The direct thrombin inhibitors (argatroban, bivalirudin and lepirudin) and the indirect Xa-inhibitor (danaparoid) increase fibrin network porosity and thus facilitate fibrinolysis

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Summary

The present study aimed to assess whether the fibrin network structure is modified by the direct thrombin-inhibitors lepirudin, argatroban or bivalirudin and by the indirect Xa-inhibitor danaparoid. Using an in vitro assay that imitates the physiological process of coagulation from thrombin generation to fibrin formation, we examined a normal plasma pool spiked with one of the inhibitors. At concentrations considered to be the plasma levels observed during therapy, almost no influence was detected for lepirudin despite clear-cut effects on clotting time. However, argatroban, bivalirudin and danaparoid increased the fibrin gel permeability (Ks) to a similar extent. At concentrations higher than the therapeutic levels, the dose-response curve in the Ks assay became very steep for lepirudin while those were shallow for the others. In parallel with the drug-induced increases of Ks, larger network pores in 3D-microscopic images and significant shortening in “clot lysis time” induced by addition of rtPA were observed. Recombinant factor VIII (rFVIII) added to danaparoid-treated samples profoundly counteracted the increase of Ks but had only a slight or no effect on the other drugs. Thus, in vitro, argatroban, bivalirudin and danaparoid have comparable anticoagulating effects, rendering the fibrin network more permeable and less resistant to fibrinolysis. For lepirudin, the steep dose-response curve supports previous clinical findings, i.e. this thrombin inhibitor has a narrow therapeutic window. Furthermore, our data suggest that the haemostatic agent, rFVIII, might be effective in treatment of bleeding complications induced by danaparoid.

Keywords
Thrombin inhibitor, factor-Xa inhibitor, fibrin network porosity, fibrinolysis, recombinant FVIII

Introduction

The need for more effective anticoagulating agents – combined with an increasing recognition for the central role of thrombin in the “coagulation cascade” – has led to development of highly selective thrombin- or activated factor X (Xa)-inhibitors (1–3). In recent years, these new types of anticoagulants have been evaluated in large clinical trials of venous thrombosis, coronary artery disease, atrial fibrillation and ischaemic stroke. Though the therapeutic benefits in most clinical trials are evident, the drug dosing and the risk of bleeding complications partly remain uncertain.

The end product of the “coagulation cascade” is the fibrin network which together with platelet aggregates and other entrapped blood cells builds up the haemostatic plug or thrombus. Theoretically, the structure of the fibrin network may vary between two extremes, a network consisting of thin fibrin fibers lying close together, or thick fibrin fibers with fewer branches. For a tight fibrin network, the smaller pores are unfavourable for transportation of fibrinolysis-promoting components into the clot. The thin fibrin fibers provide few binding sites to t-PA and plasminogen and as a result, degradation of fibrin by plasmin is reduced (4–6). In contrast, in a permeable fibrin network with larger pores and thick fibrin fibers, the interaction between fibrin and plasmin should be facilitated, leading to an increased fibrinolysis. Therefore, the fibrin network porosity is an important determinant for the strength of the haemostatic plug (7–10). Since the polymerisation of fibrin monomers and the scaffold formation are critically dependent on thrombin generation potential or thrombin concentrations in plasma (9, 11), we thought that fibrin gel porosity may be of interest in the context of antithrombotic effects of thrombin- or Xa-inhibitors on the fibrin network structure.

Flow measurement and three-dimensional (3D) microscopic analyses are useful to determine the fibrin network porosity (12, 13). In

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older versions of these methods (11–13), fibrin networks were prepared by adding exogenous thrombin to plasma samples. In order to better resemble the physiology of haemostasis, we have modified the method by using tiny amounts of tissue factor and phospholipids to initiate the “coagulation cascade”, imitating the thrombin generation in vivo and the consequent fibrin formation (14).

We recently used the modified method to examine a normal plasma pool spiked with one of the inhibitors, assessing whether and how the fibrin network porosity is modified by danaparoid (a monovalent reversible thrombin-inhibitor), argatroban (a monovalent reversible thrombin-inhibitor), bivalirudin (a bivalent reversible thrombin-inhibitor), and danaparoid (a heparinoid which inhibits factor Xa through an antithrombin-mediated mechanism and also to some extent has thrombin inhibiting effects). The initial findings of drug-induced enhancements in fibrin network permeability have been reported at a symposium in Geneva (2007) (14). In the present study, we in addition demonstrate data from 3D-microscopic images, and “initial clotting time” and “clot lysis time” experiments that support our earlier findings. We also show that recombinant factor VIII (rFVIII) can reverse impairment in haemostasis caused by some of the drugs, especially by danaparoid. Our methods may be valuable in dose-finding studies of anticoagulating agents.

