited DNase I [12] and pancreatic elastase [13].

E-mail address: ralhoran@xula.edu.

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Accordingly, CS (and other sulfated oxysterol) appears to be a key player in many biological pathways affecting human health and disease. Recessive X-linked ichthyosis has been linked to cholesterol sulfatase deficiency resulting in the accumulation of CS, which was found to inhibit serine proteases involved in cell dissociation during skin development. Furthermore, the ability of CS to inhibit thrombin and plasmin suggested that CS can act as an endogenous modulator of hemostasis [7]. Moreover, CS has been found to promote divalent cation-independent adhesion of both activated and inactivated platelets [14].

In this study, we employed a series of biochemical and biophysical techniques to further investigate the *in vitro* effects of CS on human thrombin and other serine proteases of the coagulation and fibrinolysis processes. Results indicate that CS does not only inhibit human thrombin, but it also inhibits factors Xa, XIa, and XIIa in the intrinsic coagulation pathway as well as plasmin of the fibrinolysis process. The inhibition of thrombin amidolytic activity by CS is uniquely noncompetitive and it translates into partial inhibition of thrombin-mediated activation of fibrinogen as well as into prolongation of the activated partial thromboplastin clotting time (APTT) of human plasma. These results encourage further *in vitro* (for example using thrombo-elastography) and *in vivo* (for example using animal models of venous and arterial thrombosis) investigation of CS to better understand its (patho)physiological roles in coagulation and hemostasis.

2. Materials and methods

Materials. Cholesterol, anhydrous acetonitrile, DMSO-d_6 for NMR, and sulfur trioxide:trimethylamine (SO_3:$N(CH_3)_3$) were from Millipore-Sigma (St. Louis, MO, USA). Human plasma clotting enzymes and plasmin as well as fibrinogen were purchased from Haematologic Technologies (Essex Junction, VT). Factor XIIa was from Enzyme Research Laboratories (South Bend, Indiana). The chromogenic substrates for thrombin, factor Xa, factor IXa, factor XIIa, and plasmin were purchased from Biomedica Diagnostics (Windsor, NS Canada). Factor XIa chromogenic substrate (S-2366) was purchased from Diapharma (West Chester, OH). Reagents for clotting assays including thromboplastin-D, APTT reagent, and thrombin time reagent were all from Fisher Scientific (Pittsburgh, PA, USA). Human plasma was from George King Bio-Medical, Inc. (Overland Park, KS, USA). Bovine unfractionated heparin (UFH) was from Sigma Aldrich, hirudin peptide (HirP, [5 F]-Hir-(54–65)-(SO_3^-)) was from Anaspec (Fremont, CA). Stock solutions of thrombin, factor XIa, factor XIIa, and plasmin were all prepared in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% PEG8000, and 150 mM NaCl. A stock solution of factor IXa was prepared in 20 mM Tris-HCl buffer, pH 7.4, containing 0.1% PEG8000, 2.5 mM $CaCl_2$, 100 mM NaCl, and 33% v/v ethylene glycol. A stock solution of factor Xa was prepared in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% PEG8000.

A Bruker-400 MHz spectrometer was used to record the ^{1}H and ^{13}C NMR in DMSO-d_6. Signals, in part per million (ppm), are relative to the residual peak of the solvent. The NMR data are reported as chemical shift (ppm), integration, multiplicity of signal (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublet, m = multiplet), and coupling constants (Hz). CS was purified using Sephadex G10 size exclusion chromatography. The quaternary ammonium counter ion of sulfate groups present was exchanged for sodium ion using SP Sephadex-Na cation exchange chromatography. Sephadex G10 and SP Sephadex-Na chromatographies were performed using Flex columns (KIMBLE/KONTES, Vineland, NJ) of dimensions 170 × 1.5 cm and 75 × 1.5 cm, respectively. For regeneration of the cation exchange column, 1 L of 2 M NaCl solution was used. Water was used as an eluent in the two chromatographies [15].

Chemical preparation of sodium cholesteryl sulfate (CS). The sulfonation reaction was performed in CEM® microwave by modifying reported conditions [16,17]. To a solution of cholesterol (50 mg, 0.13 mmol) in CH_3CN (2 mL), SO_3:$N(CH_3)_3$ (90 mg, 0.65 mmol) was added under a flow of nitrogen gas in an 8 mL-microwave tube. The tube was then sealed and put in a CEM® microwave reactor. Microwave-assisted sulfonation was carried out at 100 °C for 1 h. The reaction was allowed to cool down before the organic solvent was removed under vacuum by a rotary evaporator. The organic residue was suspended in H_2O:CH_3OH (1:2) mixture and loaded into the short pad of G-10 size exclusion matrix to remove the excess of SO_3:$N(CH_3)_3$ as well as unreacted cholesterol, if any. Fractions containing the trimethylammonium salt of cholesteryl sulfate were collected into a 10-mL centrifuge tube. The salt was then frozen at −80 °C for 2 h and lyophilized overnight to afford a white-yellowish solid salt of mono-sulfonated cholesterol in about 92% yield. To obtain the sodium form (sodium cholesteryl sulfate, CS), the solid was suspended in H_2O:CH_3OH (1:2) and passed through as a short column of sodium exchange matrix. Turbid fractions were collected and lyophilized to afford a white solid of CS in a quantitative yield.

Characterization of CS salts. Trimethylammonium salt. ^{1}H NMR (400 MHz, DMSO-d_6): 5.28 (d, J = 4.96 Hz, 1 H), 3.89–3.81 (m, 1 H), 2.79 (s, 9 H), 2.40–2.36 (dd, J = 13.08 Hz, J = 2.84 Hz, 1 H), 2.18–2.11 (m, 1 H), 1.97–1.87 (m, 3 H), 1.81–1.74 (m, 2 H), 1.55–0.97 (m, 21 H), 0.95 (s, 3 H), 0.88 (d, J = 6.32 Hz, 3 H), 0.85 (d, J = 1.8 Hz, 3 H), 0.83 (d, J = 1.8 Hz, 3 H), 0.65 (s, 3 H). ^{13}C NMR (100 MHz, DMSO-d_6): 140.67, 121.08, 75.36, 56.13, 55.58, 49.56, 44.23, 41.85, 36.86, 36.06, 35.63, 35.13, 31.43, 31.35, 28.78, 27.73, 27.33, 23.83, 23.15, 22.62, 22.36, 20.56, 19.02, 18.54, 11.65. **Sodium salt.** ^{1}H NMR (400 MHz, DMSO-d_6): 5.29 (t, J = 2.44 Hz, 1 H), 3.88–3.80 (m, 1 H), 2.40–2.36 (dd, J = 13.24 Hz, J = 4.76 Hz, J = 2.04 Hz, 1 H), 2.18–2.11 (m, 1 H), 1.98–1.91 (m, 3 H), 1.90–1.78 (m, 2 H), 1.55–1.03 (m, 21 H), 0.96 (s, 3 H), 0.89 (d, J = 6.30 Hz, 3 H), 0.86 (d, J = 1.84 Hz, 3 H), 0.85 (d, J = 1.84 Hz, 3 H), 0.66 (s, 3 H).^{13}C NMR (100 MHz, DMSO-d_6): 140.76, 121.01, 75.20, 56.14, 55.59, 49.58, 41.86, 36.89, 36.08, 35.64, 35.15, 31.45, 31.37, 28.79, 27.74, 27.34, 23.84, 23.17, 22.62, 22.36, 20.56, 19.02, 18.54, 11.66.

