Fucosylated Chondroitin Sulfate Inhibits Plasma Thrombin Generation via Targeting of the Factor IXa Heparin-Binding Exosite

Yang Buyue and John P. Sheehan*

University of Wisconsin-Madison, Departments of Medicine/Hematology-Oncology and Pathology, Madison, WI 53706

Running Title: DHG inhibition of plasma thrombin generation

Scientific Heading: Thrombosis and Hemostasis

*Address correspondence to: John P. Sheehan, M.D.
Department of Medicine/Hematology
University of Wisconsin
1300 University Avenue
Medical Sciences Center, Room 4285
Madison, WI 53706
Phone: (608) 262-1964
FAX: (608) 263-4969
email: jps@medicine.wisc.edu
ABSTRACT:

Depolymerized holothurian glycosaminoglycan (DHG) is a fucosylated chondroitin sulfate with antithrombin-independent antithrombotic properties. Heparin cofactor II (HCII)-dependent and independent mechanisms for DHG inhibition of plasma thrombin generation were evaluated. When thrombin generation was initiated with 0.2 pM tissue factor (TF), the EC50 for DHG inhibition was identical in mock- or HCII-depleted plasma, suggesting a serpin-independent mechanism. In the presence of excess TF, the EC50 for DHG was increased 13-27 fold, suggesting inhibition was dependent on intrinsic tenase (factor IXa-factor VIIIa) components. In factor VIII-deficient plasma supplemented with 700 pM factor VIII or VIIIa, and factor IX-deficient plasma supplemented with plasma-derived factor IX or 100 pM factor IXa, the EC50 for DHG was similar. Thus, cofactor and zymogen activation did not contribute to DHG inhibition of thrombin generation. Factor IX-deficient plasma supplemented with mutant factor IX(a) proteins demonstrated resistance to DHG inhibition of thrombin generation (factor IX(a) R233A> R170A> WT) that inversely correlated with protease-heparin affinity. These results replicate the effect of these mutations with purified intrinsic tenase components, and establish the factor IXa heparin-binding exosite as the relevant molecular target for inhibition by DHG. Glycosaminoglycan-mediated intrinsic tenase inhibition is a novel antithrombotic mechanism with physiologic and therapeutic applications.
Introduction:

Depolymerized holothurian glycosaminoglycan (DHG) is a low molecular weight (average MW 12,500) fucosylated chondroitin sulfate isolated from the sea cucumber *Stichopus japonicus*, and prepared by partial oxidative depolymerization with hydrogen peroxide \(^1\). DHG demonstrates antithrombotic efficacy in models of murine thrombin-induced pulmonary thromboembolism, thrombin-induced venous thrombosis in the rat, and canine dialysis during renal failure \(^3\)-\(^6\). DHG does not bind antithrombin with high affinity, and exhibits antithrombin-independent antithrombotic efficacy in vivo \(^3\),\(^7\). Compared to equitherapeutic doses of unfractionated or low molecular weight heparins, DHG demonstrates significantly reduced effects on tail transection and template bleeding assays in rat and dog models \(^4\),\(^5\),\(^8\). Thus, DHG has potential as an antithrombotic agent with reduced bleeding risk relative to heparin. *In vitro* testing has suggested that DHG accelerates thrombin inhibition by heparin cofactor II (HCII), inhibits factor VIII activation by thrombin, and inhibits factor X activation by the intrinsic tenase complex \(^9\)-\(^11\). Herein, we investigate the relevant mechanism(s) for the antithrombotic effect of DHG in human plasma.

In vitro and ex vivo modeling of the coagulation cascade indicates that factor X activation by the intrinsic tenase complex (factor IXa-factor VIIIa) is the rate-limiting step for thrombin generation \(^12\)-\(^15\). The heparin-binding exosite on factor IXa is the interactive site for the factor VIIIa A2 domain, contributing to stabilization of cofactor activity and allosteric activation of the protease within the enzyme complex \(^16\)-\(^18\). The
physiologic importance of this exosite is demonstrated by its critical role in the regulation of thrombin generation in human plasma, and saphenous vein thrombosis in the mouse\textsuperscript{19}. In an experimental system with purified components, the factor IXa heparin-binding exosite is the molecular target for antithrombin-independent inhibition of the intrinsic tenase complex by both low molecular weight heparin (LMWH) and DHG\textsuperscript{11,17}.

Since in vitro data demonstrates that DHG inhibits the intrinsic tenase complex by interacting with heparin-binding exosite of factor IXa, and this exosite is a critical regulator of plasma thrombin generation and murine venous thrombosis, we hypothesized that DHG regulates thrombin generation via interaction with the factor IXa heparin-binding exosite. The effect of DHG on plasma thrombin generation was evaluated by fluorogenic substrate cleavage and Western blot analysis in HCII- or mock-immunodepleted plasma, factor VIII or IX-deficient human plasma, and factor IX-deficient plasma reconstituted with recombinant factor IX(a) possessing selected mutations in the heparin-binding exosite. The results demonstrate that DHG inhibits plasma thrombin generation by targeting the heparin-binding exosite of factor IXa. Inhibition of plasma thrombin generation by DHG was independent of effects on factor VIII or IX activation, or acceleration of thrombin inhibition by HCII. These data provide proof of principle that glycosaminoglycan-mediated targeting of the factor IXa heparin binding exosite is a novel antithrombotic mechanism.
Materials and Methods:

