Pergamon

Thrombosis Research 109 (2003) 333–339

THROMBOSIS RESEARCH

Regular Article

Additive thrombin inhibition by fast moving heparin and dermatan sulfate explains the anticoagulant effect of sulodexide, a natural mixture of glycosaminoglycans

Benilde Cosmi[a,*], Michela Cini[a], Cristina Legnani[a], Claudia Pancani[a], Fiorella Calanni[b], Sergio Coccheri[a]

[a] *Divisione di Angiologia e Malattie della Coagulazione, Policlinico S.Orsola-Malpighi, Università degli Studi di Bologna, Bologna, Italy*
[b] *Alfa Wassermann, Bologna, Italy*

Received 20 December 2002; received in revised form 14 April 2003; accepted 23 April 2003

Abstract

Introduction: The aim of the study was to evaluate the mechanism of the anticoagulant action of sulodexide, a mixture of glycosaminoglycans (GAGs) composed of dermatan sulfate (DS) and fast moving heparin (FMH), in vitro. *Materials and methods*: Thrombin clotting time (TCT) was measured in human platelet poor plasma (PPP). A chromogenic substrate assay was used to determine the pseudo-first order constant kinetic of thrombin inhibition ($k' = k_{obs}$/min) either in defibrinated PPP or antithrombin (AT) or heparin cofactor II (HCII) depleted defibrinated PPP in the absence and presence of sulodexide or its components, alone and in combination. The interaction between DS and FMH was analysed by both the algebraic fractional and isobole graphical methods. *Results*: Sulodexide, DS and FMH produced a dose-dependent prolongation of TCT with unclottable TCT at sulodexide above 4 μg/ml and at DS or FMH above 5 μg/ml. Sulodexide and its components alone and in combination produced a dose-dependent linear increase in the rate of thrombin inhibition in defibrinated PPP. The algebraic fractional and the isobole graphical methods indicated an additive effect between DS and FMH. In AT depleted PPP, the dose-dependent increase in k' produced by sulodexide was significantly lower than in PPP, while the dose-dependent increase in k' produced by DS was similar to the increase produced in PPP. In HCII depleted PPP, the dose-dependent increase in k' produced by sulodexide was significantly lower than in PPP, while the dose-dependent increase in k' produced by FMH was similar to the increase produced in PPP. *Conclusions*: Thrombin inhibition produced by sulodexide is due to the additive effect of its components, namely, HCII catalysis by DS and AT catalysis by FMH.

Keywords: Glycosaminoglycans; Thrombin; Interaction; Anticoagulants

1. Introduction

Sulodexide, a natural mixture of glycosaminoglycans (GAGs) composed of dermatan sulfate (DS) and fast moving heparin (FMH), is the active ingredient of an available drug for clinical use. Sulodexide has been shown to be effective both parenterally and orally in vascular diseases with thrombotic risk [1–5]. However, the mechanism of the anticoagulant and antithrombotic action of this mixture of GAGs is not completely elucidated.

GAGs exert their antithrombotic action by interacting with naturally occurring serine protease inhibitors (SERPINS) such as antithrombin (AT) and heparin cofactor II (HCII) [6]. As a result, the inhibition of the activated serine proteases of the coagulation system by SERPINS is accelerated by 1000-fold or more [7,8]. Thrombin is the pivotal serine protease of the coagulation system as it promotes the deposition of thrombi by cleaving fibrinogen which polymerises into fibrin [9]. Thrombin also exerts a positive feedback on its own generation by activating FVIII and FV

Abbreviations: AT, antithrombin; HCII, heparin cofactor II; FMH, fast moving heparin; DS, dermatan sulfate; PPP, platelet poor plasma; TCT, thrombin clotting time; GAGs, glycosaminoglycans; SERPINS, serine protease inhibitors.