Inhibition of human thrombin by CS under different conditions. Direct thrombin inhibition was measured using a chromogenic substrate hydrolysis assay using a microplate reader (FlexStation III, Molecular Devices), as reported earlier [18–20]. These studies were performed using a 20 mM TrisHCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% PEG 8000 at 25 °C or 37 °C in the presence or absence of 0.02% Tween80. Generally, 185 μL of pH 7.4 buffer was added to the wells, and 5 μL of CS (or DMSO) and 5 μL of thrombin (6 nM final concentration) were sequentially added. After 10 min incubation (or no incubation), 5 μL of

thrombin substrate (Spectrozyme® TH, 50 μM final concentration) was rapidly added and the residual thrombin activity was measured from the initial rate of increase in absorbance at 405 nm. Relative residual thrombin activity at each concentration of the inhibitor was calculated from the ratio of thrombin activity in the presence and absence of CS. Logistic Equation (1) was used to fit the dose dependence of residual protease activity to obtain the potency (*IC50*) and efficacy (ΔY%) of inhibition.

$$Y = Y_0 + \frac{Y_M - Y_0}{1 + 10^{(\log\ [I]_0 - \log\ IC_{50})(HS)}} \quad (1)$$

In the above equation, Y is the ratio of residual thrombin activity in the presence of CS to that in its absence, Y_M and Y_0 are the maximum and minimum possible values of the fractional residual human thrombin activity, *IC50* is the concentration of the inhibitor that leads to 50 % inhibition of enzyme activity, and HS is the Hill slope. Y_M, Y_0, IC_{50}, and HS values are determined by nonlinear curve fitting of the data.

Michaelis-Menten kinetics of thrombin inhibition by CS. The initial rate of the tripeptide chromogenic substrate (Spectrozyme® TH) hydrolysis by human thrombin (6 nM) was obtained from the linear increase in A_{405} nm relevant to the consumption of less than 10% of the chromogenic substrate, as documented previously [20–22]. The initial rate was measured as a function of different concentrations of the substrate (0–125 μM) in the presence of a fixed concentration of CS in 20 mM TrisHCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% PEG 8000 at 25 °C with no incubation. The experiment was conducted at four concentrations of CS (0, 17, 125, and 490 μM). The data were fitted using the standard Michaelis–Menten Equation (2) to determine the V_{MAX} (the maximum hydrolysis reaction velocity; y-axis) and the K_M (the affinity of the substrate to the active site of thrombin; x-axis).

$$V = \frac{V_{MAX}\ [S]}{K_M + [S]} \quad (2)$$

CS binding to thrombin. The thrombin – CS dissociation constant was calculated by measuring the change in intrinsic fluorescence emission of thrombin as a function of the concentration of CS in 20 mM Tris Buffer pH 7.4, containing 100 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% PEG 8000 at 25 °C, as previously described using a QM4 fluorimeter (Photon Technology International, Birmingham, NJ) [19–22]. CS solution was titrated into a 200 μL solution of thrombin (300 nM) and the change in the fluorescence at 348 nm (λ_{EX} = 280 nm) was monitored, with the excitation and emission slit width set to 1–1.5 mm. Using the quadratic equilibrium binding Equation (3), the saturable change in the fluorescence signal was fitted to obtain the K_D of interaction. In this equation, ΔF is the change in fluorescence due to the formation of the complex following each addition of CS from the initial fluorescence $F_\circ$, ΔF_{max} represents the maximum change in fluorescence observed on saturation of thrombin and Hill coefficient (n) is a measure for the cooperativity of binding.

$$\frac{\Delta F}{F_O} = \Delta F_{max} \times \frac{[CS]^n}{(K_{D,app})^n + [CS]^n} \quad (3)$$

Competition studies with UFH and HirP. Thrombin inhibition by CS was studied in the presence of the exosite 1 competitor hirudine peptide (HirP) and exosite 2 competitor (UFH), in a manner similar to that described for direct thrombin inhibition [18,20]. Briefly, to a solution of thrombin (6 nM final concentration) in 20 mM TrisHCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% PEG 8000 at 25 °C, was added HirP (75 nM) or UFH (50 and 250 μM). Then, different concentrations of CS were added and after adding the substrate Spectrozyme® TH, the residual thrombin activity was measured using the chromogenic substrate hydrolysis protocol. The dose dependence of the fractional residual thrombin activity at each concentration of CS was fitted using Equation (1) to obtain the apparent *IC50*, HS, Y_O, and Y_M.

Inhibition of thrombin-mediated fibrinogen activation by CS. Direct thrombin-mediated fibrinogen activation inhibition by CS was measured using a turbidity assay in a microplate reader. The study was performed using a 20 mM TrisHCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% PEG 8000. Generally, 88 μL of pH 7.4 buffer was added to the wells, and 5 μL of CS (or DMSO) and 5 μL of thrombin (240 nM) were sequentially added. After 10 min incubation (or no incubation), 2 μL of fibrinogen (5 mg/mL) was rapidly added and the residual thrombin activity was measured from the initial rate of increase in absorbance at 600 nm. Relative residual thrombin activity at each concentration of the inhibitor was calculated from the ratio of thrombin activity in the presence and absence of CS. Logistic Equation (1) was used to fit the dose dependence of residual protease activity to obtain the potency (IC_{50}) and efficacy (ΔY%) of inhibition.