Materials: Human pooled plasma, factor IX-deficient and factor VIII-deficient patient plasmas were purchased from George King (Overland Park, KS). HCII- or mock-immunodepleted human plasma prepared from the same parent pooled plasma was purchased from Affinity Biologicals (Ancaster, Ontario, Canada). Corn trypsin inhibitor (CTI) was obtained from Haematologic Technologies (Essex Junction, VT). Human plasma-derived factor IX, IXa, and thrombin were purchased from Enzyme Research (South Bend, IN). Recombinant human factor VIII (Kogenate FS) was generously provided by Andreas Mueller-Beckhaus of Bayer HealthCare, LLC (Richmond, CA). Phosphatidylserine (PS) and phosphatidylcholine (PC) were purchased from Avanti Lipids (Alabaster, AL). Cholesterol was purchased from Calbiochem (San Diego, CA). Phosphatidylcholine: phosphatidylserine:cholesterol (molar ratio 75:25:1) phospholipid vesicles (PC:PS vesicles) were prepared by extrusion through a 100 nm polycarbonate filter 20. Bovine serum albumin (A-9647) was purchased from Sigma (St. Louis, MO). Dimethylsulfoxide (DMSO) was purchased from Mallinckrodt (St. Louis, MO). Lyophilized bovine thrombin-α2-macroglobulin complex was purchased from Thrombinscope BV (Maastricht, The Netherlands). Thromborel S, a human thromboplastin from Dade Behring (Deerfield, IL), was used as the source of relipidated human tissue factor (200 ng/ml) 19. The fluorogenic substrate Z-Gly-Gly-Arg-AMC·HCl was obtained from Bachem (King of Prussia, PA). DHG was generously provided by Kazuhisa Minamiguchi of Taiho Pharmaceuticals (Saitama, Japan).
Expression and purification of recombinant factor IX: Stable HEK 293 cell lines expressing human factor IX wild type and R233A were constructed as previously described 17,18. A HEK 293 cell lines stably transfected with human factor IX R170A was provided by Darrel Stafford (University of North Carolina-Chapel Hill) 21. Recombinant factor IX proteins were purified to homogeneity from conditioned media, and quantitated by absorbance at 280 nm. A portion of the factor IX was activated to factor IXa with human factor XIa, and factor IXa catalytic sites quantitated by titration with antithrombin III, as previously described 18.

Fluorogenic assay for detection of plasma thrombin generation- Thrombin generation in human plasma was detected as previously described 19,22. Thrombin activity was determined by AMC (7-amino-4-methycoumarin) release from the fluorogenic substrate Z-Gly-Gly-Arg-AMC, detected by a 360/40-nm-excitation and 460/40-nm-emission filter set in a Biotek Synergy HT fluorescent plate reader equipped with Gen 5 software (Biotek Instruments, Inc., Winooski, VT). Calibration was performed as previously described 19. The raw data was imported into Technothrombin TGA evaluation software from Technoclone (Vienna, Austria) to construct calibration curves for each plasma.

For tissue factor-triggered assays, the initiator solution was composed of 0.12 mg/CTI, 25 µM PC:PS vesicles, 0.6 pM or 12 pM tissue factor and 0-50 µM DHG in TGA buffer. Prior to each assay, a fresh fluorogenic substrate and calcium solution (FluCa substrate) containing 2.5 mM Z-Gly-Gly-Arg-AMC and 100 mM CaCl₂ was prepared as described 19. Plasma (60 µl) and initiator solution (20 µl) were added to each well and preheated at
37°C for 10 min. FluCa substrate (20 µl) preheated to 37°C was then added, mixed at medium intensity for 5 sec, and readings were obtained at 30 sec intervals for one hour. Final concentrations (extrapolated to the 60 µl plasma volume) were 0.2 pM (limiting) or 4 pM (excess) tissue factor, 8.3 µM PC:PS vesicles, and 40 µg/ml CTI. DHG (0-10 µM) concentrations were based on overall assay volume (100 µl). In factor VIII-deficient plasma, thrombin generation was triggered with 0.2 pM tissue factor in the presence of a final plasma concentration of 700 pM factor VIII or thrombin-activated factor VIIIa. Recombinant factor VIII (2.1 nM) was added to the tissue factor initiator solution, or factor VIII was activated with a 1:2 molar ratio of thrombin in 0.15 M NaCl, 5 mM CaCl₂, 20 mM HEPES, pH 7.4, and 0.01% Tween-80 at 37°C for 30 sec, and immediately added to assay wells followed by FluCa substrate to initiate the reaction. In factor IX deficient plasma, thrombin generation was triggered with 0.2 pM tissue factor in the presence of 100% plasma levels (90 nM) of plasma-derived or recombinant factor IX, or 100 pM factor IXa in the absence of tissue factor. For factor IXa-triggered assays, tissue factor was omitted from the initiator solution and protease was added to the assay well just prior to the FluCa substrate.

Fluorescent signal data were exported to Technothrombin® TGA evaluation software, and thrombin generation over time was determined using the appropriate calibration curve for each plasma. Thrombin generation parameters lag time (start until first burst in thrombin formation), peak thrombin concentration, time to thrombin peak, and velocity index (slope between the end of lag time and peak thrombin) were determined using the software. To determine the relative potency of DHG for inhibition of plasma thrombin
generation under each experimental condition, inhibitor concentration was plotted versus the relative velocity index (the ratio of the velocity index at each DHG concentration to velocity index in the absence of DHG). Data was fitted to a binding equation to obtain the EC$_{50}$ as previously described $^{18,23}$.