* Corresponding author. Dipartimento Cardiovascolare, Divisione di Angiologia, Ospedale S.Orsola-Malpighi, Università di Bologna, Via Massarenti 9, 40138 Bologna, Italy. Tel.: +39-51-6363420; fax: +39-51-341642.

E-mail address: bcosmi@med.unibo.it (B. Cosmi).

0049-3848/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0049-3848(03)00246-9

[10]. The inhibition of thrombin is a key mechanism by which drugs such as GAGs exert their anticoagulant and antithrombotic action [11]. In particular, FMH and DS accelerate the inhibition of thrombin by their specific interaction with AT and HCII, respectively [12]. As a result, they directly inhibit thrombin and also thrombin generation by inhibiting the feedback activation of prothrombin [10].

The aim of the study was to evaluate the mechanism of the anticoagulant action of a mixture of GAGs, sulodexide, in vitro. The specific objectives were the following: (a) to evaluate the interaction between the components of sulodexide (FMH and DS) on the inhibition of thrombin in human plasma and (b) to define whether the interaction between the components of sulodexide (FMH and DS) on the inhibition of thrombin is additive or synergistic. To this purpose, a chromogenic assay was employed to measure the rate of thrombin inhibition in human plasma in the absence or presence of sulodexide or its components, alone or in combination.

2. Materials and methods

2.1. Materials

Sulodexide (lot. no. 990406) and its components, dermatan sulfate fraction (DS OP 2047/1; MW:25,000 ± 2000 Da) and fast moving heparin fraction (Fm OPH 2046; MW: 7000 ± 2000 Da) (1:4; w/w), separated by Opocrin (Corlo, Modena, Italy) by means of size-exclusion chromatography, were provided by Alfa Wassermann (Bologna, Italy).

Human α-thrombin (prepared according to the method of Fenton et al. [13] with a specific activity of 3261 U/mg) and purified AT and HCII were purchased from Enzyme Research Laboratories, South Bend, IN, USA, through Cabru, Milan, Italy.

CNBr-Sepharose was purchased from Pharmacia, Milan, Italy.

A monospecific sheep IgG against human AT (10 mg/ml), an affinity purified goat anti-human HCII (5 mg/ml) antibody and purified human AT and HCII were obtained from Affinity Biologicals, Hamilton, Ontario, Canada, through Cabru.

Immunopure gentle elution buffer was obtained from Celbio Pierce, Milan, Italy.

Ultrafiltration concentrators were purchased from Amicon Grace, Amicon Division, W.R. Grace, Beverly, CT, USA.

AT and HCII activities were measured by commercial assays (STA Antithrombin III; Stachrom HCII) purchased from Diagnostica Stago, Asniéres-sur-Seine, France.

The defibrinating agent Ancrod (Arvin), the chromogenic substrate *N*-*p*-Tosyl-Gly-Pro-Arg-*p*-Nitroanilide and polybrene (hexadimethrine bromide, Sigma) were purchased from Sigma-Aldrich, Milan, Italy.

2.2. Methods

2.2.1. Collection and preparation of pooled platelet poor plasma (PPP)

Blood was drawn from the antecubital veins of 20 healthy volunteers into plastic syringes prefilled with 1/10 vol of 0.13 M trisodium citrate. Plasma was separated by centrifugation at 1700 × *g* for 15 min at 4 °C, pooled and frozen in aliquots at − 70 °C. Before pooling, prothrombin time and activated partial thromboplastin time were determined to ensure that they were in the normal range.

2.2.2. Measurement of thrombin clotting time (TCT)

Sulodexide or its components DS and FMH separately were added to PPP (1/10 v/v) and incubated at room temperature for 5 min. TCT was measured with a mechanic coagulometer (KC10, Amelung, M. Brunelli, Milan, Italy) using human α-thrombin at a final concentration of 1.0 U/ml (control value: 21–24 s) [14].