Effect of CS on clotting times of human plasma. Plasma clotting assays of APTT and PT are commonly used to investigate the effects of enzyme inhibitors on human clotting times. In this study, APTT assay is used to measure the effect of CS on the contact/intrinsic pathway-driven clotting which involves factor IXa, factor XIa, and factor XIIa. PT assay is used to measure the effect of CS on the extrinsic pathway of coagulation which involves factor VIIa. These experiments were conducted using the BBL Fibrosystem fibrometer (Becton−Dickinson, Sparles, MD, USA), as reported by our earlier studies [23–26]. In the APTT assay, 90 μL of human plasma was mixed with 10 μL of CS solution (or DMSO) and 100 μL of prewarmed 0.2% ellagic acid. After incubation for 4 min at 37 °C, clotting was initiated by adding 100 μL of prewarmed 0.025 M $CaCl_2$, and the time to clotting was noted. In the PT assay, 90 μL of human plasma was mixed with 10 μL of CS solution (or DMSO) and was subsequently incubated for 30 s at 37 °C. Following the addition of 200 μL of prewarmed thromboplastin-D reagent, the time to clotting was noted. In the two assays, several concentrations of CS were used to construct concentration vs. effect profiles. The data were plotted to a quadratic trendline, which was used to estimate the concentration needed to double the clotting time.

Inhibition of other serine proteases including clotting factors by CS. The inhibition potential of CS toward factors IXa, Xa, XIa,

XIIa, and plasmin were also evaluated using the corresponding chromogenic substrate hydrolysis assays as reported in our recent studies [23–26]. For example, to each well of a 96-well microplate containing 85 μL or 185 μL of 20–50 mM Tris-HCl buffer, pH 7.4, containing 100–150 mM NaCl, 0.1% PEG8000, and 0.02% Tween80 at either 25 °C or 37 °C was added 5 μL of CS (or DMSO) and 5 μL of the enzyme. The final concentrations of the enzymes were: 89 nM for factor IXa, 1.09 nM for factor Xa, 0.77 nM for factor XIa, 5 nM for factor XIIa, and 20 nM for plasmin. Following 5–10 min incubation, 5 μL of the corresponding substrate was rapidly added and the residual enzyme activity was measured from the initial rate of increase in absorbance at λ_{405} nm. The effective concentrations of substrates in microplate cells were: 850 μM for factor IXa, 125 μM for factor Xa, 125 μM for factor XIIa, and 50 μM for plasmin. Relative residual enzyme activity as a function of the concentration of CS was calculated. Data were plotted using Equation (1) above to obtain the corresponding IC_{50} values and other inhibition parameters.

3. Results and discussion

Chemical synthesis and characterization of sodium cholesteryl sulfate (CS). CS was synthesized using a microwave-assisted synthesis, as reported before for the sulfonation of aliphatic and aromatic alcohols [16,17]. Briefly, cholesterol was dissolved in acetonitrile and 5 equivalents of the sulfonating agent sulfur trioxide:trimethylamine (SO_3:$N(CH_3)_3$) (Fig. 1). The reaction was carried out in a microwave tube at 100 °C for 1 h. The resulting trimethylammonium salt of sulfonated cholesterol was isolated and passed through a sodium exchange column to afford the corresponding CS, which was subsequently desalted and lyophilized. It was then dissolved in DMSO-d_6 and characterized by proton and carbon NMR.

Inhibition of human thrombin by CS under different conditions. CS was evaluated for its potential to inhibit thrombin hydrolysis of Spectrozyme® TH, a chromogenic small peptide substrate (H-D-CHA-Ala-Arg-pNA.2AcOH), at 25 °C and pH 7.4, as reported in our previous studies [18,20] The presence of CS resulted in a dose-dependent reduction in thrombin activity (Fig. 2A). The dose-dependence inhibition of thrombin activity could be fitted using the logistic Equation (1), which resulted in an IC_{50} value of 140.8 ± 21.8 μM with an efficacy of ~83.5 ± 5.1% and a Hill slope of 1.6 ± 0.3 (Table 1), at a salt concentration of 100 mM. The inhibition potency did not significantly change when inhibition was evaluated at 37 °C. The presence of 0.02% Tween80 led to a 2-fold loss of inhibition potency (IC_{50} = 279.9 ± 7.1 μM, HS = 2.8 ± 0.4, and efficacy = 76.9 ± 3.3%), suggesting the significance of hydrophobic interactions for the inhibition. Lastly, 10-min incubation of CS with thrombin resulted in a marginal increase in potency (IC_{50} = 228.6 ± 10.2 μM, HS = 3.3 ± 1.2, and efficacy = 71.3 ± 4.2%) in the presence of 0.02% Tween80. Given the limited effect of temperature and incubation, we chose to perform subsequent experiments without incubation at 25 °C and with no Tween80.

CS is an allosteric inhibitor of human thrombin. To identify the mechanistic basis of inhibition, Michaelis–Menten kinetics of H-D-CHA-L-Ala-L-Arg-*p*-nitroaniline diacetate (SPECTROZYME TH®) hydrolysis by thrombin was performed in the presence of CS at pH 7.4. Fig. 2B shows the initial rate profiles in the presence of CS (0–490 μM). Each curve shows a characteristic rectangular hyperbolic dependence, which could be fitted using the Michaelis–Menten equation (Equation (2)) to obtain the apparent K_M and V_{MAX} (Table 2). The K_M for the substrate remained essentially unchanged in the presence or absence of CS, whilst the V_{MAX} decreased steadily from 39.4 ± 3.2 mAU/min in the absence of CS to 10.5 ± 0.8 mAU/min at 490 μM of CS (~4-fold decrease). Therefore, CS appears to bring about structural changes in the active site of thrombin, which does not affect the formation of the Michaelis complex, but induces a significant dysfunction in the catalytic apparatus. This indicates that CS is an allosteric inhibitor of human thrombin using a chromogenic substrate that is more specific to thrombin. A previous study provided similar results using thrombin from bovine plasma and using the substrate t-butyloxycarbonyl-[(2*S*)-2-amino-3-(benzyloxycarbonyl) propionyl]-L-Pro-L-Arg-MCA [7].

