Heparin-induced thrombocytopenia: in vitro studies on the interaction of dabigatran, rivaroxaban, and low-sulfated heparin, with platelet factor 4 and anti-PF4/heparin antibodies

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Heparin is a widely used anticoagulant. Because of its negative charge, it forms complexes with positively charged platelet factor 4 (PF4). This can induce anti-PF4/heparin IgG Abs. Resulting immune complexes activate platelets, leading to the prothrombotic adverse drug reaction heparin-induced thrombocytopenia (HIT). HIT requires treatment with alternative anticoagulants. Approved for HIT are 2 direct thrombin inhibitors (DTI; lepirudin, argatroban) and danaparoid. They are niche products with limitations.

We assessed the effects of the DTI dabigatran, the direct factor Xa-inhibitor rivaroxaban, and of 2-O, 3-O desulfated heparin (ODSH; a partially desulfated heparin with minimal anticoagulant effects) on PF4/heparin complexes and the interaction of anti-PF4/heparin Ab with platelets. Neither dabigatran nor rivaroxaban had any effect on the interaction of PF4 or anti-PF4/heparin Abs with platelets. In contrast, ODSH inhibited PF4 binding to gel-filtered platelets, displaced PF4 from a PF4-transfected cell line, displaced PF4/heparin complexes from platelet surfaces, and inhibited anti-PF4/heparin Ab binding to PF4/heparin complexes and subsequent platelet activation.

Introduction

Heparin remains a widely used parenteral anticoagulant for prevention and treatment of thrombotic events because of several favorable pharmacologic properties, leading to a rapid onset of action, short half-life, and reversibility. It is of low cost and available in all medical systems, making it an essential drug according to the World Health Organization. Except for bleeding, its most important adverse effect is heparin-induced thrombocytopenia (HIT).1,2 In HIT, immunogenic PF4/heparin complexes initiate anti-PF4/heparin Ab production,3-5 which trigger platelet activation through immune-complex engagement of FcyIIa receptors.6-8 This results in thrombocytopenia and a heightened prothrombotic state with an increased risk for new thrombosis9 that necessitates treatment with alternative anticoagulants.

Dabigatran and rivaroxaban are 2 new, oral anticoagulants which are already approved or are awaiting approval for thrombosis prophylaxis after major orthopedic surgery, for stroke prevention in patients with atrial fibrillation, and for treatment of deep vein thrombosis in several countries in North America and Europe. Both drugs have predictable pharmacokinetic and pharmacodynamic profiles.10 It is expected that both drugs will soon be widely used. Dabigatran is an oral, direct, and reversible thrombin inhibitor used as prodrug mesilate, a methanesulfonic acid salt, which is converted into the active form immediately after absorption.11 Rivaroxaban is an oral direct and reversible factor Xa inhibitor, which chemically belongs to the group of oxazolidinones. Because of their molecular structure, neither drug would be expected to interact with PF4 or PF4 binding to platelets. Although rivaroxaban did not induce platelet activation in the presence of anti-PF4/heparin Abs in a functional assay,12 systematic studies for dabigatran are lacking.

Another new, recently described compound, 2-O, 3-O desulfated heparin (ODSH), is derived from unfractionated heparin (UFH) by desulfation at the 2-O and 3-O position. Through this chemical modification, ODSH lacks most of the anticoagulant effects of heparin.13 It has a low affinity for antithrombin, and therefore low anti-Xa and anti-IIa activities and does not activate factor XII.13 ODSH was initially developed to separate the anticoagulant and the anti-inflammatory effects of heparin.14 The latter are dependent on 6-O sulfation15 and are almost unaffected by 2-O, 3-O desulfation. ODSH has also been implicated to have the potential to inhibit PF4/heparin complex formation.13

In this study, we systemically assessed the interactions of dabigatran, rivaroxaban, and ODSH with PF4/heparin complex formation, PF4 binding to platelets, and their effects on platelet activation by anti-PF4/heparin Abs in the presence of heparin. We found that dabigatran and rivaroxaban did not interact with PF4 or anti-PF4/heparin Abs, making them an attractive therapeutic option in patients with a history of HIT who require anticoagulation. In contrast, ODSH did inhibit formation of PF4/heparin complexes and platelet activation by anti-PF4/heparin Abs in the presence of heparin. If administered in combination with heparin, it might prevent the adverse effect of HIT.
Methods