**Material and methods**

**Plasma samples and reagents**

A normal platelet-poor plasma pool (NPP) was obtained from CryoCheck (Dartmouth, NS, Canada).

One vial of recombinant human tissue factor (rTF) – Innovin® from Siemens Healthcare Diagnostics, Marburg, Germany, was reconstituted with 10 ml distilled H2O (stock solution: a theoretical concentration = 6,000 pM) (15) and then kept at −20°C in small portions. The purified phospholipid mixture (PPL, 250 μM, containing highly purified phosphatidylcholine, phosphatidylserine and sphingomyelin) was from Rossix (Mölndal, Sweden).

Argatroban was from Mitsubishi–Tanabe Pharma Corporation (Tokyo, Japan; lot no. L008ATS, MW=527). A stock solution of the inhibitor was prepared according to instructions from the manufacturer: 5.15 mg argatroban was mixed with 10.3 ml distilled water and placed in an ultrasonic bath at room temperature for 10 minutes (min), followed by a further incubation in another water bath without ultrasonic wave at 30°C for 3 min. The stock solution containing 500 μg/ml (949 μM) was stored at room temperature (22 − 24°C) for no longer than one month and protected from light.

Bivalirudin (Angiox®, MW=2,180), 50 mg/ml, was from Nycomed (Zurich, Switzerland). Danaparoid (Orgaran®, 1,250 U/ml, was from Organon (Oss, Netherlands). Lepirudin (Refludan®, MW = 6,980) from Pharmion (Cambridge, UK), 50 μg, was prepared into 50 mg/ml with distilled water. The three stock solutions of inhibitors were kept in small aliquots at −70°C.

Monoclate-P®, i.e. rFVIII from CSL Behring, Marburg, Germany, 300 IU, was prepared into 100 IU/ml with distilled water and stored in small aliquots at −70°C.

Recombinant tissue plasminogen activator (rt-PA; Actilyse®, Boehringer Ingelheim, Germany) was prepared into 1 mg/ml with the manufacturer’s solvent and stored in small aliquots at −70°C.

**Preparation of buffers**

- Diluting buffer: 50 mM Tris (hydroxymethyl-aminomethane) and 130 mM NaCl in distilled water, adjusted to pH 7.4 with HCl.
- Percolating buffer: 20 mM Tris, 100 mM NaCl, 20 mM imidazol and 5 KIU/ml aprotinin in distilled water, adjusted to pH 7.4 with HCl. This buffer was passed through a sterile filter (Millipore, 0.22 μm) and degassed before use.
- Buffer for FTTC (prepared before use): 280 ml NaCO3 solution (0.1 M in distilled water) was mixed with 120 ml NaHCO3 solution (0.1 M in distilled water) to obtain 400 ml of sodium carbonate-sodium bicarbonate buffer (pH = 9.5).

**Measurement of the fibrin gel permeability**

A solution for triggering the coagulation (”trigger solution”) was prepared as follows: 20 μl Innovin® (6,000 pM), 40 μl PPL (250 μM) and 200 μl CaCl2 (1 M) were added into 224 μl of the diluting buffer. Stock solutions of the different inhibitors were prepared in the diluting buffer, 5 μl of which was mixed with 200 μl NPP to obtain an “inhibitor-treated plasma sample” (total 205 μl). The sample mixtures (Table 1) contained lepirudin at 12 concentrations (0–1.4 μl), argatroban at nine concentrations (0–15.2 μl), bivalirudin at nine concentrations (0–16.5 μl) or danaparoid at nine concentrations (0–2.8 U/ml; U = unit of anti-Xa activity).

In a plastic test tube, 45 μl of the “trigger solution” was mixed with 205 μl of each “inhibitor-treated plasma sample”; final concentrations were 5 pM of Innovin, 4 μl of PPL and 20 μM of CaCl2. Using a gel-loading tip (diameter 0.5 mm; Labdesign, Stockholm, Sweden), 180 μl of the sample mixture was immediately pipetted into a plastic cylinder which had been coated with purified fibrinogen, where a fibrin gel was formed during the incubation period (overnight, at room temperature) (12, 16).