The time course of plasma thrombin generation by Western Blotting- Thrombin generation was initiated in 1.5 ml eppendorf tubes with 0.2 pM or 4 pM human tissue factor, 8.3 µM PC:PS vesicles, and 40 µg/ml CTI (plasma concentrations) in mock-depleted plasma with or without 0.5 µM or 2.5 µM DHG at 37°C. Individual reactions were quenched by addition of 100 µl 2X SDS-PAGE loading buffer containing 5 M urea at the indicated time points. Quenched samples were incubated at 37°C for 5 min, boiled for 5 min, diluted 1:20 in non-reducing SDS-PAGE loading buffer, and subjected to 10% (for 0.2 pM tissue factor condition) or 12.5 % (for 4 pM tissue factor condition) SDS-PAGE and then immunoblotted as previously described $^{19}$.
Results:

Effect of tissue factor concentration on thrombin generation in factor IX-deficient plasma. Thrombin generation was triggered by 0.2 or 4 pM tissue factor (TF) in factor IX-deficient plasma with or without addition of 100% (90 nM) plasma-derived factor IX (Fig 1). Control reactions in the absence of tissue factor demonstrated that factor IX-deficient plasma alone failed to generate detectable thrombin, while addition of 90 nM factor IX resulted in a modest, delayed thrombin peak (Fig 1A, B). In the presence of 0.2 pM TF, minimal thrombin generation was observed in factor IX-deficient plasma alone, while peak thrombin was approximately 400 nM at 6 min when supplemented with 100% plasma-derived factor IX (Fig 1A). In the presence of 4 pM TF, peak thrombin was approximately 550 nM with or without addition of 100% plasma-derived factor IX, with modest shortening of time to peak thrombin when factor IX was present (Fig 1B). Thus, the magnitude of plasma thrombin generation was highly dependent on factor IX in the presence of limiting tissue factor (0.2 pM), and largely independent of factor IX in the presence of excess tissue factor (4 pM).

Effect of DHG on tissue factor triggered thrombin generation in mock- or HCII-immunodepleted plasma. Plasma thrombin generation was triggered by limiting tissue factor (0.2 pM) in the presence of increasing concentrations of DHG (0-5 µM) in either mock- or HCII-immunodepleted plasma (Fig 2A, B). The parent pooled plasma underwent identical immunodepletion protocols to minimize pre-analytical plasma variables. In the absence of tissue factor, no detectable thrombin was generated in
either plasma (Fig 2A, B). In mock-depleted plasma, increasing DHG concentration resulted in a dramatic dose-dependent decrease in velocity index (slope) and peak thrombin concentration, with a more gradual prolongation of the lag time and time to peak thrombin generation (Fig 2A). At the highest DHG concentration, thrombin generation approached the level observed in the absence of tissue factor. In HCII-depleted plasma, increasing DHG concentration demonstrated very similar effects on plasma thrombin generation (Fig 2B). The potency of DHG inhibition was estimated by velocity index for plasma thrombin generation at each DHG concentration relative to the absence of glycosaminoglycan (Fig 2C). The mean EC$_{50}$ for reduction in the velocity index by DHG was identical for both mock-depleted plasma and HCII-depleted plasma (Table I).

Compared to limiting tissue factor conditions, thrombin generation triggered by excess tissue factor (4 pM) demonstrated enhanced peak thrombin concentration and shortening of time to peak in both mock- and HCII-depleted plasma. Significantly higher concentrations of DHG were required to inhibit thrombin generation in mock-depleted plasma (Fig 3A) under these conditions. This inhibition was primarily reflected in reduction of velocity index and peak thrombin concentration, with no discernable effects on the lag time or time to peak thrombin generation. The mean EC$_{50}$ for reduction in relative velocity index by DHG inhibition was increased 13-fold (Table I) for mock-depleted plasma in the presence of excess versus limiting tissue factor concentration, suggesting that components of the intrinsic tenase complex contribute to this inhibition. In HCII-depleted plasma, even higher concentrations were required to inhibit thrombin
generation, with incomplete inhibition even at 10 μM DHG (Fig 3B). Similarly, this inhibition was characterized by reduction in velocity index and peak thrombin concentrations, with minimal effects on the time to peak thrombin generation. The mean EC₅₀ for reduction in the velocity index by DHG in HCII-depleted plasma triggered by excess tissue factor was more than two-fold higher than mock-depleted plasma (Fig 3C) in the presence of excess tissue factor, and 27-fold higher than limiting tissue factor conditions (Table I).

**Effect of DHG on tissue factor triggered plasma thrombin generation detected by Western Blot.** To confirm that DHG primarily inhibits plasma thrombin generation instead of accelerating thrombin inhibition, the time course of thrombin activation and inhibition products was monitored by Western blot. Thrombin generation was initiated by 0.2 pM tissue factor in mock-depleted plasma with or without 0.5 μM DHG. The reaction products were analyzed by SDS-PAGE under non-reducing conditions using a primary antibody that simultaneously detected prothrombin, thrombin-related cleavage products, HCII-thrombin complex and the thrombin-antithrombin complex (TAT) (Fig 4A). When thrombin generation was triggered by 0.2 pM tissue factor, mock-depleted plasma demonstrated appearance of free thrombin and the TAT complex at 6 min, peak free thrombin at 9 min, and progressive accumulation of TAT over time in the absence of DHG. In the presence of 0.5 μM DHG, peak thrombin generation was delayed until approximately 15 min, and the intensity of free thrombin and TAT bands was reduced relative to control. Likewise, depletion of the prothrombin/meizothrombin band occurred more slowly, and to a lesser extent than in the absence of DHG (Fig 4A). The time
course of plasma thrombin generation by Western blotting correlated well with results of the fluorogenic substrate assay under these conditions. Finally, a very modest (relative to TAT) thrombin-HCII band was noted only in the presence of DHG.