Dabigatran, rivaroxaban, and ODSH

Dabigatran, the active form of dabigatran etexilate (Pradaxa), was provided by Boehringer Ingelheim and dissolved in 95% DMSO + 5mM HCl to obtain a stock solution with a concentration of 5 mg/mL. Rivaroxaban (Xarelto) was provided by Bayer HealthCare and dissolved in 100% DMSO (0.5 mg/mL); ODSH was provided as a solution (50 mg/mL) by ParinGenix Inc. Unfractionated heparin (UFH; 150 IU/mg; Braun), dabigatran, rivaroxaban, and ODSH (per concentrations described above) were used at 0.07, 0.13, 0.26, 0.52, 1.04, 2.08, 4.17, 8.33, 16.67, and 33.33 μg/mL if not indicated otherwise. To check vehicle effects, we additionally used 6.7%, 3.3%, and 1.7% DMSO as final concentrations in all experiments performed with platelets or cells.

Preparation of platelets

Platelet-rich plasma (PRP) was obtained from hirudinized (10 μg/mL, lepirudin [Refludan]; Pharmion) whole blood of healthy volunteers by centrifugation (120g, 20 minutes, 30°C). Gel-filtered platelets (GFPs) were obtained by adding PRP onto a Sepharose CL-2B liquid chromatography column (30 mL; Sigma-Aldrich) precultivated with buffer (137mM NaCl, 2.7mM KCl, 2mM MgCl_2 × 6 H_2O, 2mM CaCl_2 × 2 H_2O, 12mM NaHCO_3, 0.4mM NaH_2PO_4, 0.4% BSA, 0.1% glucose, pH 7.2).

Influence of dabigatran, rivaroxaban, and ODSH on PF4 binding to platelets

GFPs (40000/μL) were incubated (30 minutes, 37°C) with 25 μg/mL platelet factor 4 (PF4; ChromaTec) in the presence of increasing concentrations of UFH, dabigatran, rivaroxaban, ODSH, or buffer. Then GFPs were fixed (1% paraformaldehyde; Merck; 20 minutes, 4°C), washed twice with buffer (600g, 7 minutes, 4°C), incubated with rabbit anti-human PF4-FITC (30 minutes, 37°C), and washed, before flow cytometric analysis.

Influence of dabigatran, rivaroxaban, and ODSH on a PF4-transfected cell line

A human embryonal kidney (HEK-293) cell line transfected with, and expressing, human PF4 and the PF4 receptor, kindly provided by Dr P. Romagnani (University of Florence, Italy), was cultured in DMEM (Invitrogen) 10% FCS until reaching confluence, harvested, adjusted to 3 × 10^7/mL with buffer (PBS + Ca_2+/Mg_2+, 0.2% BSA, pH 7.2), incubated with UFH, dabigatran, rivaroxaban, ODSH, or buffer (30 minutes, 37°C), washed with buffer (190g, 10 minutes, room temperature), incubated with rabbit anti-human PF4-FITC (30 minutes, 37°C), and washed, before flow cytometric analysis.

Influence of dabigatran, rivaroxaban, and ODSH on PF4/heparin complex binding to platelets

GFPs (40000/μL) were preloaded with PF4/heparin complexes (25 μg/mL PF4 and 0.52 μg/mL UFH). UFH, 30 minutes, 37°C), allowing binding of PF4/heparin complexes at experimentally determined optimal concentrations. Then, platelets with bound PF4/heparin complexes were washed (137mM NaCl, 2.7mM KCl, 12mM NaHCO_3, 0.4mM NaH_2PO_4, 0.4% BSA, 0.1% glucose, 2.5 U/mL apyrase, 0.1 μg/mL hirudin, pH 6.3; 600g, 7 minutes, 37°C) to remove unbound PF4 and heparin, incubated with UFH, dabigatran, rivaroxaban, ODSH, or buffer, fixed, and processed as described above, before flow cytometric analysis. The amount of PF4 bound after preincubation of platelets with PF4/heparin complexes (formed at optimal ratios) was defined as 100% PF4 binding.