The flow measurement for assay of the fibrin gel permeability (Ks) was performed as described earlier (12). The obtained variables, i.e. eluate volume (Q), percolating time (t), viscosity mainly dependent of the environmental temperature (η), fibrin gel length (L), cylinder inter-area (A) and difference of hydrostatic pressure (ΔP) were included in a formula to calculate the permeability constant of Ks (Ks = Q.L. η / t. A. ΔP). In our laboratory, the highest level of Ks that can be measured is 40 (x 10−9, cm2), above which the fibrin network is highly porous.

To assess whether rFVIII can reverse the haemostatic potential impaired by the inhibitors, 205 μl of the “inhibitor-treated plasma...
In the samples without addition of rt-PA, plasma “initial clotting time” was defined as time-to-start of persistently detectable fibrin formation (15). In the samples with addition of rt-PA, “clot lysis time” was defined as the time (min) needed to show a 50% decrease of the maximum OD (17).

### Statistical methods

Statistical calculations were performed using GraphPad Prism for Windows. A level of Ks, “initial clotting time” or “clot lysis time” was shown as mean ± standard deviation (SD) from at least two repeated experiments. Differences between two groups with independent variables were identified with Student’s t-test. The associated variables – inhibitor concentrations (x) and Ks levels (y) – were analysed by the linear regression test to obtain a slope value. A p-value < 0.05 was considered statistically significant.

### Results

**Effects of different inhibitors on fibrin network permeability (Ks)**

A normal plasma pool without addition of the inhibitors was examined to obtain the control level of Ks 8.2 ±1.5 cm² x 10⁻⁹, which was set to “1” (Fig. 1).

Each of the inhibitors was used at a wide concentration range, principally covering the estimated plasma levels during therapy and “supratherapeutic” plasma levels (1–2 folds higher than the “therapeutic” levels). The results may indicate how the different inhibitor concentrations alter the fibrin network structure, favouring selection of proper dosages in clinical application and estimation of the bleeding risk due to over-dosing.

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**Table 1: The different inhibitor levels used in the present study.**

<table>
<thead>
<tr>
<th>Lepirudin μM (ref. 18–20 &amp; industry information)</th>
<th>Lower than “therapeutic” concentrations</th>
<th>Estimated “therapeutic” concentrations</th>
<th>“Supratherapeutic” concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 – 0.07</td>
<td>0.1 – 0.6</td>
<td>0.7 – 1.4</td>
<td></td>
</tr>
<tr>
<td>Argatiban μM (ref. 18, 21–23 &amp; industry information)</td>
<td>0 – 0.4</td>
<td>0.8 – 7.6</td>
<td>11.4 – 15.2</td>
</tr>
<tr>
<td>Bivalirudin μM (ref. 24–27 &amp; industry information)</td>
<td>0 – 0.9</td>
<td>2.8 – 7.3</td>
<td>11.0 – 16.5</td>
</tr>
<tr>
<td>Danaparoid anti-Xa U/ml (ref. 28–30 &amp; industry information)</td>
<td>0 – 0.2</td>
<td>0.4 – 0.9</td>
<td>1.4 – 2.8</td>
</tr>
</tbody>
</table>

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**Analysis of fibrin network structure by 3D-confocal microscopy**

In a plastic test tube, the “inhibitor-treated plasma sample” was mixed with the “trigger solution”, giving the same final concentrations as in the Ks assay. The sample mixture (120 μl) was added into a micro-chamber (made in our laboratory) which had been coated with purified fibrinogen (12). After incubation in a wet box overnight at room temperature, soluble substances in the network were washed away with the percolating buffer. To label the fibrin fibers, similar percolation was performed during the following one hour with a FITC solution (1.07 mM FITC in the sodium carbonate-sodium bicarbonate buffer). After further extensive washing for 1–2 hours, the 3D structure of the labelled fibrin network was observed by a confocal laser scanning microscope (Zeiss LSM510, Carl Zeiss, Germany). The 488 nm line from an argon ion laser was used for excitation, and fluorescence was collected through a 500–550 nm band pass filter. Image stacks measuring 71.4 x 71.4 x μm were collected and presented as 3D-projections, rendered with the software supplied with the confocal microscopy.