2.2.3. Immunodepletion of AT and/or HCII from plasma

A monospecific sheep anti-human AT IgG and a goat anti-human HCII IgG were each coupled to CNBr-Sepharose at concentrations of 10 and 1 mg/ml, respectively. Two and a half milliliter aliquots of plasma were subjected to affinity chromatography at 23 °C on a 7 × 1.5 cm column containing anti-AT IgG immobilized on Sepharose or a 7 × 1.5 cm column containing anti-HCII IgG coupled to Sepharose at an elution rate of 1 ml/3 min. The fractions were collected, pooled and concentrated to the starting protein concentration by ultrafiltration using Amicon concentrators with a molecular weight cut-off of 10,000 Da. The total protein concentration was determined using the method of Bradford [15] with human albumin as a standard.

While two affinity chromatography steps were sufficient to remove all of the HCII from the plasma, three passages over a regenerated column containing the anti-AT IgG complexed to Sepharose were needed to ensure complete removal of AT. Between passages, the column was regenerated with 5 ml of 0.2 M glycine–HCl and 0.5 M NaCl, pH 2.5 followed by 5 ml of 0.1 M Tris–HCl, pH 8.0 and was then equilibrated with 0.15 M NaCl buffered to pH 7.4 with 0.02 M Tris–HCl (TBS). The column containing immobilized anti-HCII IgG was regenerated with 10 ml of Immunopure gentle elution buffer followed by 5 ml of 0.1 M Tris–HCl, pH 8.0 and was then equilibrated with TBS. To characterize plasma depleted of AT or HCII, AT or HCII activities were measured using commercial assays. The levels of these proteins in the immunodepleted plasma preparations were then compared with those in the starting plasma.

As a control, 2.5 ml of citrated platelet poor plasma (Seph-PPP hereafter) was passed over a 8.5 × 1.5 cm column containing activated and ethanolamine blocked CNBr-Sepharose; this was followed by the addition of 5 column volumes of 0.2 M Gly–HCl and 0.5 M NaCl (pH

2.5) and Tris–HCl 0.1 M (pH 8.0) and the column was equilibrated with TBS (pH 7.4). Plasma fractions were collected, pooled and concentrated as mentioned above. Purified human AT and HCII at a concentration in the physiological range (2 and 1 μM, respectively [16,17], assuming the molecular weight of AT and HCII as 58 and 65.6 kDa, respectively [18,19]) were also added back to the immunodepleted plasma.

2.2.4. Preparation of defibrinated plasma

To allow the measurement of thrombin activity by the chromogenic method, plasma was defibrinated by incubation with Ancrod (0.34 IU/ml plasma) for 20 min at 37 °C [20]. After wringing out the clot with a wooden stick, the plasma was left on ice for 15 min and any remaining fibrin was removed by centrifugation at 10,000 × *g* for 5 min. The addition of Ancrod does not change significantly the levels of clotting factors [20].

2.2.5. Measurement of the rate of thrombin inhibition in plasma

The ability of plasma to inhibit thrombin activity was measured in defibrinated plasma in the absence and in the presence of GAGs after the addition of preformed thrombin by means of a discontinuous method [21,22].

In the absence of GAGs, 2 μl of 3504 nM α-thrombin was added to 98 μl of defibrinated plasma to obtain a final concentration of 70 nM α-thrombin.

To measure the effect of GAGs, the appropriate concentration of each agent was added to plasma in a volume of 100 μl (1:50, v/v). When the rate of thrombin inhibition was measured in the presence of GAGs, *N-p*-Tosyl-Gly-Pro-Arg-*p*-Nitroanilide was also added to obtain a final synthetic substrate concentration of 0.44 mM (1:50, v/v) and to compete with AT or HCII for thrombin. This effectively slows the inhibition rate and thus permits the measurement of the residual enzyme over a 60-s interval [23,24].