In a second series of experiments, GFPs were also incubated with PF4 (25 μg/mL), UFH (0.52 μg/mL), and ODSH or buffer concurrently before fixation. In addition, GFPs were preincubated with the monoclonal GPIIIa/IIa-specific Ab abxicinab (4 μg/mL; ReoPro; Centocor) to block platelet aggregation before they were incubated with PF4 (25 μg/mL), UFH (0.52 μg/mL), and ODSH (per concentrations described above) and UFH, and convulxin (100 ng/mL; kindly provided by Dr K. J. Clemetson, University of Bern, Switzerland), concurrently before fixation. Convulxin was used to activate platelets.

PF4/heparin Ab assays

An in-house ELISA that detects anti-PF4/heparin IgG was used as previously described. In addition, UFH, dabigatran, rivaroxaban, or ODSH (per concentrations described above) was added to the patient serum containing anti-PF4/heparin IgG before incubation with PF4/heparin coated onto a microtiter plate. The plate was washed and incubated with peroxidase-conjugated goat anti-human IgG, washed again, tetramethylbenzidine was added, and the reaction was stopped with 1M H_2SO_4. Six sera of patients with serologically confirmed HIT were tested.

The heparin-induced platelet activation (HIPA) test was performed as described using the low-molecular-weight heparin (LMWH) reviparin or UFH, both at 0.2 IU/mL (reviparin induces greater maximal platelet activation in the presence of anti-PF4/heparin Abs compared with UFH), or various concentrations of ODSH (as per the concentrations given above) to test for any cross-reactivity.

To assess whether the new anticoagulants inhibit platelet activation by anti-PF4/heparin Abs, in addition to the optimal concentration of LMWH required for platelet activation, UFH, dabigatran, rivaroxaban, or ODSH were added in increasing concentrations (per concentrations described above). In all functional experiments, high concentrations of UFH (100 IU/mL) were used to show inhibition.

Anticoagulant efficacy of ODSH mixed with UFH

Activated partial thromboplastin time (aPTT) and anti-factor Xa activity (anti-Xa; UFH standard curve) were analyzed by standard methods (BCS XP; Siemens Healthcare) in pooled plasma (n = 25 healthy blood donors) spiked with UFH (2 μg/mL ~ 0.3 IU/mL), ODSH (1.04, 2.08, 4.17, 8.33, 16.67 μg/mL) or the combination of UFH (2 μg/mL) and ODSH (1.04, 2.08, 4.17, 8.33, 16.67 μg/mL). The concentration of UFH was chosen to best identify changes in the PTT by additional ODSH. Saline 0.9% was added as buffer control to measure the baseline activity of the pooled plasma.

Statistical analysis

Comparison between groups was performed by paired samples t test. P values < .05 were considered statistically significant.

Ethics

All volunteers gave written informed consent in accordance with the Declaration of Helsinki. The study was approved by the University of Greifswald Institutional Ethics Review Board.

Results

Influence of dabigatran, rivaroxaban, and ODSH on PF4 binding to platelets

PF4 binding to platelets without any anticoagulants was arbitrarily set at 100% (baseline). UFH showed the typical PF4 binding curve with increasing PF4 binding at low concentrations, reaching maximal binding at 0.52 μg/mL (4-fold, P = .0009), with decreasing levels of bound PF4 at higher UFH concentrations added (Figure 1A). At concentrations of 8.33 μg/mL or higher, UFH
binding was even lower than baseline ($P = .0068$). Dabigatran had no effect on PF4 binding to platelets at all concentrations tested. Increased PF4 binding was seen with rivaroxaban only at concentrations $> 8.33 \mu g/mL$. However, when we tested the solution in which rivaroxaban is dissolved and which contains DMSO, even without added rivaroxaban, the same effect was seen at equivalent concentrations. Thus enhancement of PF4 binding was most likely caused by alteration of platelets by DMSO. As DMSO is not present when rivaroxaban is administered orally, these in vitro effects are without clinical relevance.

ODSH enhanced PF4 binding maximally at $0.52 \mu g/mL$ ($P = .0024$) but only by 2-fold and significantly lower than observed with UFH ($P = .0005$). Compared with UFH, ODSH was more effective in inhibiting PF4 binding to platelets, showing strong inhibition already at $2.08 \mu g/mL$ ($P = .0039$).