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**Determination of “initial clotting time” and “clot lysis time”**

To each of duplicate wells on a microtiter plate (Thermo Scientific, Waltham, MA, USA), 20 μl of the diluting buffer or a rt-PA solution was added into 100 μl of the “inhibitor-treated plasma sample”, followed by a 2-min shaking. The sample was then mixed with 25 μl of a solution containing Innovin, PPL and CaCl₂, giving final concentrations for the three reagents similar to those in Ks assay while that for rt-PA was 222 ng/ml. After another shaking for 10 seconds, fibrin optical density (OD) was kinetically recorded (240 reading circles within 240 min; 405 nm; 24°C), by using a spectrophotometer from Tecan (Gröding, Austria).

In the samples without addition of rt-PA, plasma “initial clotting time” was defined as time-to-start of persistently detectable fibrin formation (15). In the samples with addition of rt-PA, “clot lysis time” was defined as the time (min) needed to show a 50% decrease of the maximum OD (17).
Lepirudin (Table 1 & Fig. 1A)

At “therapeutic” concentrations of lepirudin, i.e. 0.1–0.6 μM ([18–20] & industry information), there was no increase – or even slight decrease – in the Ks levels, as compared to the control. However, at “supratherapeutic” concentrations, i.e. 0.7–1.4 μM, Ks was sharply increased, reaching values even beyond the highest measurable level in our laboratory (Ks > 40 cm² x 10⁻⁹ or > 4.9 fold of the control).

Argatroban and bivalirudin (Table 1 & Fig. 1B & C)

At “therapeutic” concentrations of argatroban, i.e. 0.8–7.6 μM ([18, 21–23] & industry information), Ks gradually increased from 16.4 ± 1.0 to 22.0 ± 0.8, which is about 2.0 and 2.7 fold of the control, respectively. For bivalirudin at “therapeutic concentrations”, i.e. 2.8–7.3 μM ([24–27] & industry information), Ks increased from 13.5 ± 2.1 to 22.1 ± 2.0, which is about 1.7 fold and 2.7 fold of the control, respectively.

At “supratherapeutic” concentrations, i.e. argatroban at 7.6–15.2 μM or bivalirudin at 7.3–16.5 μM, Ks increased from 22.0 ± 0.8 to 23.5 ± 0.6 for argatroban which is about 2.7 and 2.9 fold of the control, respectively, while Ks increased from 22.1 ± 2.0 to 31.3 ± 2.8 for bivalirudin, which is about 2.7 and 3.8 fold of the control, respectively.

Danaparoid (Table 1 & Fig. 1D)

Within the range of 0.4–0.9 U/ml, which corresponds to “therapeutic concentrations” of danaparoid ([28–30] & industry information), Ks increased from 14.2 ± 2.9 to 23.1 ± 0.9, which is about 1.6 fold and 2.8 fold of the control, respectively. At higher drug concentrations of 0.9–2.8 U/ml, Ks increased from 23.1 ± 0.9 to 27.5 ± 0.7, which is about 2.8 and 3.3 fold of the control, respectively.

Figure 1: Dose-response curves of lepirudin (lep), argatroban (arg), bivalirudin (biv), and danaparoid (dan) on the fibrin gel permeability (Ks). A normal plasma pool without addition of the inhibitors was used to obtain the control level of Ks 8.2 ± 1.5 cm² x 10⁻⁹, which was set to “1”, grey circles, within the “therapeutic” range. The highest detectable level of Ks in our laboratory is 40 x 10⁻⁹ cm² i.e., 4.9 fold of control. The linear regression analysis was made between the inhibitor concentrations (x) and Ks levels (y) to obtain a slope value. *** = p<0.001 (lep vs. arg, biv or dan); * = p<0.05 (arg vs. biv or dan); ns = p>0.05 (biv vs. dan).