After incubation of the mixture of plasma containing the GAGs and the synthetic substrate at room temperature for at least 5 min, thrombin was added and a 10-μl aliquot of the mixture was withdrawn immediately and then every 10 s for 1 min. Each aliquot was transferred to a well of a 96-well microplate prefilled with a quenching solution composed of: (1) 25 μl of 1 mg/ml Polybrene, (2) 25 μl of a solution of 2.2 mM chromogenic substrate for thrombin, (3) 190 μl of TBS buffer. The residual thrombin activity was measured in the microplate by reading the change in absorbance at 405 nm in a microplate reader (Titertek Multiskan) at room temperature for 3 min every 15 s.

The efficacy of the quenching solution in preventing further enzyme inhibition by inhibitors or GAGs during the measurement of the residual enzyme activity was demonstrated by: (1) a constant rate of thrombin activity for at least 5 min following the addition to the quenching solution, and (2) similar thrombin activity both in control assays without plasma and in assays where an equivalent amount of thrombin was added to the quenching solution containing plasma.

Analysis of the enzyme inactivation was based on the first order rate equation:

$$[E]_t = [E]_0 e^{-k_{obs}t}$$

This can be transformed into: $\log[E]_t = \log[E]_0 - k_{obs}t/2.303$, where $[E]_t$ and $[E]_0$ are the enzyme concentrations at time t and time 0, respectively, and k_{obs} is the pseudo-first order rate constant given by: $k_{obs} = k[I]_0$, where $[I]_0$ is the initial concentration of the inhibitor. The value of k_{obs} is determined from the slope of the semilogarithmic plot of the residual enzyme activity, given by the initial rate of substrate hydrolysis (over 60 s) versus time or alternatively by fitting the data by nonlinear least squares regression to the exponential function: $[E]_t/[E]_0 = e^{-k't}$, where E_0 is the thrombin activity at time 0, E_t is thrombin activity at time t and k' is the pseudo-first order rate constant [21,22].

3. Statistical analysis

Data are expressed as mean ± confidence intervals as appropriate. Data analyses included *t*-test and linear regression when appropriate. The analysis was performed with the statistical package GraphPad Prism (version 3.00 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com). A two-sided *P* value of .05 or less was considered statistically significant.

The algebraic fractional and the isobole graphical methods were used to analyse the interaction between DS and FMH [25,26]. Equieffective concentrations are doses of FMH (designated Ae) or DS (designated Be) that when used separately produce the same rate of thrombin inhibition (k') as combined doses of FMH (designated A) and DS (designated B) in combination in sulodexide. The equieffective concentrations are substituted in the equation: $A/\mathrm{Ae} + B/\mathrm{Be} = C$. If C is <1, the combination is synergistic, if $C = 1$, the agents are additive, and if C is >1, antagonism is suggested. To establish whether C is significantly different from 1, 95% confidence intervals of each equieffective concentration were calculated on the basis of the linear dose-dependent increase in the rate of thrombin inhibition. Upper and lower 95% confidence limits were substituted in the equation: $A/\mathrm{Ae} + B/\mathrm{Be} = C$ to obtain confidence limits of C.

In the isobole graphical method, the equieffective doses of FMH alone are plotted on the *y*-axis, whereas the equieffective doses of DS alone are plotted on the *x*-axis. When the coordinates of the upper and lower 95% confidence limits of the combination in sulodexide giving the same degree of inhibition as the individual agents are plotted in each Cartesian system [26], an additive effect is indicated if they include the straight line connecting the equieffective concentrations of the individual agents,