Platelet P-selectin (CD62P) and PF4 expression was minimal after gel filtration as assessed by flow cytometry (CD62P: 5.8 MFI ± 0.9; PF4: 13.2 MFI ± 2.8; $n = 14$), which makes release of additional PF4 from the nonactivated platelet $\alpha$-granules unlikely. However, both markers were strongly enhanced on platelets activated with convulxin (CD62P: 250.0 MFI ± 13.2, PF4: 36.6 MFI ± 15.5; $n = 4$).

Influence of dabigatran, rivaroxaban, and ODSH on PF4-transfected HEK-293 cells

PF4 not only binds to platelets but also to other cells like endothelial cells and monocytes. To show that the effects of the anticoagulants are not limited to the platelet surface, we analyzed their potency for dissolving PF4 from a constitutively PF4-expressing HEK-293 cell line. The signal of surface-bound PF4 without addition of any anticoagulant was set as 100% baseline. Dabigatran and rivaroxaban did not affect PF4 expression on their potency for dissolving PF4 from a constitutively PF4-expressing HEK-293 cell line. The signal of surface-bound PF4 when rivaroxaban is administered orally, these in vitro effects are without clinical relevance.

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Influence of dabigatran, rivaroxaban, and ODSH on PF4/heparin complex binding to platelets

Binding of PF4/heparin complexes to the platelet surface might be necessary for anti-PF4/heparin Ab-induced platelet activation. Dabigatran and rivaroxaban had no effect on PF4 or PF4/heparin complex binding to platelets (except the above-described DMSO-induced artifact). In contrast, ODSH was not only effective in displacing PF4 but also displaced PF4/heparin complexes from the platelet surface starting at $0.13 \mu g/mL$ and reaching significance at a concentration of $1.04 \mu g/mL$ ($P = .0253$; Figure 1B). UFH reduced PF4 binding at higher concentrations, reaching significance at a concentration of $2.08 \mu g/mL$ ($P = .0248$).

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Platelet P-selectin (CD62P) and PF4 expression were minimal after washing platelets (CD62P: 6.4 MFI ± 2.2, PF4: 10.7 MFI ± 4.4; $n = 13$), which makes release of additional PF4 from the platelet $\alpha$-granules unlikely. However, they were strongly enhanced on platelets activated with convulxin (CD62P: 250.0 MFI ± 13.2, PF4: 36.6 MFI ± 15.5; $n = 4$).

Figure 1. ODSH but not dabigatran or rivaroxaban displaces PF4 or PF4/heparin complexes from cell surfaces. (A) UFH strongly increased PF4 binding to the platelet surface at low concentrations and inhibited PF4 binding at high concentrations. ODSH was even more effective in displacing PF4 from platelets starting at concentrations of 2.08 $\mu g/mL$ while there was only a weak enhancement at lower concentrations. Dabigatran had no effect. The increase of PF4 binding by rivaroxaban at higher concentrations was because of the solvent carrier DMSO. GFPs were incubated with 25 $\mu g/mL$ PF4 in the presence of increasing concentrations of UFH, ODSH, dabigatran, or rivaroxaban. PF4 binding was detected with a FITC-labeled anti–human PF4 Ab using flow cytometry and Ab binding was quantified by geometric MFI. PF4 binding without anticoagulant was defined as 100%. Data are mean ± SD of 4 (DMSO = 3) independent experiments.

(B) ODSH displaced PF4 from a PF4-expressing HEK-293 cell line more efficiently than UFH. Dabigatran and rivaroxaban showed no effect. PF4-transfected HEK-293 cells were incubated with increasing concentrations of UFH, ODSH, dabigatran, or rivaroxaban. PF4 expression was detected with a FITC-labeled anti–human PF4 Ab using flow cytometry and Ab binding was quantified by geometric MFI. Surface-bound PF4 without anticoagulant was defined as 100%. Data are mean ± SD of 4 (UFH = 5) independent experiments. (C) ODSH and UFH equally displaced PF4/heparin complexes from the platelet surface. Dabigatran had no effect. The increase of PF4 binding with high concentrations of rivaroxaban was because of the solvent carrier. GFPs preincubated with PF4/heparin complexes (formed at optimal ratios) and then washed, were incubated with increasing concentrations of UFH, ODSH, dabigatran, and rivaroxaban. PF4 binding was detected with a FITC-labeled anti–human PF4 Ab using flow cytometry and Ab binding was quantified by geometric MFI. Preincubation of platelets with PF4/heparin complexes was defined as 100% binding. Data are mean ± SD of 4 (DMSO = 3) independent experiments.
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Influence of ODSH mixed with UFH on PF4/heparin complex binding to platelets