Trend of Ks increase along with increasing inhibitor concentrations (Fig. 1)

In the present study, the slope value is assumed to be a parameter serving as a quantitative indication for the trend of Ks increase when increasing drug doses are given; a higher slope value incriminates a steeper trend. Within the “therapeutic” range, the slope values were non-significantly changed (p>0.05) between samples treated with bivalirudin (2.0 ± 0.002) or danaparoid (2.2 ± 0.5); both were significantly higher (p>0.05) than for argatroban (0.3 ± 0.07). At the higher concentrations, the slope value was 0.02 ± 0.009 for argatroban which was significantly lower (p<0.05) than those for bivalirudin (0.1 ± 0.02) and for danaparoid (0.3 ± 0.07);
however, difference between the latter two values was non-significant (p>0.05). For lepirudin, a linear correlation between Ks levels and drug concentrations was only shown at “supratherapeutic levels” (but not at “therapeutic concentrations”), where the slope value was 5.7 ± 0.58, significantly higher than those for other inhibitors (p<0.001).

3D-microscopic images of fibrin network derived from the inhibitor-treated plasma samples (Fig. 2)

Only the thrombin inhibitors were enrolled in 3D microscopic assays, since we did not have enough NPP with the same “lot No.” to make the experiments for danaparoid. The 3D-microscopic images from the samples containing lepirudin at concentrations within the “therapeutic” interval (0.1 μM or 0.3 μM) showed that the fibrin network structure was comparable with the control or even slightly tighter (Fig. 2). However, the network became more porous or extremely porous at the inhibitor concentrations of 1.0 μM or 1.3 μM, respectively; the latter network was broken down by the pressure of liquid percolation (impossible to make 3D-microscopic images). For argatroban- or bivalirudin-treated samples, the fibrin fibers were less branched and thus the network pores became larger, in a drug dose-dependent way.

Reversal effect of recombinant FVIII (rFVIII) on haemostatic impairment by the inhibitors (Fig. 3)

rFVIII, an important coagulant in the coagulation cascade, was added into the plasma sample containing one of the inhibitors (final concentration 1 IU/ml), which may contribute to thrombin generation, giving reversal effects on the inhibitor-induced increase of fibrin network permeability (Fig. 3). In samples containing lepirudin, almost no influence of rFVIII on Ks was detected. For the other inhibitors examined, addition of rFVIII made Ks decrease to different extents in terms of the minus percentages, i.e. –17% for argatroban at 7.6 μM, –14% for bivalirudin at 7.3 μM, and –107% for danaparoid at 0.9 U/ml. For the higher drug concentrations, the corresponding decreases were –22% for argatroban (15.2 μM), –25% for bivalirudin (16.5 μM) and –92% for danaparoid (2.8 U/ml).

“Initial clotting time” and “clot lysis time” in the inhibitor-treated plasma samples (Figs. 4 & 5)

“Initial clotting time” (lag phase until fibrin formation could be detected) is usually considered as an indication of the rate of fibrinogen cleaving by thrombin; a prolonged “initial clotting time” in principle reflects increased thrombin inhibition. In the control
plasma, “initial clotting time” was 3.0 ± 0.5 min. In the thrombin inhibitor-treated samples, “initial clotting time” was increased with increasing drug concentrations (Fig. 4). This action was most evident for lepirudin, where the dose-response curve was clearly the steepest. Until the end of the observation period (240 min), no clotting occurred at the drug concentrations higher than 0.4 μM; the “initial clotting time” was thus “undetectable”.

“Clot lysis time” is usually considered as an indication of the rate of fibrin dissolution by plasmin; a shorter “clot lysis time” reflects that the fibrinolytic potential is higher. Similar “clot lysis time” was observed between the samples treated by 0.04 or 0.1 μM lepirudin, or by the diluting buffer alone (control). Unexpectedly, higher concentrations of the inhibitor from 0.2 to 0.3 and 0.4 μM brought noticeable prolongation of “clot lysis time” which was 1.5 – 1.8 fold of the control level (Fig. 5). At lepirudin concentrations higher than 0.4 μM, “clot lysis time” could not be determined, since no clotting occurred during the observation period (240 min). However, in samples treated with argatroban or bivalirudin, shortening of “clot lysis time” were markedly drug dose-dependent (Fig. 5).