A similar analysis was undertaken for thrombin generation initiated by 4 pM tissue factor in mock-depleted plasma with 0, 0.5 or 2.5 µM DHG present (Fig 4B). Peak free thrombin was observed at 3 min under all conditions, but was reduced with increasing DHG. Prothrombin/meizothrombin depletion was rapid and dramatic in the presence of 0 or 0.5 µM DHG, with little or no detectable prothrombin/meizothrombin band from 6 min onward. In the presence of 2.5 µM DHG, the prothrombin band decreased with time but persisted through 9 min. The TAT complex appeared at 3 min under all conditions, and predominated in the absence of DHG. Minimal thrombin-HCII complex was noted in the absence of DHG, but an increasing proportion of thrombin was found in thrombin-HCII relative to TAT complex with increasing DHG concentration. In contrast, HCII-thrombin was barely detectable under limiting tissue factor conditions in the presence of 0.5 µM DHG (Fig 4A). Thus, higher concentrations of DHG were associated with enhanced formation of thrombin-HCII complex when plasma thrombin generation was triggered by excess tissue factor.

Effect of factor VIII or factor IX activation on inhibition of plasma thrombin generation by DHG. The markedly increased EC₅₀ for inhibition of plasma thrombin generation by DHG in the presence of excess versus limiting tissue factor concentration suggests that components of the intrinsic tenase complex contribute to this inhibition (Figs 1, 2A &
3A). The potential contribution of factor VIII or factor IX activation to the DHG inhibition mechanism was examined in factor VIII-deficient and factor IX-deficient plasma, respectively. Thrombin generation was triggered by 0.2 pM tissue factor in factor VIII-deficient plasma supplemented with either 700 pM recombinant factor VIII or thrombin-activated factor VIIIa (Fig 5A-B). No significant thrombin response was observed in the absence of factor VIII(a) and tissue factor, while 0.2 pM tissue factor or factor VIII alone generated only very modest increases over baseline thrombin. As expected, supplementing the factor VIII deficient plasma with 700 pM factor VIII resulted in a marked increase in thrombin generation in the presence of 0.2 pM tissue factor (Fig 5A). To bypass factor VIII activation, 700 pM thrombin-activated factor VIIIa was added to the factor VIII-deficient plasma under identical conditions, which generated a similar thrombin response (Fig 5B). Controls demonstrated no significant effect of “carryover” thrombin from the factor VIII activation mixture (data not shown). In both cases, addition of increasing concentration of DHG (0-5 µM) reduced the velocity index and peak thrombin concentration, and modestly prolonged lag time and time to peak thrombin (Fig 5A & Fig 5B). The mean EC₅₀ for inhibition of the velocity index for thrombin generation by DHG in factor VIII deficient plasma was 0.41 ± 0.02 µM in the presence of 700 pM recombinant factor VIII, and 0.44 ± 0.05 µM in the presence of 700 pM thrombin-activated factor VIIIa. Representative curves are shown (Fig 5C).

To evaluate the potential role of factor IX activation, thrombin generation was compared in factor IX-deficient plasma supplemented with 0.2 pM tissue factor and 100% levels of plasma-derived factor IX, or 100 pM plasma-derived factor IXa in the absence of tissue
factor (Fig 5D & 5E). The magnitude of thrombin generation in factor IX deficient plasma was similar under both conditions, and no significant levels of thrombin generation were noted in the absence of factor IX or factor IXa. Addition of increasing DHG concentration (0-10 µM) inhibited plasma thrombin generation in a similar fashion in the presence of either 100% plasma-derived factor IX, or 100 pM plasma-derived factor IXa, although the lag time and time to peak were slightly prolonged in the latter case (Fig 5E). The mean EC₅₀ (± SEM) for inhibition of the velocity index for thrombin generation by DHG in factor IX deficient plasma was 0.36 ± 0.01 µM for 100% plasma-derived factor IX, and 0.34 ± 0.02 µM for plasma-derived FIXa. Representative curves are shown (Fig 5F).

**Effect of mutations in the heparin-binding exosite of recombinant factor IX on the ability of DHG to inhibit tissue factor triggered plasma thrombin generation.** The effect of mutations in the heparin-binding exosite of factor IXa on the ability of DHG to inhibit plasma thrombin generation was assessed with recombinant factor IX wild type, R170A, and R233A. The respective proteases derived from these zymogens demonstrate a progressive decrease in heparin affinity (WT>R170A>R233A), and increase in resistance to inhibition by both DHG and LMWH using purified intrinsic tenase components. The ability of DHG to inhibit thrombin generation triggered by 0.2 pM tissue factor was evaluated in factor IX deficient plasma supplemented with 100% levels of recombinant factor IX wild type, R170A, and R233A (Fig 6A-C). In the absence of DHG, factor IX R170A (Fig 6B) demonstrated increased peak thrombin concentration (1.5-fold) and velocity index (2-fold), while the response for factor IX R233A (Fig 6C) was blunted and...
delayed relative to wild type protease, as previously described. Minimal thrombin response was noted in the absence of factor IX. For all three recombinant factor IX proteins, addition of increasing DHG concentrations to factor IX deficient plasma resulted in a progressive reduction in peak thrombin, and prolongation of the time to peak thrombin concentration triggered by 0.2 pM tissue factor. In the presence of factor IX wild type, plasma thrombin generation was completely inhibited by 5 µM DHG and the mean EC$_{50}$ for inhibition of the velocity index was 0.38 ± 0.01 µM (Fig 6D, Table II), similar to plasma-derived factor IX. In the presence of factor IX R170A, up to 10 µM DHG failed to completely suppress thrombin generation and the mean EC$_{50}$ for inhibition of the velocity index was more than 2-fold higher than in the presence of factor IX wild type (Fig 6D, Table II). In the presence of factor IX R233A, the magnitude of baseline thrombin generation was reduced relative to factor IX wild type, but demonstrated remarkable resistance to inhibition by DHG (Fig 6C). The mean EC$_{50}$ for inhibition of the velocity index was more than 9-fold higher than in the presence of factor IX wild type (Fig 6D, Table II).