Next, we analyzed whether ODSH can prevent PF4/heparin complex formation and binding to platelets in combination with UFH. PF4 binding in the presence of UFH alone (at optimal ratios) was defined as 100% binding. ODSH reduced PF4/heparin complex binding at 0.26 µg/mL or higher, with values reaching statistical significance at 2.08 µg/mL UFH, increasing concentrations of ODSH, and 100 ng/mL convulxin (open symbols). PF4 binding was detected with a FITC-labeled anti-human PF4 Ab using flow cytometry and Ab binding was quantified by geometric means. PF4 binding in the presence of PF4 and UFH alone was defined as 100%. Data are mean ± SD of at least 3 independent experiments.

Influence of dabigatran, rivaroxaban, and ODSH on Ab binding to PF4/heparin complexes

Dabigatran and rivaroxaban had no effect on Ab binding to PF4/heparin complexes. After showing that ODSH can displace PF4 or PF4/heparin complexes from cell surfaces using flow cytometry, we investigated its influence on anti-PF4/heparin Ab binding to PF4/heparin complexes coated onto a solid phase using a PF4/heparin ELISA. The signal of anti-PF4/heparin Ab binding without any anticoagulants was set at 100%. ODSH reduced Ab binding starting at 2.08 µg/mL and reaching significance at concentrations > 4.17 µg/mL (P = .0124) with the same efficacy as UFH (Figure 2). This indicates that ODSH directly interferes with the PF4/heparin complexes.

Influence of dabigatran, rivaroxaban, and ODSH on anti-PF4/heparin Ab-induced platelet activation

Dabigatran and rivaroxaban had almost no effect on platelet activation by anti-PF4/heparin Abs (the inhibition of platelet activation with high concentrations of rivaroxaban was again an artifact because of DMSO). ODSH did not induce platelet activation in the presence of anti-PF4/heparin Abs without LMWH at any concentrations tested (n = 3 sera). However, it inhibited platelet activation by anti-PF4/heparin Abs in the presence of 0.2 IU/mL LMWH starting at ~ 1.04 µg/mL ODSH (Figure 4 filled squares). The inhibitory effect was even more pronounced in the presence of UFH (Figure 4 open squares).

Anticoagulant efficacy of ODSH mixed with UFH

The former experiments showed that a mixture of UFH and ODSH could be an option to lower the risk for developing HIT. However, it must also be considered that ODSH could enhance the anticoagulant effect of heparin. We therefore assessed the aPTT and anti-Xa activity in pooled plasma spiked with UFH at a fixed concentration of 2 µg/mL (~ 0.3 IU/mL) and ODSH in increasing concentrations that had been effective in PF4/heparin complex displacing from platelets. Up to 4 µg/mL, ODSH had only minimal effects on the aPTT and anti-Xa activity (Table 1).

Discussion

In this in vitro study on the interaction of new anticoagulants with PF4 or anti-PF4/heparin Abs and platelets, we found no influence of dabigatran and rivaroxaban whereas partially desulfated ODSH inhibited fundamental reactions that are key to the pathogenesis of HIT.