Also due to lack of the NPP, we could not perform the experiments of “clotting time” and “clot lysis time” experiments for danaparoid.
Discussion

The fibrin gel is the final product of coagulation cascade. Its network characteristics are critically dependent on thrombin generation rate and thrombin concentrations in plasma (11, 12). If the fibrin network permeability is increased following treatment of the plasma sample with a thrombin- or Xa-inhibitor, then we may consider that this is due to inhibitor-induced suppression in thrombin generation or thrombin activity. In fact, this idea concerning thrombin information is supported by other studies (31–33). Alike what we did in the present study when preparing the fibrin network, the authors employed tiny amounts of rTF and PPL to initiate the coagulation cascade in normal plasma spiked with one of the inhibitors; the results demonstrated that endogenous thrombin potential (ETP) was decreased in a drug dose-dependent way.

When we used the drug concentrations considered to be the plasma levels during therapy (Fig. 1), the maximum increases of the fibrin network constant (Ks) were similar for argatroban (2.7 fold of control), bivalirudin (2.7 fold of control) and danaparoid (2.8 fold of control). This suggests that the three inhibitors— if used in the dosages recommended — may give similar effects on the improvement of fibrin network permeability.

At “therapeutic concentrations” (Fig. 1), the slope value derived from the argatroban dose-response curve (0.3 ± 0.07) was significantly lower than for bivalirudin (2.0 ± 0.002) and danaparoid (2.2 ± 0.5). This implies that the antithrombotic effect of argatroban is less altered than for bivalirudin and danaparoid when the drug dosages were increased within the recommended range. In other words, a lower dose of argatroban is probably effective enough against thromboembolism, as recently suggested by some clinicians (according to industry information). At the “supratherapeutic” concentrations (Fig. 1), the slope values for argatroban (0.02 ± 0.009) was also lower than those for the other two inhibitors (0.1 ± 0.02 and 0.3 ± 0.07, respectively). Clinical investigations may be required to assess whether the different steepness of dose-response curves reflects different risks of bleeding complications, particularly in administration of over-dosing.

In the ETP measurements made by other groups (32, 33), “therapeutic” concentrations of lepirudin added to normal plasma caused a prolonged depression of thrombin generation (prolonged “lag phase”). However, after the delay, thrombin generation was detected, eventually showing an ETP level similar to the control. In agreement with these findings, a significantly prolonged “initial clotting time” and “unaffected” fibrin network porosity were shown in our investigation (Figs. 1 & 5). However, either based on the ETP assay or the Ks measurement, above findings are in conflict with the concept regarding a negative relationship between “lag phase” and ETP, or a positive relationship between “initial clotting time” and the fibrin network permeability, respectively. Although the underlying mechanisms are not yet confirmed, the effect of surface-activated coagulation following the tissue factor pathway activation is most probably contributing. Thus, lepirudin — when used at “therapeutic” concentrations — is effective in blocking the thrombin generated from the activation of tissue factor pathway by rTF (Fig. 5); later on, the plastic surfaces applied in the experiment and perhaps the exogenous PPL may trigger activation of the contact pathway, obtaining a full thrombin “burst”. At that moment, lepirudin — the irreversible thrombin inhibitor — cannot dissociate from its complex with thrombin; the subsequently generated thrombin molecules can then act on fibrinogen, leading to formation of a “normal” fibrin network.

At the “supratherapeutic” concentrations of lepirudin, great decrease or full suppression in ETP was detected by other groups (32, 33). This may help us to understand why the steep dose-response curve of lepirudin in the Ks assay is shown at concentrations above the “therapeutic” range. A possible explanation is that at the “supratherapeutic” concentrations of lepirudin, sufficient amounts of lepirudin molecules may be kept in a “free form” (not bound to thrombin), which inhibits thrombin stemming from the activation of contact pathway and thus increase the fibrin network permeability.

In the images of 3D-confocal microscopy, argatroban and bivalirudin modified the network structure of fibrin gel in a dose-dependent way, shown as fewer fiber branches and larger network
pores (Fig. 2). For lepirudin at concentrations within the “therapeutic” range, the fibrin network was almost unaffected or even tighter. Nevertheless, the higher drug concentrations made the network looser where the fiber branches were visibly reduced and the pores became larger. Thus, above information shown in the microscopic images did verify the results of liquid flow-measurement, confirming that the investigated inhibitors can change the architecture of fibrin network.