CS binds human thrombin inducing a protein conformational change. To investigate the interaction between CS and human thrombin, the intrinsic fluorescence of thrombin was monitored as a function of CS concentration. Fig. 3A shows a characteristic sigmoidal dependence of thrombin's intrinsic fluorescence on the concentration of CS. The profile can be fitted well by the standard, three-parameter Hill equation, which gives the maximal fluorescence change (ΔF_{MAX}), the Hill coefficient (n) and the apparent

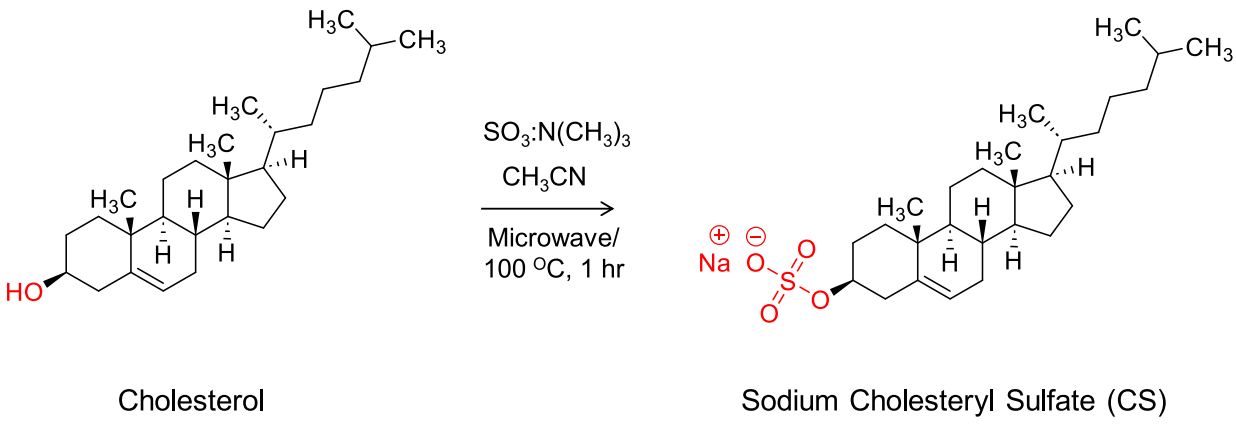

Fig. 1. Microwave-assisted chemical synthesis of CS.

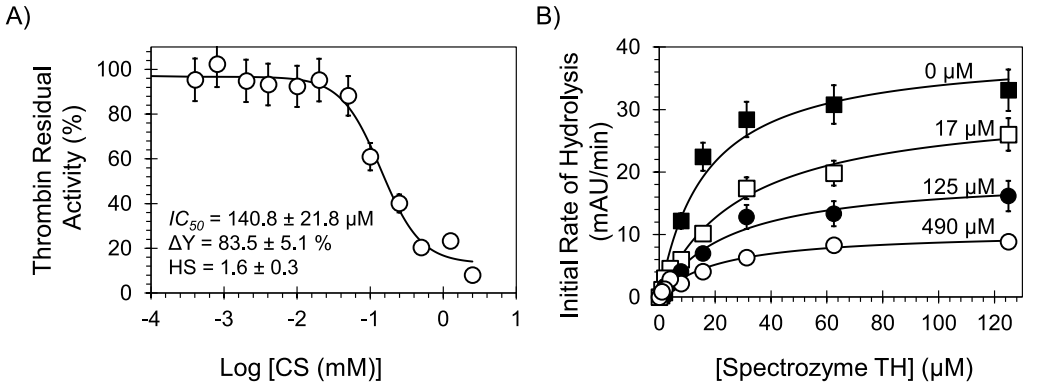

Fig. 2. A) Direct inhibition of human thrombin by CS. The inhibition studies were performed using the corresponding chromogenic substrate hydrolysis assays as described in the experimental part. Solid lines represent sigmoidal dose–response fits (Equation (1)) of the data to obtain the values of IC_{50}, HS, and ΔY. B) Michaelis–Menten kinetics of Spectrozyme® TH hydrolysis by human thrombin in the presence of CS. The initial rate of hydrolysis at various substrate concentrations was measured in pH 7.4 buffer as described in the experimental part using thrombin. CS concentrations are 0 (■), 17 (□), 125 (●), 490 μM (○). Solid lines represent nonlinear regressional fits to the data using the standard Michaelis–Menten equation to calculate the V_{MAX} and K_M.

Table 1
Inhibition of human thrombin by CS under different conditions.[a]

Inhibitor	IC_{50} (μM)	HS	ΔY (%)
CS No Tween 0 Incubation 25 °C	140.8 ± 21.8[b]	1.6 ± 0.3	83.5 ± 5.1
CS No Tween 0 Incubation 37 °C	143.6 ± 20.2	1.3 ± 0.5	83.3 ± 10.2
CS 0.02% Tween80 0 Incubation 25 °C	279.9 ± 7.1	2.8 ± 0.4	76.9 ± 3.3
CS 0.02% Tween80 10 min Incubation 25 °C	228.6 ± 10.2	3.3 ± 1.2	71.3 ± 4.2

[a] The values of IC_{50}, HS, and ΔY were obtained following non-linear regression analysis of direct inhibition of human thrombin in appropriate Tris–HCl buffers of pH 7.4. Inhibition was monitored by spectrophotometric measurement of residual enzyme activity.[b] Errors represent ±1 S E.

Table 2
Michaelis-Menten kinetics of chromogenic substrate hydrolysis by thrombin in the presence of CS.[a]

CS (μM)	K_M (mM)	V_{MAX} (mAU/min)
0	0.020 ± 0.004[b]	39.4 ± 3.2
17	0.030 ± 0.004	31.3 ± 1.8
125	0.024 ± 0.005	19.5 ± 1.3
490	0.021 ± 0.005	10.5 ± 0.8

[a] K_M and V_{MAX} values of the chromogenic substrate hydrolysis by thrombin were measured as described under (Materials and Methods). mAU indicates milliabsorbance units.
[b] Error represents ±1 S E.

dissociation constant (K_D) of binding. Using this equation, CS was found to bind with an affinity of 180.9 ± 18.9 μM, which compares favorably with the IC_{50} measured above. The Hill coefficient was calculated to be 1.7 ± 0.3, which supports a positive cooperative interaction. Similar to cooperativity in proteins containing multiple ligand-binding sites or multiple domains or subunits, cooperativity in monomeric single-site enzymes is often linked to protein conformational changes [22]. Fig. 3B shows the emission profile of