Not unexpectedly, the 2 new agents dabigatran and rivaroxaban, which have no similarities in their molecular structure with heparin, did not show any interaction with PF4. In this regard, these newer anticoagulants are very similar to the established direct thrombin inhibitors, lepirudin22 and argatroban,23 which also do not interfere with the PF4/heparin Ag or anti-PF4/heparin Ab binding.24 Dabigatran and rivaroxaban are already approved for several indications25-27 and many additional studies are ongoing. Based on our present in vitro experiments, both drugs have no risk for inducing a HIT-like syndrome at clinically achieved concentrations (~ 0.05-0.5 µg/mL).28,29 Therefore, they seem to be appropriate anticoagulants in patients with a history of HIT. In countries that do not have access to any of the alternative anticoagulants approved for acute HIT (lepirudin,22 argatroban,23 or danaparoid30), dabigatran or rivaroxaban might also represent an option to treat patients with acute HIT.
In contrast to dabigatran and rivaroxaban, ODSH interferes with PF4 in several ways, as it inhibits PF4 binding to platelets, prevents PF4/heparin complex binding to platelets, and reduces anti-PF4/heparin Ab binding to PF4/heparin complexes. Through one or all of these mechanisms, ODSH inhibits platelet activation by anti-PF4/heparin Abs in the presence of heparin. To reveal these mechanisms of ODSH, we compared different concentrations of ODSH in the various experiments simulating in vivo reactions which might be important for the pathogenesis of HIT (Table 2). First, we analyzed PF4 or PF4/heparin complex binding to platelets or cells because patients expressing high platelet surface PF4 levels may have a higher risk of developing HIT. The effective ODSH concentration was dependent on the amount of PF4 present on the cell surface as well as on free PF4 in the fluid phase. As shown in Figure 1B and C, transfected HEK-293 cells express more PF4 than PF4/heparin-loaded platelets (906.0 MF1 ± 25.5 SD, n = 9 vs 382.9 MF1 ± 225.9 SD, n = 13) and consequently more ODSH was required to detach PF4 from these HEK cells than from platelets. Even higher ODSH concentrations were needed for PF4 displacement in experiments in which free PF4 was additionally present in the fluid phase (eg, when platelets where incubated with added PF4 and ODSH [Figure 1]; when PF4, ODSH, and UFH were coincubated at the same time [Figure 2 filled symbols], or when platelets were activated and released further PF4 from the α-granules [Figure 2 open symbols]). This was likely caused by binding of ODSH to free PF4 which left less ODSH for binding to surface bound PF4. However, the highest amount of ODSH was required to inhibit anti-PF4/heparin Ab binding to immobilized PF4/heparin complexes in the ELISA (Figure 3). This inhibition is likely because of disruption of the PF4/heparin complexes rather than their displacement from the microtiter plate. As in our experiments, PF4/heparin complexes are linked covalently to the microtiter plate via PF4 molecules at the basis of the complexes; increasing concentrations of heparin and ODSH results in disruption of the coated PF4/heparin complexes (with increasing release of PF4 into the fluid phase) and consequently to a decrease in anti-PF4/heparin Ab binding. We have previously shown that even very high concentrations of heparin do not detach the covalently bound basis PF4 molecules. We propose that increasing ODSH concentrations reduce Ab binding to PF4/heparin complexes by competitively binding to PF4 thereby disrupting the complexes. In this regard, our observation that, in the functional assay, similar ODSH concentrations are required to prevent platelet activation by anti-PF4/heparin Abs as needed to inhibit anti-PF4/heparin Ab binding to coated PF4/heparin complexes in the ELISA has interesting implications. The inhibition by high heparin/ODSH concentrations in the HIRA is more likely caused by disruption of PF4/heparin complexes than just by displacement of the complexes from the platelet surface. The inhibition of high heparin is not caused by interference of heparin with binding of immune complexes to FcγRIIa as heat-aggregated IgG induces FcγRIIa-dependent platelet activation even in the presence of high heparin concentrations. This indicates that PF4 displacement from platelets alone is not sufficient for inhibition of platelet activation as the immune complexes consisting of anti-PF4/heparin Abs and PF4/heparin complexes in the fluid phase likely still activate platelets by cross-linking FcγRIIa.

From this we conclude the following sequential steps of in vitro inhibition of platelet activation by anti-PF4/heparin Abs depending on the ODSH concentration. In the initial phase of in vitro activation of platelets by PF4/heparin Abs, small amounts of PF4 are released from the platelet α-granules and rebind to the platelet surface. There they form complexes with added heparin. At the same time, PF4/heparin complexes formed in the fluid phase bind to platelets. Anti-PF4/heparin Abs bind to PF4/heparin complexes, forming immune complexes. These immune complexes activate platelets by FcγRIIa cross-linking. In the presence of low concentrations of ODSH, PF4 or PF4/heparin complexes are displaced from the platelet surface but PF4/heparin complexes remain intact. PF4/heparin Abs still bind to the PF4/heparin complexes in the fluid phase, still allowing FcγRIIa cross-linking and platelet activation. Higher amounts of ODSH then also disrupt PF4/heparin complexes and thereby inhibit PF4/heparin Ab binding to isolated PF4/heparin complexes in the ELISA and platelet activation by PF4/heparin Abs in the functional assay.