For newly developed antithrombotic compounds such as thrombin- or Xa-inhibitors, it is of importance to evaluate different haemostatic agents used as antidotes in bleeding complications. Agents like prothrombin complex concentrates (Feiba) and recombinant activated factor VIIa (rFVIIa, NovoSeven) have been assessed by other authors (34). In the present study, we were interested to examine FVIII. FVIII is an essential component of the contact pathway in blood coagulation cascade which increases factor Xa generation by the protease factor IXa. Similar to the ETP measurement and a global haemostasis assay set up by our group (15, 31), coagulation development in the present method should “bypass” the activation of tissue factor pathway and contact pathway, where FVIII up-regulates thrombin burst and in turn manipulate the fibrin network structure. As expected, in the samples containing argatroban or bivalirudin (Fig. 3), the enhancement of Ks was somewhat restored by addition of the recombinant FVIII (rFVIII; usually used to treat haemophilia A). Noteworthy, this coagulant displayed a great potential against the effect of danaparoid; the inhibitor-induced increase of fibrin gel permeability was fully or almost turned over at least when low or moderately high concentrations were used. Nevertheless, above mentioned influence on the fibrin network permeability was almost undetectable for lepirudin. Thus, rFVIII may be clinically significant for treatment of bleeding complication in therapy with danaparoid, and perhaps with argatroban or bivalirudin, although the induced risk of thrombotic events remains undetermined.

The assay of “clot lysis time” (Fig. 5) demonstrated that in the samples treated with argatroban or bivalirudin, the fibrinolytic potential, shown as time needed to obtain a 50% proteolysis of the clot, was reduced in parallel with the increases of fibrin network porosity. By view of this, it can be substantiated that fibrinolysis is up-regulated by the porosity of the fibrin network. As regards lepirudin, further studies are required to explain why the obvious prolongation of “clot lysis time” was shown in the plasma samples containing this inhibitor from 0.2 to 0.4 μM (Fig. 5) where the Ks levels are normal or only slightly decreased (Fig. 1).

In conclusion, this is the first work using a physiologically relevant system to evaluate the effects of different thrombin-inhibitors or the special Xa-inhibitors on fibrin formation. The data indicate that argatroban, bivalirudin and danaparoid have similar effects to increase the fibrin network porosity. This facilitates fibrinolysis, as an uncommonly recognised mechanism of the antithrombotic therapy. The lack of effect on fibrin network porosity by lepirudin at concentrations within the “therapeutic range” disputes the fact that lepirudin is a highly potent thrombin inhibitor. Thus, the fibrin gel permeability assay seems to be suitable to monitor the therapeutic effects for argatroban, bivalirudin and danaparoid except for irreversible thrombin inhibitors like lepirudin. However, the steep dose-response curve found at the higher drug concentrations indicates a narrow therapeutic window for lepirudin therapy, which does agree with the high risk of haemorrhagic complications in patients receiving excess doses of this drug (35–38). Furthermore, our data suggest that rFVIII, a haemostatic agent, may be effective for magement of bleeding complication in danaparoid therapy.

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References

What is known about this topic?
- The fibrin gel network porosity is an important determinant of the haemostatic plug strength including resistance to fibrinolysis.
- Fibrin gel porosity is of great interest in the context of the antithrombotic effects of the new Xa- or thrombin-inhibitors, with regard to the fibrin network structure (the final step in haemostasis) and the related increase of the fibrinolytic potential we have found. Only one paper has reported the effect of a single Xa inhibitor, fondaparinux, on clot structure and the consequent.
- For newly developed antithrombotic compounds such as thrombin- or Xa-inhibitors, it is of importance to test different haemostatic agents used as antidotes in case of bleeding complications. Agents like prothrombin complex concentrates (Feiba) and recombinant activated factor VIIa (NovoSeven) have been investigated by other authors.

What does this paper add?
- Several thrombin and Xa inhibitors using a large range of drug concentrations were evaluated with fibrin gel measurement (flow measurement), clotting time, fibrinolysis time and 3D confocal microscopy images.
- This study using the tiny dose of tissue factor to initiate coagulation is more physiologically relevant than our old versions of the method and those used by other authors.
- This is the first study which shows the possibility that rVIII can be helpful in the reversal of the haemostasis impairment by Xa inhibitors or maybe by thrombin inhibitors.
- The steep dose-response curve found at the higher drug concentrations of the thrombin inhibitor, lepirudin, indicates a narrow therapeutic window for this drug.
- This is the first study to demonstrate that larger pores of the fibrin network formed due to influence of different inhibitors, as shown in the 3D images, contribute to the fibrinolysis activity.