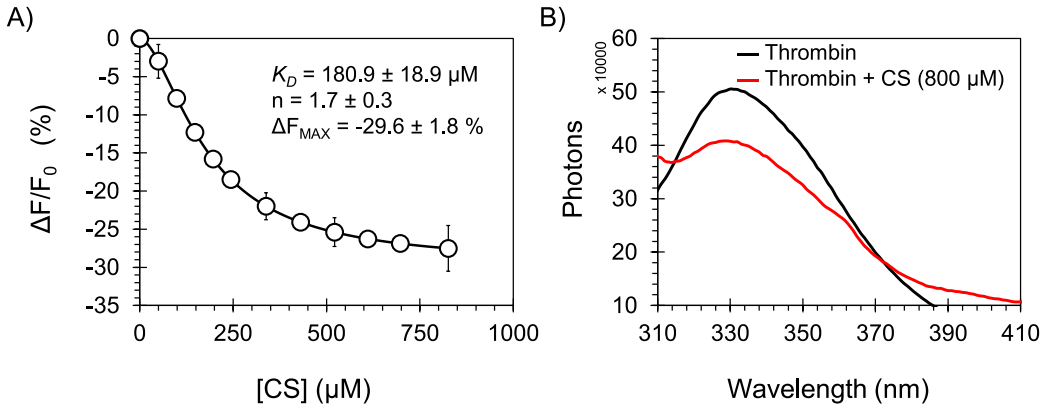

Fig. 3. A) Spectrofluorometric measurement of the affinity of human thrombin for CS at pH 7.4 and 25 °C using the intrinsic tryptophan fluorescence (λ_{EM} = 348 nm, λ_{EX} = 280 nm). Solid lines represent the nonlinear fit using Equation (3) to derive K_D. B) Thrombin emission scan in the presence and absence of CS. The binding study was repeated with the catalytically inactive thrombin (S195A) resulting in similar outcomes.

thrombin in the presence and absence of a saturating concentration of CS of 800 μM. While there was no change in the λ_{Max} of emission (332 nm), there was a decrease in the intensity of the fluorescence by 29.6 ± 1.8%.

CS does not compete with unfractionated heparin (UFH) or HirP for binding to thrombin. Given the allosteric nature of CS inhibition of thrombin, we studied its potential to recognize the two known allosteric sites of thrombin: exosite 1 and exosite 2. Exosite 1 is the site for interaction with fibrinogen, which propagates the clotting signal. It is also the site where hirudin and bivalirudin bind and inhibit thrombin. Exosite 2 is the site where UFH, γ′-fibrinogen, and glycoprotein Ibα bind [20]. We performed competitive inhibition studies using prototypical ligands, hirugen (exosite 1) and heparin (exosite 2). On one hand, HirP, a hirudin-based dodecapeptide, that binds to exosite 1 with a K_D value of 28 nM [21], did not affect the apparent IC_{50} of thrombin inhibition by CS at a concentration nearly 3 times higher than the affinity (Table 3). Likewise, when thrombin inhibition was studied in the presence of both CS and UFH (50 and 250 μM), the apparent IC_{50} values of thrombin inhibition by CS also did not change (Table 3). These results imply that CS does not engage exosites 1 or 2 of thrombin, and it potentially recognizes a third allosteric exosite.

CS partially inhibits thrombin-mediated fibrinogen activation. Thrombin is a pivotal enzyme in the coagulation process with multiple physiological substrates, including fibrinogen, factor XI, and factor XIII among others. Therefore, to determine whether the inhibition of the amidolytic activity of thrombin using the chromogenic substrate translates into inhibition of thrombin's physiological proteolytic activity, we assessed the inhibition potential of CS toward thrombin-mediated fibrinogen activation. Activation of fibrinogen by thrombin generally results in the formation of fibrin, which is a less soluble protein, resulting in a more turbid solution. As exhibited by Fig. 4A, CS inhibited thrombin-mediated fibrinogen activation dose-dependently with an IC_{50} value of 175.5 ± 17.5 μM, *albeit* partially with an efficacy of 26.0 ± 6.6%. This indicates that CS inhibition of thrombin is physiologically relevant.

CS prolongs the APTT of human plasma. Given its effect on thrombin activity, we next evaluated the potential effect of CS on clotting times of normal human plasma: activated partial thromboplastin time (APTT) and prothrombin time (PT). The former time is measured under conditions similar to those that provoke the intrinsic coagulation pathway via the use of ellagic acid, whereas the latter is measured under conditions similar to those that initiate the extrinsic coagulation pathway via the use of thromboplastin-D. Interestingly, CS was found to dose-dependently prolong the APTT (Fig. 4B). The concentration of CS needed to double the APTT was found to be about 521 μM. Nevertheless, CS did not double the PT at the highest concentration tested of 3000 μM. These results suggest that CS can be a physiologically relevant molecule.

Inhibition of other human clotting factors by CS. All human clotting factors, except factor XIIIa, are serine proteases. Therefore, we attempted to identify the effect of CS on other clotting factors via the use of the corresponding chromogenic substrate hydrolysis

Table 3
Inhibition of human thrombin by CS in the presence of UFH and HirP.[a]

Competitor	Conc. (μM)	IC_{50} (μM)	HS	ΔY (%)
UFH	**0**	140.8 ± 21.8[b]	1.6 ± 0.3	83.5 ± 5.1
	50	135.5 ± 5.3	2.6 ± 0.4	69.8 ± 3.1
	250	140.5 ± 5.1	2.0 ± 0.3	87.5 ± 3.1
Competitor	**Conc. (nM)**	**IC_{50} (μM)**	**HS**	**ΔY (%)**
HirP	**0**	166.7 ± 18.9	1.9 ± 0.7	83.9 ± 9.2
	75	143.6 ± 20.3	1.3 ± 0.5	83.3 ± 10.2

[a] [n] IC_{50} values for inhibition of thrombin in the presence of different potential competitors.
[b] Error represents ±1 S E.