Effect of mutations in the heparin-binding exosite of recombinant factor IXa on the ability of DHG to inhibit factor IXa triggered plasma thrombin generation- To exclude glycosaminoglycan effects on the activation of factor IX by the tissue factor-factor VIIa complex, the ability of DHG to inhibit thrombin generation was evaluated in factor IX deficient plasma triggered by 100 pM recombinant factor IXa wild type, R170A, and R233A (Fig 7A-C). Under these conditions, factor IXa wild type supported a slightly delayed but similar magnitude of thrombin generation to that triggered by 0.2 pM tissue
factor in the presence of 100% levels of the zymogen factor IX (Fig 7A). In the absence of factor IXa, no significant thrombin generation was observed. In the absence of DHG, factor IXa R170A demonstrated a 2-fold increase in peak thrombin concentration and up to 2-fold increase in velocity index (Fig 7B), while the response for factor IXa R233A (Fig 7C) was similar to wild type protease. Addition of increasing DHG concentrations to factor IX deficient plasma again resulted in a progressive reduction in peak thrombin, and prolongation of the time to peak thrombin concentration in all cases. In the presence of factor IXa wild type, the mean EC₅₀ for inhibition of the velocity index by DHG was essentially identical to that observed for the factor IX wild type zymogen (Fig 7D, Table II). In the presence of factor IXa R170A, the enhanced level of thrombin generation demonstrated increased resistance to DHG, with a mean EC₅₀ for inhibition of the velocity index nearly 3-fold higher than the wild type protease (Fig 7D, Table II). In the presence of factor IXa R233A, marked resistance to inhibition of plasma thrombin generation by DHG was observed, with a mean EC₅₀ for inhibition of the velocity index nearly 8-fold higher than factor IXa wild type (Fig 7D, Table II).
Discussion:

DHG is a fucosylated chondroitin sulfate that demonstrates antithrombin-independent antithrombotic activity in animal models of thrombosis, with apparent reduced bleeding risk relative to equitherapeutic heparin doses 3-6. Although a number of in vitro activities have been described, the relevant mechanism for the antithrombotic efficacy of DHG was previously undefined. To determine the relevant mechanism of action, the effect of DHG on tissue factor triggered thrombin generation in human plasma was examined. Plasma thrombin generation has been proposed as a useful phenotypic characterization of thrombotic tendency, and accumulating evidence suggests that increased magnitude of thrombin generation is associated with elevated risk of recurrent or unprovoked venous thrombosis 26-31. Our results demonstrate that DHG inhibits plasma thrombin generation in a serpin-independent manner, by interacting with the heparin-binding exosite on the factor IXa protease domain.

The magnitude of thrombin generation in factor IX deficient plasma was dependent on addition of factor IX when triggered by limiting (0.2 pM) tissue factor, and independent of factor IX when triggered by excess (4 pM) tissue factor (Fig 1). These results are consistent with previous characterizations of thrombin generation as dependent on the intrinsic tenase complex in the presence of limiting or dilute tissue factor 12,13,32. Furthermore, Western blot analysis demonstrated that substantial prothrombin persisted throughout the thrombin time course triggered by limiting tissue factor, whereas rapid and complete prothrombin depletion occurred with excess tissue factor (Fig 4A-B).
Thus, the time course of plasma thrombin generation triggered by excess tissue factor was both independent of the intrinsic tenase (factor IXa-factor VIIIa) complex and influenced by substrate depletion, suggesting limited physiologic relevance. However, comparison of tissue factor concentrations provided insight into the mechanism of DHG action in plasma. DHG inhibited the velocity index (slope) of plasma thrombin generation with 13-fold higher potency in the presence of limiting versus excess tissue factor, suggesting that the intrinsic tenase complex contributed significantly to the mechanism of action for this glycosaminoglycan (Figs 2C & 3C, Table I). Likewise, Western blot analysis in the presence of limiting tissue factor demonstrated that DHG delayed conversion of prothrombin to free thrombin and TAT complex, confirming that this glycosaminoglycan inhibits plasma thrombin generation primarily by reducing prothrombin activation rather than accelerating inhibition of thrombin by HCII (Fig 4A).

The contribution of HCII to the inhibition of plasma thrombin generation by DHG was likewise compared in the presence of both limiting and excess tissue factor. With limiting tissue factor, the potency of inhibition by DHG was equivalent (identical EC50 values) in both mock- and HCII-depleted plasma (Fig 2C, Table I), and thrombin-HCII complex was barely detectable by Western blot at a DHG concentration in excess of the EC50 (Fig 4A), indicating that HCII did not significantly contribute to the inhibition mechanism. In the presence of excess tissue factor, HCII depletion resulted in an additional 2-fold decrease in DHG potency relative to mock-depleted plasma (Fig 3A-C, Table 1), and Western blot analysis demonstrated predominance of thrombin-HCII relative to TAT complex at a DHG concentration in excess of the EC50 (Fig 4B). Thus,
HCII contributed modestly to inhibition of plasma thrombin generation when triggered by excess tissue factor, but required substantially higher DHG concentrations. In contrast, HCII did not significantly contribute to inhibition of plasma thrombin generation by DHG in the more physiologic limiting tissue factor condition.