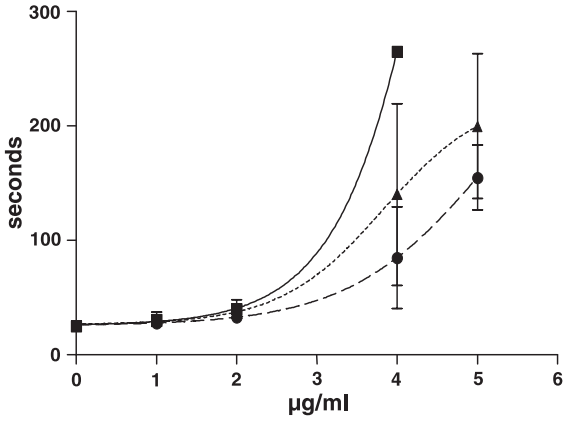

Fig. 1. The effect on thrombin clotting time (seconds) produced by increasing concentrations of sulodexide (full squares), FMH (full triangles) and DS (full circles). Each point represents the mean of six experiments ± 95% confidence intervals.

whereas a synergistic or antagonist effect is indicated if they are both below or above the straight line connecting the equieffective concentrations of the individual agents.

4. Results

4.1. The effect of GAGs on TCT

Sulodexide, FMH and DS all produced a dose-dependent prolongation of TCT (Fig. 1).

Sulodexide produced a greater prolongation of TCT than equivalent concentrations of both FMH and DS. FMH produced a greater prolongation of TCT than equivalent concentrations of DS.

Concentrations of sulodexide greater than 4 μg/ml and concentrations of both FMH and DS greater than 5 μg/ml produced a prolongation of TCT above 350 s.

4.2. The rate of thrombin inhibition in PPP

Both sulodexide and the combination of FMH 80% and DS 20% (DS/FMH = 1:4 w/w) produced similar slope when their dose-dependent increase in the rate of thrombin inhibition (k') was measured (Table 1; P=.51). Both DS and FMH produced a dose-dependent increase in the rate of thrombin inhibition (k') when added separately to PPP. The slope of the dose-dependent increase in k' produced by sulodexide was significantly higher than the slopes of the dose-dependent increase in k' produced by equivalent amounts of either FMH or DS in PPP (Table 1; $P < .0001$).

The equieffective concentrations of FMH and DS on the rate of thrombin inhibition (k') were calculated based on the dose response characteristics when tested alone and in combination in sulodexide. When these concentrations were substituted in the equation: $A/\mathrm{Ae} + B/\mathrm{Be} = C$ for $k' = 0.75$, $k' = 1$ and $k' = 1.5$, C resulted to be 0.92, 0.87 and 0.85, respectively. The upper and lower 95% confidence intervals of C were also calculated and they included 1 in all cases (0.64 and 1.25 for $k' = 0.75$, 0.67 and 1.12 for $k' = 1$ and 0.70 and 1.03 for $k' = 1.5$). As a result, it can be concluded that C is not significantly different from 1, thus indicating an additive effect for all the combinations tested. When the graphical isobole method was applied, the coordinates of the upper and lower 95% confidence limits of the combination of FMH and DS in sulodexide giving the same degree of inhibition as the individual agents (calculated for $k' = 0.75$, $k' = 1$ and $k' = 1.5$), included the straight line connecting the equieffective concentrations of the individual agents in a Cartesian system (data not shown). As a result, an additive effect was indicated as well for all the combinations tested.

4.3. The characterization of AT depleted and HCII depleted plasma

The AT depleted plasma was shown to be devoid of AT because AT activity was less than the lowest detectable levels (less than 5%). In contrast, the immunodepletion process had no effect on HCII activity.

The HCII depleted plasma was shown to be devoid of HCII because HCII activity was less than the lowest detectable levels (less than 5%). In contrast, the immunodepletion process had no effect on AT activity.