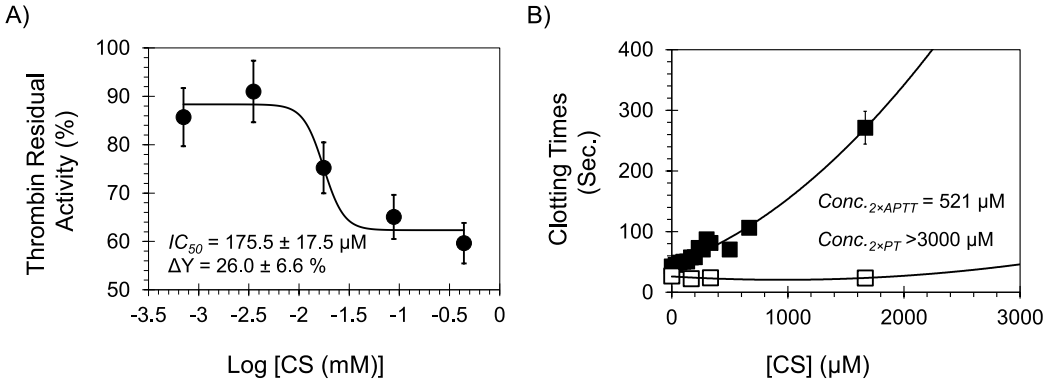

Fig. 4. A) CS inhibition of thrombin-mediated fibrinogen activation. The inhibition study was performed using a turbidity assay at an absorbance wavelength of 600 nm, as described in the experimental part. Solid lines represent sigmoidal dose−response fits (Equation (1)) of the data to obtain the values of IC_{50}, HS, and ΔY. B) Effect of CS on human plasma clotting times. The time to clot was measured in either the APTT assay (■) or the PT assay (□) in the presence of varying concentrations of CS. Solid lines are trend lines, which were used to calculate the concentration of CS to double APTT.

assays. Interestingly, we found that CS also inhibited other factors with variable potencies and efficacies (Fig. 5 and Table 4). For example, CS inhibited factors Xa and XIa with IC_{50} values of 249.3 ± 36.3 μM and 227.4 ± 28.1, respectively, and efficacies of 94.0 ± 11.9% and 99.0 ± 17.3%, respectively. However, CS did not inhibit factor IXa at the highest concentration tested. Surprisingly, we found that CS most potently inhibited factor XIIa with an IC_{50} value of 16.5 ± 1.0 μM and efficacy of 60.3 ± 3.6%. Factor XIIa is the topmost clotting factor that belongs to the intrinsic pathway of coagulation, or more accurately the contact activation system. Besides its role in clotting, factor XIIa contributes to inflammation through the activation of the inflammatory bradykinin-producing kallikrein-kinin system. In addition to the clotting factors, we also found that CS inhibited plasmin, a fibrinolytic enzyme, with an IC_{50} value of 84.9 ± 15.6 μM and efficacy of 95.6 ± 11.3%. Previously, CS was reported to inhibit plasmin hydrolysis of t-butyloxycarbonyl-L-valyl-L-leucyl-L-lysine 4-methyl coumaryl-7-amide (Boc-Val-Leu-Lys-MCA) [7].

4. Conclusion

This study is the first to report a microwave-assisted protocol for the synthesis of a sterol sulfate, which can be used to promote the commercial availability of novel oxysterol sulfates to be used as standards. Furthermore, this study has also offered a better understanding of thrombin inhibition by CS. Inhibition was independent of temperature or incubation time (Table 1). Michaelis-Menten kinetics indicated that CS inhibition of thrombin is noncompetitive (allosteric) in nature and exhibits a positive cooperativity which is indicative of conformational change-related allostery (Table 2 and Fig. 2). Moreover, fluorescence spectroscopy studies indicated that CS binds to thrombin in a manner that changes its intrinsic fluorescence and the intensity of its fluorescence emission profile (Fig. 3). Remarkably, competitive inhibition studies, in the presence of UFH and HirP, revealed that CS recognizes a different allosteric site, different from exosites 1 and 2 (Table 3).

While the inhibitory potential of CS toward a range of serine proteases including trypsin, chymotrypsin, thrombin, and plasmin was reported before, this is the first study that reports on the inhibition potential of CS towards other clotting factors of Xa, IXa, XIa, and XIIa (Table 4 and Fig. 5). To our surprise, CS did not inhibit factor IXa at the highest concentration tested which indicates that the inhibitory activity of CS is unlikely to be nonspecific. In fact, we tried sodium dodecyl sulfate and found that it did not inhibit thrombin at the highest concentration tested of 600 μM (4-fold of CS IC_{50} of thrombin, data not shown), suggesting that both the sulfate group as well as the cholesterol alicyclic backbone are essential for CS's inhibitory activity. Furthermore, CS potently inhibited factor XIIa with an IC_{50} value of 17 μM which makes it even more (patho-)physiologically relevant given that CS becomes elevated under the pathological conditions of cirrhosis [27], hypercholesterolemia [27], and hypothyroidism [28]. In addition, plasma cholesterol sulfate is significantly increased during the course of normal pregnancy, an increase associated with its elevated placental production [29]. Previously, it was reported that CS was found to trigger the activation of factor XII and prekallikrein in the presence of high molecular weight kininogen [30]. It is very interesting that CS acts as an activator for the zymogen i.e., factor XII, and as an inhibitor for the enzyme i.e., factor XIIa.

Interestingly, we also found that CS partially inhibits the physiological function of thrombin and that is activation of fibrinogen to fibrin. Not only that but it also selectively prolonged the APTT which promotes clotting via the intrinsic coagulation pathway that involves both factor XIa and factor XIIa (Fig. 4). Given the results of this study, we strongly recommend further *in vitro* (for example, thrombo-elastography) and *in vivo* (for example, animal models of venous and arterial thrombosis) investigation of CS to better understand its (patho-)physiological roles in coagulation and hemostasis which may intersect with the other reported biological effects of CS [31–39].

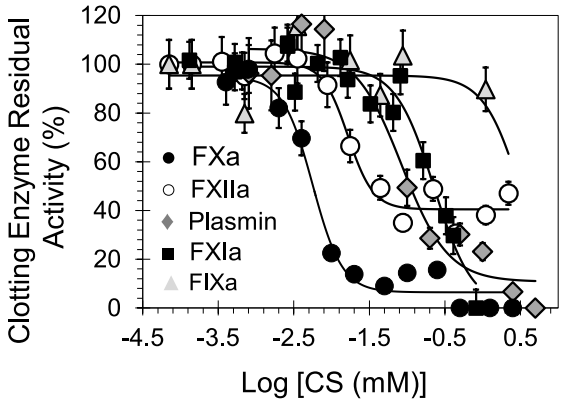

Fig. 5. Direct inhibition of human clotting factors by CS. The inhibition profiles of (●) factor Xa, (○) factor XIIa, (◆) plasmin, (■) factor XIa, and (▲) factor IXa were studied using the corresponding chromogenic substrate hydrolysis assays as described in the experimental part. Solid lines represent sigmoidal dose–response fits (Equation (1)) of the data to obtain the values of IC_{50}, HS, and ΔY.