The specific mechanism for DHG inhibition of plasma thrombin generation was further defined by evaluating the effect of this glycosaminoglycan on intrinsic tenase complex assembly versus activity. The EC₅₀ for DHG inhibition of the velocity index for plasma thrombin generation was similar in factor VIII-deficient plasma supplemented with factor VIII or factor VIIIa (Fig 5A-C), and factor IX-deficient plasma supplemented with factor IX or factor IXa (Fig 5D-F) demonstrating that the inhibition mechanism does not involve reduced cofactor or protease activation. To evaluate the molecular target for DHG on the intrinsic tenase complex, the dose response for DHG inhibition of thrombin generation was compared in factor IX-deficient plasma supplemented with factor IX(a) wild type, R170A or R233A (Figs 6 & 7). The rank order of the EC₅₀ for DHG in the presence of either zymogen or protease forms (factor IX(a) R233A > R170A > WT) correlated with heparin affinity (lower to higher), and the resistance to inhibition of intrinsic tenase activity by LMWH or DHG (higher to lower) with purified components (Table II)¹¹,¹⁷. The higher DHG concentrations required for inhibition in plasma likely results from extensive protein-glycosaminoglycan binding, and the less dramatic differences between factor IX(a) mutants and wild type likely reflects the substantial influence of plasma inhibitors on the more complex endpoint of thrombin generation. The EC₅₀ values for zymogen and protease forms of the recombinant proteins were not
significantly different (Fig 6D, 7D, Table II), confirming that factor IX activation is not involved in the DHG inhibition mechanism. Further, while the amount of plasma thrombin generation supported by the mutant factor IXa proteases varied significantly based on cofactor affinity and stability \(^{17,19}\), these properties did not correlate with the relative resistance to DHG inhibition (Figs 6 & 7). Thus, the factor IXa heparin-binding exosite is the relevant molecular target for DHG inhibition of plasma thrombin generation.

The factor IXa heparin-binding exosite plays an essential role in regulation of the coagulation response. Factor X activation by the intrinsic tenase complex is the rate-limiting step for thrombin generation \(^{12-15}\), and mutations in the heparin-binding exosite regulate the rate of plasma thrombin generation and formation of saphenous vein thrombi in response to FeCl\(_3\)-induced injury in the mouse \(^{19}\). This enzymatic exosite interacts with the factor VIIIa A2 domain, forming a relatively unstable protein-protein interaction that is critical for cofactor activation of the protease, and is specifically targeted in vitro by glycosaminoglycans such as DHG and heparin \(^{11,17,18}\). The recognized mechanisms for glycosaminoglycan-mediated regulation of the coagulation response in human plasma are predominantly serpin-dependent \(^{33}\). The unique serpin-independent mechanism for inhibition of plasma thrombin generation by DHG further supports the critical role of the factor IXa heparin-binding exosite in the regulation of ex vivo and in vivo coagulant responses \(^{19}\). Furthermore, this mechanism may potentially be used by other glycosaminoglycans that bind factor IXa with sufficient affinity. Therapeutic concentrations of unfractionated and low molecular-weight heparin inhibit factor X
activation by the intrinsic tenase complex in purified systems, but the contribution of this mechanism to antithrombotic efficacy is unclear due to their concomitant antithrombin-dependent activities in plasma. The lack of therapeutically relevant serpin-dependent mechanisms for the fucosylated chondroitin sulfate DHG provides proof of principle that the factor IXa heparin binding exosite is a novel antithrombotic target.

The major limiting factor in the application of current antithrombotic agents to human disease remains the relatively narrow therapeutic range and increased risk of bleeding, especially in high-risk populations. In the setting of atrial fibrillation and venous thromboembolic disease, extended use of the antithrombin-dependent pentasaccharide idraparinux has been associated with an increased risk of major hemorrhage relative to vitamin K antagonists. In contrast, animal models suggest that targeting of the intrinsic tense complex may improve the risk/benefit ratio of antithrombotic therapy. Treatment with active site-blocked factor IXai in canine coronary thrombosis, murine stroke, or baboon cardiopulmonary bypass models is as effective as UFH, and demonstrates a significant reduction in template bleeding or blood loss. Similarly, monoclonal antibody versus the factor IXa Gla domain is at least as effective as LMWH in a rat carotid thrombosis model, with markedly reduced APTT prolongation and blood loss with injury. These animal models suggest that selective inhibition of factor IX(a), in the presence of intact tissue factor-induced coagulation, may reduce bleeding risk. Likewise, targeting enzymatic exosites is an important strategy that may enhance safety by providing a more graded effect on function, as opposed to the all or none nature of active site inhibition. Thus, targeting the intrinsic tenase complex via the
antithrombin-independent, exosite-mediated inhibition mechanism demonstrated for DHG represents a promising approach to antithrombotic therapy.
Acknowledgements

This research was supported by National Institutes of Health grant HL080452 (J.P.S). We would like to thank Darrell Stafford for providing the factor IX R170A cell line, Kazuhisa Minamiguchi of Taiho Pharmaceuticals (Saitama, Japan) for DHG, Andreas Mueller-Beckhaus of Bayer HealthCare, LLC for recombinant factor VIII (Kogenate FS); and Technoclone, Ltd. for the Technothrombin TGA software for analysis of plasma thrombin generation.

Authorship

Contribution:  B.Y. performed the research, analyzed and interpreted data, and wrote the manuscript.  J.P.S. designed the research, analyzed and interpreted data, and wrote the manuscript.