When AT or HCII at a concentration in the physiological range were added back to the immunodepleted plasma,

Table 1
The slope, the intercept and r^2 of the lines produced by increasing concentrations (0, 1, 2.5, 5 and 7.5 μg/ml) of FMH 80%–DS 20% combination, sulodexide, FMH and DS in Seph-PPP

	FMH 80%–DS 20%	Sulodexide	FMH	DS
Slope (95% CI)[a]	0.307 (0.280–0.333)	0.297 (0.260–0.333)	0.257 (0.252–0.262)	0.152 (0.139–0.164)
Y-intercept (95% CI)[a]	0.192 (0.079–0.304)	0.219 (0.067–0.372)	0.309 (0.287–0.330)	0.148 (0.097–0.199)
r^2	0.998	0.996	0.999	0.998

FMH 80%–DS 20% vs. sulodexide: P=.51.
Sulodexide vs. FMH: P=.013.
Sulodexide vs. DS: P=.001.
FMH vs. DS: P=.001.
For each concentration, the mean of six experiments was calculated.
[a] 95% CI indicates 95% confidence intervals.

Table 2
The slope, the intercept and r^2 of the lines produced by increasing concentrations (0, 1, 2.5, 5 and 7.5 μg/ml) of sulodexide in Seph-PPP, in HCII depleted plasma and in AT depleted plasma

	Sulodexide in Seph-PPP	Sulodexide in HCII depleted plasma	Sulodexide in AT depleted plasma
Slope	0.332	0.285	0.141
(95% CI)[a]	(0.288 to 0.375)	(0.242 to 0.327)	(0.124 to 0.159)
Y-intercept	0.140	0.070	0.077
(95% CI)[a]	(−0.043 to 0.324)	(−0.108 to 0.248)	(0.002 to 0.152)
r^2	0.995	0.993	0.995

Sulodexide in Seph-PPP vs. sulodexide in AT depleted plasma: $P < .0001$.
Sulodexide in Seph-PPP vs. sulodexide in HCII depleted plasma: $P = .049$.
For each concentration, the mean of six experiments was calculated.
[a] 95% CI indicates 95% confidence intervals.

thrombin inhibition in the presence of GAGs was identical to that of the starting plasma (data not shown), thus indicating that the immunodepletion did not significantly affect the characteristics of PPP.

To ensure that the immunodepletion did not affect significantly other plasma components, the activity of sulodexide, DS and FMH on thrombin inhibition was measured both in normal PPP and in Seph-PPP. The activity of sulodexide, DS and FMH and of the combination of FMH 80% and DS 20% was similar in normal PPP and in Seph-PPP (data not shown) thus indicating that the manipulation of plasma during the immunodepletion procedure did not affect significantly plasma components other than AT and HCII.

4.4. The mechanism of the additive effect of DS and FMH

To determine whether FMH can potentiate the anti-thrombin activity of DS in sulodexide through HCII or AT, thrombin inhibition produced by sulodexide was also measured in AT depleted plasma. The dose-dependent increase in the rate of thrombin inhibition produced by sulodexide was significantly lower in AT depleted plasma than in Seph-PPP (Table 2; $P < .0001$). The rate of thrombin inhibition produced by increasing amounts of DS was similar in AT depleted plasma and in Seph-PPP (Table 3; $P = .34$). These results indicate that in the absence of AT, the inhibition of thrombin by sulodexide is due solely to the activity of its DS fraction on HCII.

To determine whether DS can potentiate the anti-thrombin activity of FMH through HCII or AT, the rate of thrombin inhibition produced by sulodexide was also measured in HCII depleted plasma. The dose-dependent increase in the rate of thrombin inhibition produced by sulodexide was significantly lower in HCII depleted plasma than in Seph-PPP (Table 2; $P = .049$). The rate of thrombin inhibition produced by increasing amounts of FMH was similar in HCII depleted plasma and in Seph-PPP (Table 3; $P = .22$).These results indicate that in the absence of HCII, the inhibition of thrombin by sulodexide is due solely to the activity of its FMH fraction on AT.

5. Discussion

The objective of this study was to investigate the mechanism by which a mixture of GAGs, sulodexide, potentiates the rate of thrombin inhibition in vitro in human plasma.