Table 4
Inhibition of other clotting enzymes by CS.[a]

Enzyme	IC_{50} (μM)	HS	ΔY (%)
Thrombin	140.8 ± 21.8[b]	1.6 ± 0.33	83.5 ± 5.1
Factor Xa	249.3 ± 36.3	1.02 ± 0.27	94.0 ± 11.9
Factor XIa	227.4 ± 28.1	1.71 ± 0.52	99.0 ± 17.3
Factor IXa	>3224	*ND*[c]	*ND*
Factor XIIa	16.5 ± 1.0	2.6 ± 0.9	60.3 ± 3.6
Plasmin	84.9 ± 15.6	1.4 ± 0.9	95.6 ± 11.3

[a] [n] IC_{50} values for inhibition of other clotting enzymes.
[b] Error represents ±1 S E.
[c] Not determined.

Ethical approval

Not applicable.

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Data availability

Data included in article/supp. Material/referenced in the article.

Key points

- CS noncompetitively inhibits thrombin without competing with heparin or hirudin.
- CS also inhibits human factors Xa, XIa, XIIa, and plasmin, but not factor IXa.
- CS moderately binds thrombin and changes its fluorescence emission profile.
- CS inhibits thrombin-mediated fibrinogen activation and selectively prolongs APTT.

CRediT authorship contribution statement

Rami A. Al-Horani: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

Activated partial thromboplastin clotting time (APTT)
Cholesterol sulfate (CS)
Dimethylsulfoxide (DMSO)
Hirudin peptide (HirP)
Prothrombin time (PT)
Unfractionated heparin (UFH)

References

[1] L.D. Sanchez, L. Pontini, M. Marinozzi, L.C. Sanchez-Aranguren, A. Reis, I.H.K. Dias, Cholesterol and oxysterol sulfates: pathophysiological roles and analytical challenges, Br. J. Pharmacol. 178 (16) (2021) 3327–3341.
[2] C.A. Strott, Y. Higashi, Cholesterol sulfate in human physiology: what's it all about? J. Lipid Res. 44 (7) (2003) 1268–1278.
[3] J. Prah, A. Winters, K. Chaudhari, J. Hersh, R. Liu, S.H. Yang, Cholesterol sulfate alters astrocyte metabolism and provides protection against oxidative stress, Brain Res. 1723 (2019) 146378.
[4] J.S. Park, I.J. Chung, H.R. Kim, C.D. Jun, The immunosuppressive potential of cholesterol sulfate through T cell microvilli disruption, Immune Netw 23 (3) (2023) e29.
[5] J.H. Park, J. Lee, G.R. Lee, M. Kwon, H.I. Lee, N. Kim, H.J. Kim, M.O. Lee, W. Jeong, Cholesterol sulfate inhibits osteoclast differentiation and survival by regulating the AMPK-Sirt1-NF-κB pathway, J. Cell. Physiol. 238 (9) (2023) 2063–2075.
[6] K. Morino, K. Kunimura, Y. Sugiura, Y. Izumi, K. Matsubara, S. Akiyoshi, R. Maeda, K. Hirotani, D. Sakata, S. Mizuno, S. Takahashi, T. Bamba, T. Uruno, Y. Fukui, Cholesterol sulfate limits neutrophil recruitment and gut inflammation during mucosal injury, Front. Immunol. 14 (2023) 1131146.
[7] M. Iwamori, Y. Iwamori, N. Ito, Regulation of the activities of thrombin and plasmin by cholesterol sulfate as a physiological inhibitor in human plasma, J. Biochem. 125 (3) (1999) 594–601.
[8] M.L. Williams, M. Hughes-Fulford, P.M. Elias, Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and sterol synthesis by cholesterol sulfate in cultured fibroblasts, Biochim. Biophys. Acta 845 (3) (1985) 349–357.
[9] R. Woscholski, T. Kodaki, R.H. Palmer, M.D. Waterfield, P.J. Parker, Modulation of the substrate specificity of the mammalian phosphatidylinositol 3-kinase by cholesterol sulfate and sulfatide, Biochem 34 (36) (1995) 11489–11493.
[10] M. Iwamori, Y. Iwamori, N. Ito, Sulfated lipids as inhibitors of pancreatic trypsin and chymotrypsin in the epithelium of the mammalian digestive tract, Biochem. Biophys. Res. Commun. 237 (2) (1997) 262–265.
[11] J. Sato, M. Denda, J. Nakanishi, J. Nomura, J. Koyama, Cholesterol sulfate inhibits proteases that are involved in the desquamation of stratum corneum, J. Invest. Dermatol. 111 (2) (1998) 189–193.
[12] M. Iwamori, H. Suzuki, T. Kimura, Y. Iwamori, Shedding of sulfated lipids into gastric fluid and inhibition of pancreatic DNase I by cholesterol sulfate in concert with bile acids, Biochim. Biophys. Acta 1487 (2–3) (2000) 268–274.
[13] N. Ito, Y. Iwamori, K. Hanaoka, M. Iwamori, Inhibition of pancreatic elastase by sulfated lipids in the intestinal mucosa, J. Biochem. 123 (1) (1998) 107–114.
[14] M. Merten, J.F. Dong, J.A. Lopez, P. Thiagarajan, Cholesterol sulfate: a new adhesive molecule for platelets, Circulation 103 (16) (2001) 2032–2034.
[15] R.A. Al-Horani, P. Ponnusamy, A.Y. Mehta, D. Gailani, U.R. Desai, Sulfated pentagalloylglucoside is a potent, allosteric, and selective inhibitor of factor XIa, J. Med. Chem. 56 (3) (2013) 867–878.
[16] R.A. Al-Horani, U.R. Desai, Chemical sulfation of small molecules - advances and challenges, Tetrahedron 66 (16) (2010) 2907–2918.
[17] R.A. Al-Horani, R. Karuturi, S. Verespy 3rd, U.R. Desai, Synthesis of glycosaminoglycan mimetics through sulfation of polyphenols, Methods Mol. Biol. 1229 (2015) 49–67.
[18] D.K. Afosah, S. Verespy 3rd, R.A. Al-Horani, R.S. Boothello, R. Karuturi, U.R. Desai, A small group of sulfated benzofurans induces steady-state submaximal inhibition of thrombin, Bioorg. Med. Chem. Lett 28 (6) (2018) 1101–1105.
[19] R. Chiles, D.K. Afosah, R.A. Al-Horani, Investigation of the anticoagulant activity of cyclic sulfated glycosaminoglycan mimetics, Carbohydr. Res. 529 (2023) 108831.
[20] S. Verespy 3rd, A.Y. Mehta, D. Afosah, R.A. Al-Horani, U.R. Desai, Allosteric partial inhibition of monomeric proteases. Sulfated coumarins induce regulation, not just inhibition, of thrombin, Sci. Rep. 6 (2016) 24043.
[21] I.M. Verhamme, S.T. Olson, D.M. Tollefsen, P.E. Bock, Binding of exosite ligands to human thrombin. Re-evaluation of allosteric linkage between thrombin exosites I and II, J. Biol. Chem. 277 (9) (2002) 6788–6798.
[22] A. Cornish-Bowden, M.L. Cárdenas, Co-operativity in monomeric enzymes, J. Theor. Biol. 124 (1) (1987) 1–23.
[23] S. Kar, M. Mottamal, R.A. Al-Horani, Discovery of benzyl tetraphosphonate derivative as inhibitor of human factor xia, Chemist 9 (11) (2020) 1161–1172.
[24] R.A. Al-Horani, D. Clemons, M. Mottamal, The in vitro effects of pentamidine isethionate on coagulation and fibrinolysis, Molecules 24 (11) (2019) 2146.
[25] S. Kar, P. Bankston, D.K. Afosah, R.A. Al-Horani, Lignosulfonic acid sodium is a noncompetitive inhibitor of human factor XIa, Pharmaceuticals 14 (9) (2021) 886.
[26] R.A. Al-Horani, E. Parsaeian, M. Mohammad, M. Mottamal, Sulfonated non-saccharide molecules and human factor XIa: enzyme inhibition and computational studies, Chem. Biol. Drug Des. 100 (1) (2022) 64–79.
[27] N. Tamasawa, A. Tamasawa, K. Takebe, Higher levels of plasma cholesterol sulfate in patients with liver cirrhosis and hypercholesterolemia, Lipids 28 (9) (1993) 833–836.
[28] J.J. van Doormaal, F.A. Muskiet, G. Jansen, B.G. Wolthers, W.J. Sluiter, H. Doorenbos, Increase of plasma and red cell cholesterol sulfate levels in induced hypothyroidism in man, Clin. Chim. Acta 155 (3) (1986) 195–200.
[29] B. Lin, K. Kubushiro, Y. Akiba, Y. Cui, K. Tsukazaki, S. Nozawa, M. Iwamori, Alteration of acidic lipids in human sera during the course of pregnancy: characteristic increase in the concentration of cholesterol sulfate, J. Chromatogr. B Biomed. Sci. Appl. 704 (1–2) (1997) 99–104.
[30] T. Shimada, H. Kato, S. Iwanaga, M. Iwamori, Y. Nagai, Activation of factor XII and prekallikrein with cholesterol sulfate, Thromb. Res. 38 (1) (1985) 21–31.
[31] W. Hu, M. Zhao, J. Lian, D. Li, J. Wen, J. Tan, Lithium cholesterol sulfate: a novel and potential drug for treating alzheimer's disease and autism spectrum disorder, CNS Neurol. Disord.: Drug Targets (2022), https://doi.org/10.2174/1871527321666220825114236.
[32] D. Xu, R. Ma, Y. Ju, X. Song, B. Niu, W. Hong, R. Wang, Q. Yang, Z. Zhao, Y. Zhang, Y. Zheng, Q. Bai, M. Lv, N. Sun, X. Li, Cholesterol sulfate alleviates ulcerative colitis by promoting cholesterol biosynthesis in colonic epithelial cells, Nat. Commun. 13 (1) (2022) 4428.