Conflict-of-interest disclosure:  The authors declare no competing financial interests.
References:


Table I: The EC$_{50}$ (± SEM) for DHG inhibition of the mean velocity index for thrombin generation (µM) in immunodepleted plasma

<table>
<thead>
<tr>
<th>TF(pM)</th>
<th>Mock-depleted plasma</th>
<th>HCII-depleted plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.16 ± 0.01</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>2.02 ± 0.09</td>
<td>4.31 ± 0.23</td>
</tr>
</tbody>
</table>

a Thrombin generation was initiated with 0.2 or 4 pM human tissue factor, 8.3 µM PC:PS vesicles, and 40 µg/ml CTI (plasma concentrations) in mock- or HCII-depleted plasma in the presence of increasing DHG (0-10 µM). Duplicate wells were averaged to determine individual thrombin generation curves. The relative velocity index was plotted versus DHG concentration, and EC$_{50}$ values for each replicate (n=3) were determined as described in Material and Methods. The mean and standard error of the EC$_{50}$ values were determined from the 3 independent replicates.

b Statistical significance analysis: EC$_{50}$ values for DHG inhibition at 4 pM TF were significantly different from 0.2 pM in both plasma. At 4 pM TF, EC$_{50}$ value in HCII-depleted plasma was significantly different from mock-depleted plasma (Student t-test, P< 0.002).
Table II: The EC$_{50}$± SEM for DHG inhibition of the mean velocity index for thrombin generation in factor IX-deficient plasma (µM)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Factor IX(a)</th>
<th>Factor IX(a)</th>
<th>Factor IX (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>R170A</td>
<td>R233A</td>
</tr>
<tr>
<td>zymogen</td>
<td>0.38 ± 0.01</td>
<td>0.86 ± 0.06</td>
<td>3.55 ± 0.47</td>
</tr>
<tr>
<td>protease</td>
<td>0.38 ± 0.01</td>
<td>1.02 ± 0.02</td>
<td>2.98 ± 0.64</td>
</tr>
</tbody>
</table>

a Thrombin generation was initiated with 0.2 pM human tissue factor, 8.3 µM PC:PS vesicles, and 40 µg/ml CTI (plasma concentrations) in factor IX-deficient plasma supplemented with 100% (90 nM) recombinant factor IX WT, R170A and R233A in the presence of increasing DHG (0-10 µM). For factor IXa-initiated assays, tissue factor was omitted and 100 pM recombinant factor IXa was added. Duplicate wells were averaged to determine individual thrombin generation curves. The relative velocity index was plotted versus DHG concentration, and EC$_{50}$ values for each replicate (n=3) were determined as described in Material and Methods. The mean and standard error of the EC$_{50}$ values were determined from the 3 independent replicates.

b Statistical significance analysis: EC$_{50}$ values for each zymogen were not significantly different from their respective protease (Student t-test, P> 0.2). EC$_{50}$ values for factor IX(a) R170A and R233A were significantly different from factor IX(a) WT (Student t-test, P≤ 0.01). EC$_{50}$ values for factor IX(a) R170A were significantly different from factor IX(a) R233A (Student t-test, P< 0.02).
Figure Legends

Figure 1- Effect of limiting (0.2 pM) and excess (4 pM) tissue factor on plasma thrombin generation in factor IX-deficient plasma. Thrombin generation was initiated with 0.2 pM (A) or 4 pM (B) human tissue factor (TF), 8.3 µM PC:PS vesicles, and 40 µg/ml CTI (plasma concentrations) in factor IX-deficient plasma with or without addition of 100% (90 nM) of plasma factor IX (pFIX): no tissue factor or pFIX control (●), 90 nM pFIX control (○), 0.2 pM TF (■), 0.2 pM TF and 90 nM pFIX (□), 4 pM TF (▲), 4 pM TF and 90 nM pFIX (△). The time course of thrombin generation was measured as described in Material and Methods. Thrombin generation curves represent the mean thrombin concentration over the first 30 min from replicate determinations (n=3), and are identified by representative points.

Figure 2- DHG inhibition of thrombin generation triggered by limiting tissue factor in mock- or HCII-depleted pooled plasma. Thrombin generation was initiated with 0.2 pM human tissue factor, 8.3 µM PC:PS vesicles, and 40 µg/ml CTI (plasma concentrations) in mock- (A) or HCII-depleted (B) pooled plasma in the presence of increasing DHG: 0 (●), 0.05 (○), 0.1 (■), 0.25 (□), 0.5 (▲), 1 (△), 2 (◆), and 5 µM (◇). Control reactions without tissue factor or DHG are presented (▼). The time course of thrombin generation was measured as described in Material and Methods. Thrombin generation curves represent the mean thrombin concentration over the first 30 min from replicate determinations (n=3), and are identified by representative points. The relative velocity index for thrombin generation was plotted versus DHG concentration for each
condition, and the data were fit as described in *Materials and Methods* to determine the EC$_{50}$ for inhibition (C). Representative curves are presented.

**Figure 3-** DHG inhibition of thrombin generation triggered by excess tissue factor in mock- or HCII-depleted pooled plasma. Thrombin generation was initiated with 4 pM human tissue factor, 8.3 µM PC:PS vesicles, and 40 µg/ml CTI (plasma concentrations) in mock- (A) or HCII-depleted plasma (B) in the presence of increasing DHG: 0 µM (●), 0.1 µM (○), 0.25 µM (□), 0.5 µM (▲), 1 µM (△), 2.5 µM (◇), 5 µM (◆), 10 µM (◇). Control reactions without tissue factor or DHG are presented (▼). The time course of thrombin generation was measured as described in *Material and Methods*. Thrombin generation curves represent the mean thrombin concentration over the first 30 min from replicate determinations (n=3), and are identified by representative points. The relative velocity index for thrombin generation was plotted versus DHG concentration for each condition, and the data were fit as described in *Materials and Methods* to determine the EC$_{50}$ for inhibition (C). Representative curves are presented.