Thrombin exerts a pivotal role in the pathogenesis of thrombosis. Thrombin has also several other functions in blood clotting, clot lysis and tissue repair [27]. To sustain normal and pathological processes, thrombin must be continuously generated as it is consumed by various mechanisms [27] and therefore it regulates its own generation by activating Factor V and Factor VIII [27]. As a result, thrombin is a primary target of antithrombotic drugs and the inhibition of thrombin and of its generation by GAGs is a key mechanism of their anticoagulant action.

TCT is a simple and inexpensive test routinely available in coagulation laboratories which could be suitable for monitoring the anticoagulant effect of sulodexide. However, concentrations of sulodexide greater than 4 μg/ml and concentrations of FMH and DS greater than 5 μg/ml prolonged TCT above 350 s. This effect is due to the use of human thrombin which is more sensitive than bovine thrombin to the inhibitory effect of human HCII in TCT [14]. As a result, TCT seems to be unsuitable to measure the effect of sulodexide at therapeutic concentrations (up to 7–10 μg/ml).

To evaluate the anti-thrombin effect of sulodexide and of its components alone or in combination, the kinetics of thrombin inhibition were measured in a plasma system after

Table 3
The slope, the intercept and r^2 of the lines produced by increasing concentrations (0, 1, 2.5, 5 and 7.5 μg/ml) of DS in Seph-PPP, DS in AT depleted plasma, FMH in Seph-PPP and FMH in HCII depleted plasma

	DS in Seph-PPP	DS in AT depleted plasma	FMH in Seph-PPP	FMH in HCII depleted plasma
Slope (95% CI)[a]	0.130 (0.077 to 0.183)	0.108 (0.065 to 0.151)	0.285 (0.228 to 0.341)	0.330 (0.241 to 0.418)
Y-intercept (95% CI)[a]	0.141 (−0.081 to 0.363)	0.073 (−0.107 to 0.253)	0.145 (−0.092 to 0.381)	0.176 (−0.197 to 0.549)
r^2	0.953	0.955	0.989	0.979

DS in Seph-PPP vs. DS in AT depleted plasma: $P = .34$.
FMH in Seph-PPP vs. FMH in HCII depleted plasma: $P = .22$.
For each concentration, the mean of six experiments was calculated.
[a] 95% CI indicates 95% confidence intervals.

defibrination by means of a chromogenic method. In a plasma system, part of the thrombin added (approximately 25%) binds to α_2-macroglobulin and remains amidolitycally active [22] thus adding to the experimental decay constant. This correction could reduce the experimental scatter; however, we did not correct the rate of thrombin inhibition for the binding to α_2-macroglobulin as its concentration in our pooled plasma would be constant. Although measuring the effect of GAGs in pooled plasma leads to the determination of local coefficients of variation, our goal was to obtain conditions as similar as possible to normal plasma.

Sulodexide, DS and FMH all produced a linear dose-dependent increase in the pseudo-first order constant of thrombin inhibition up to 7.5 μg/ml. The effect of the FMH 80%–DS 20% combination on the rate of thrombin inhibition was similar to the effect of equivalent concentrations of sulodexide, thus indicating that the relative amounts of FMH and DS in the sulodexide mixture were those declared by the manufacturer. In PPP, the slope of the dose-dependent increase in the rate of thrombin inhibition produced by sulodexide was significantly higher than the slopes produced by equivalent amounts of either FMH or DS. These data indicate that FMH and DS in combination in sulodexide have a greater effect on thrombin inhibition than equivalent amounts of the GAGs separately added to PPP. The interaction between DS and FMH in PPP was analysed by the algebraic fractional and the graphical isobole methods which are commonly used to evaluate the interaction between different agents [25,26]. The equation of the algebraic fractional method, calculated for different rates of thrombin inhibition ($k' = 0.75$, $k' = 1$ and $k' = 1.5$) provided values for C which are close to but slightly less than 1 ($C = 0.92$, 0.87 and 0.85, respectively), thus indicating a less than additive effect. The isobole graphical method also indicated a less than additive effect. However, to establish whether C was significantly different from 1, confidence intervals [26] were also calculated and they included 1 for all the combinations tested, thus indicating that C was not significantly different from 1. As a result, we concluded that an additive effect or zero-interaction was present.