[33] T. Tatsuguchi, T. Uruno, Y. Sugiura, K. Oisaki, D. Takaya, D. Sakata, Y. Izumi, T. Togo, Y. Hattori, K. Kunimura, T. Sakurai, T. Honma, T. Bamba, M. Nakamura, M. Kanai, M. Suematsu, Y. Fukui, Pharmacological intervention of cholesterol sulfate-mediated T cell exclusion promotes antitumor immunity, Biochem. Biophys. Res. Commun. 609 (2022) 183–188.
[34] X. Zhang, D. Deng, D. Cui, Y. Liu, S. He, H. Zhang, Y. Xie, X. Yu, S. Yang, Y. Chen, Z. Su, Cholesterol sulfate exerts protective effect on pancreatic β-cells by regulating β-cell mass and insulin secretion, Front. Pharmacol. 13 (2022) 840406.
[35] T. Tatsuguchi, T. Uruno, Y. Sugiura, D. Sakata, Y. Izumi, T. Sakurai, Y. Hattori, E. Oki, N. Kubota, K. Nishimoto, M. Oyama, K. Kunimura, T. Ohki, T. Bamba, H. Tahara, M. Sakamoto, M. Nakamura, M. Suematsu, Y. Fukui, Cancer-derived cholesterol sulfate is a key mediator to prevent tumor infiltration by effector T cells, Int. Immunol. 34 (5) (2022) 277–289.
[36] T. Sakurai, T. Uruno, Y. Sugiura, T. Tatsuguchi, K. Yamamura, M. Ushijima, Y. Hattori, M. Kukimoto-Niino, C. Mishima-Tsumagari, M. Watanabe, M. Suematsu, Y. Fukui, Cholesterol sulfate is a DOCK2 inhibitor that mediates tissue-specific immune evasion in the eye, Sci. Signal. 11 (541) (2018) eaao4874.
[37] F. Wang, K. Beck-García, C. Zorzin, W.W. Schamel, M.M. Davis, Inhibition of T cell receptor signaling by cholesterol sulfate, a naturally occurring derivative of membrane cholesterol, Nat. Immunol. 17 (7) (2016) 844–850.
[38] K. Yamamoto, K. Miyazaki, S. Higashi, Pericellular proteolysis by matrix metalloproteinase-7 is differentially modulated by cholesterol sulfate, sulfatide, and cardiolipin, FEBS J. 281 (15) (2014) 3346–3356.
[39] A. Furuta, K.A. Salam, N. Akimitsu, J. Tanaka, H. Tani, A. Yamashita, K. Moriishi, M. Nakakoshi, M. Tsubuki, Y. Sekiguchi, S. Tsuneda, N. Noda, Cholesterol sulfate as a potential inhibitor of hepatitis C virus NS3 helicase, J. Enzym. Inhib. Med. Chem. 29 (2) (2014) 223–229.