**Figure 4-** Western blot analysis of limiting (A) and excess (B) tissue factor triggered thrombin generation in mock-depleted plasma with or without DHG. Thrombin generation was initiated with 0.2 (A) or 4 (B) pM human tissue factor, 8.3 µM PC:PS vesicles, and 40 µg/ml CTI (plasma concentrations) in mock-depleted plasma in the absence and presence of 0.5 µM or 2.5 µM DHG. Individual reactions were quenched over time with loading buffer containing 5 M urea, and analyzed by SDS-PAGE under non-reducing conditions as described in *Materials and Methods*. Proteins transferred to
Immobilon-P were detected with a polyclonal sheep anti-human thrombin primary antibody, followed by a peroxidase-conjugated affinity purified donkey anti-sheep IgG, and subsequent development of signal with chemiluminescent substrate. Prothrombin indicates prothrombin/meizothrombin band. TAT indicates thrombin-antithrombin complex, and HCII-thrombin indicates heparin cofactor II-thrombin complex.

**Figure 5- Effect of factor VIII and factor IX activation on inhibition of thrombin generation by DHG.** Thrombin generation was initiated with 0.2 pM human tissue factor, 8.3 µM PC:PS vesicles, and 40 µg/ml CTI (plasma concentrations) in factor VIII-deficient plasma supplemented with 700 pM recombinant factor VIII (rFVIII) (A) or 700 pM thrombin-activated factor (rFVIIIa) (B); or factor IX-deficient plasma supplemented with 100% (90 nM) plasma-derived factor IX (D) or 100 pM plasma-derived factor IXa in the absence of tissue factor (E) in the presence of increasing DHG: 0 µM (●), 0.1 µM (○), 0.25 µM (■), 0.5 µM (□), 1 µM (▲), 2.5 µM (△), 5 µM (◆), 10 µM (◇). In panels (A) & (B), control reactions are: no tissue factor or factor VIII(a) (◇), 0.2 pM tissue factor only (▼), and factor VIII(a) only (▼). Control for exogenous thrombin control used in factor VIII activation is not shown. In panels (D) and (E), control reactions are: no tissue factor or factor IX (▼), and 0.2 pM tissue factor only (▼). The time course of thrombin generation was measured as described in *Material and Methods*. Thrombin generation curves represent the mean thrombin concentration over the first 30 min from replicate determinations (n=3), and are identified by representative points. The relative velocity index for thrombin generation was plotted versus DHG concentration for each condition, and data were fit as described in *Materials and Methods* to determine the...
EC_{50} for inhibition in factor VIII-deficient plasma (C) and in factor IX-deficient plasma (F). Representative curves are presented.

Figure 6- Effect of recombinant factor IX mutants on DHG inhibition of plasma thrombin generation. Thrombin generation was initiated with 0.2 pM human tissue factor, 8.3 µM PC:PS vesicles, and 40 µg/ml CTI (plasma concentrations) in factor IX-deficient plasma supplemented with 100% (90 nM) recombinant factor IX WT (A), R170A (B) and R233A (C) in the presence of increasing DHG: 0 µM (●), 0.1 µM (○), 0.25 µM (■), 0.5 µM (□), 1 µM (▲), 2.5 µM (△), 5 µM (◆), 10 µM (◇). Control reactions are: no tissue factor or factor IX (▼), 0.2 pM tissue factor only (▽), and 90 nM recombinant factor IX only (▲). The time course of thrombin generation was measured as described in Material and Methods. Thrombin generation curves represent the mean thrombin concentration over the first 30 min from replicate determinations (n=3), and are identified by representative points. The relative velocity index for thrombin generation was plotted versus DHG concentration for each condition, and data were fit as described in Materials and Methods to determine the EC_{50} for inhibition (D). Representative curves are presented.

Figure 7- Effect of recombinant factor IXa mutants on DHG inhibition of plasma thrombin generation. Thrombin generation was initiated with 100 pM recombinant factor IXa WT (A), R170A (B), or R233A (C), 8.3 µM PC:PS vesicles, and 40 µg/ml CTI (plasma concentrations) in factor IX-deficient plasma in the presence of increasing DHG: 0 µM (●), 0.1 µM (○), 0.25 µM (■), 0.5 µM (□), 1 µM (▲), 2.5 µM (△), 5 µM


(◆), 10 µM (◇). Control reactions without recombinant factor IXa are presented (▼).

The time course of thrombin generation was measured as described in Material and Methods. Thrombin generation curves represent the mean thrombin concentration over the first 30 min from replicate determinations (n=3), and are identified by representative points. The relative velocity index for thrombin generation was plotted versus DHG concentration for each condition, and data were fit as described in Materials and Methods to determine the EC₅₀ for inhibition (D). Representative curves are presented.
Figures

Figure 1-

Figure 2-

Figure 3-
Figure 4-

A

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0 μM DHG</th>
<th>0.5 μM DHG</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>12</td>
<td>15</td>
<td>18</td>
</tr>
</tbody>
</table>

HCII-thrombin
TAT
Prothrombin

Thrombin

B

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0 μM DHG</th>
<th>0.5 μM DHG</th>
<th>2.5 μM DHG</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>6</td>
<td>9</td>
</tr>
</tbody>
</table>

HCII-thrombin
TAT
Prothrombin

Thrombin
Figure 5-

A

B

C

D

E

F

Figure 6-

A

B

C

D
Fucosylated chondroitin sulfate inhibits plasma thrombin generation via targeting of the factor IXa heparin-binding exosite

Yang Buyue and John P. Sheehan