The mechanism of the additive effect was evaluated by measuring the rate of thrombin inhibition by sulodexide or DS or FMH in PPP in the presence or in the absence of either AT or HCII. In the absence of AT, sulodexide produced a significantly lower rate of thrombin inhibition than in the presence of the inhibitor, while DS produced the same rate of thrombin inhibition as in the presence of AT. In the absence of HCII, sulodexide produced a significantly lower rate of thrombin inhibition than in the presence of the inhibitor, while FMH produced the same rate of thrombin inhibition as in the presence of HCII. These data indicate that the components of sulodexide, DS and FMH, interact separately on HCII and AT, respectively, in increasing the rate of thrombin inhibition with an additive effect.

Some limitations of our study should be pointed out. The DS and FMH components of sulodexide were separated by the manufacturer by size exclusion chromotography and we did not further purify the components. As a result, each of the DS and FMH fractions provided by the manufacturer might be contaminated with trace amounts of the other component. However, in our system, the activity of DS was similar in Seph-PPP and in AT depleted plasma. This indicates that any amounts of FMH contaminating the DS fraction have a negligible effect on HCII. Similarly, the activity of FMH was similar in Seph-PPP and in HCII depleted plasma thus suggesting that any trace amounts of DS contaminating the FMH fraction have a negligible effect on HCII.

Although in human plasma thrombin inhibition mediated through AT by FMH is predominant, HCII can also be activated by FMH. Differently from AT, which requires a specific pentasaccharide structure in heparin to be activated, HCII binds to most heparin oligosaccharides with four or more monosaccharide units in length regardless of their composition. Heparin chains that contain at least 20 monosaccharide units are required for maximal stimulation of the thrombin-HCII reaction [28]. The FMH component used in our study has a molecular weight of 7000 ± 2000 Da and could be responsible for the activation of HCII in the absence of AT. Therefore, it is possible that in AT depleted plasma, thrombin inhibition by sulodexide is due to the inactivation of HCII by both FMH and DS fractions in sulodexide. However, it can be expected that in vivo in the presence of both AT and HCII, FMH interacts predominantly with AT. In fact, in comparison with AT, HCII binds to heparin with a lower affinity, and a 10-fold higher concentration of heparin is required to accelerate thrombin inhibition by HCII [28].

Having measured the inhibition of preformed thrombin, a synergism between DS and FMH in the inhibition of thrombin generation cannot be completely excluded. Under these more physiological conditions, the combination of inhibitors with different mechanisms of action could result in a synergistic effect following activation of the coagulation system, as reported by other investigators using other inhibitors of coagulation [29].

Moreover, we chose not to evaluate the effect of FMH and of DS on the inhibition of factor Xa, as preliminary experiments indicated that the anti-Xa activity of our DS fraction, due to any contaminating FMH, was negligible up to a concentration of 50 μg/ml (data not shown).

Our observations indicate that small doses of DS can increase the anti-thrombin activity of FMH by catalysing the inhibition of thrombin by HCII. The use of a mixture of GAGs, such DS and FMH in sulodexide, could be advantageous as it could allow to obtain an antithrombotic effect with lower concentrations of the single components. A lower concentration of a single GAG could be associated with a lower frequency of bleeding side effects [30,31]. The practical implications of these experimental observations for

the prevention and treatment of venous thromboembolic disease are uncertain, but warrant further testing in appropriately designed studies in man.

6. Conclusions

The components of sulodexide, FMH and DS, show an additive effect on the rate of thrombin inhibition in human plasma. This additive effect is due to the catalysis of thrombin inhibition by two separate pathways: HCII by DS and antithrombin by FMH.

Acknowledgements

This project was supported by Alfa Wassermann, Bologna, Italy.

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