Our in vitro data indicate that these different effects of ODSH on PF4 and on PF4/heparin complexes depend on (1) the concentration of ODSH, (2) the concentration of PF4, (3) the concentration of additional heparin, and (4) the activation status of platelets. Based on the current experiments, we propose the following model:

- ODSH binds charge dependently to PF4 but because of its lower charge density, it binds less strongly than heparin.

Table 1. ODSH has only minimal anticoagulant activity

<table>
<thead>
<tr>
<th>aPTT, s</th>
<th>Anti-Xa UFH, IU/mL</th>
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<tr>
<td>+Buffer</td>
<td>+2 µg/mL UFH</td>
</tr>
<tr>
<td>Buffer</td>
<td>31.7</td>
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<tr>
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<td>31.1</td>
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<tr>
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<td>+8 µg/mL ODSH</td>
<td>35.6</td>
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<tr>
<td>+16 µg/mL ODSH</td>
<td>41.6</td>
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ODSH indicates 2-O, 3-O desulfated heparin; aPTT, activated thromboplastin time; and UFH, unfractionated heparin.

aPTT and anti-Xa activity in pooled plasma (n = 25) was analyzed in the presence of buffer (0.9% saline; baseline activity), or 2 µg/mL (~0.3 IU/mL) UFH alone (line 1). Then increasing concentrations of ODSH were added. Up to a concentration of 4 µg/mL, ODSH had only minimal effects on the aPTT or anti-Xa activity.
Inhibition of platelet activation by anti-PF4/heparin Abs in the presence of platelet-
Inhibition of Ab binding to PF4/heparin complexes by disruption of the covalently
Inhibition of PF4/heparin complex binding to activated platelets in the presence of
Inhibition of PF4/heparin complex binding to resting platelets in the presence of
Displacement of PF4/heparin complexes from platelets with no free PF4 and no free
Inhibition of PF4 binding to platelets in the presence of 25
ODSH can therefore much more easily outcompete chondroitin
Relevant reactions in HIT
Table 2. Concentrations of ODSH needed for inhibition of relevant steps in the pathogenesis of HIT

<table>
<thead>
<tr>
<th>Relevant reactions in HIT</th>
<th>ODSH concentration with &gt; 50% inhibition, μg/mL</th>
<th>See also, Figure</th>
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<tbody>
<tr>
<td>Inhibition of PF4 binding to platelets in the presence of 25 μg/mL free PF4 but no heparin in the fluid phase (resembling a clinical situation with strong platelet activation)</td>
<td>2.08</td>
<td>1A</td>
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<tr>
<td>Displacement of PF4/heparin complexes from platelets with no free PF4 and no free heparin in the fluid phase</td>
<td>0.52</td>
<td>1C</td>
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<tr>
<td>Inhibition of PF4/heparin complex binding to resting platelets in the presence of 25 μg/mL free PF4 and 0.52 μg/mL heparin in the fluid phase</td>
<td>2.08</td>
<td>2</td>
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<tr>
<td>Inhibition of PF4/heparin complex binding to activated platelets in the presence of 25 μg/mL free PF4 and 0.52 μg/mL heparin in the fluid phase (resembling strong platelet activation in a heparin-treated patient = worst case scenario)</td>
<td>4.17</td>
<td>2</td>
</tr>
<tr>
<td>Inhibition of Ab binding to PF4/heparin complexes by disruption of the covalently attached complexes</td>
<td>8.33</td>
<td>3</td>
</tr>
<tr>
<td>Inhibition of platelet activation by anti-PF4/heparin Abs in the presence of platelet-derived PF4 and heparin in the fluid phase</td>
<td>8.33</td>
<td>4</td>
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ODSH indicates 2-O, 3-O desulfated heparin; HIT, heparin-induced thrombocytopenia; and PF, platelet factor.

ODSH actively interferes with the pathogenesis of HIT by changing the structure of the PF4/heparin complexes and by displacing the Ag from the platelet surface. In this regard, ODSH has similar characteristics as danaparoid.

Danaparoid is a mixture of low sulfated polysaccharides and is the drug with the longest use in HIT, since the 1980s. However, unlike ODSH, danaparoid is a potent anticoagulant. It contains ~ 5% of highly sulfated heparin, which accounts for a large proportion of its anticoagulant effect. We have shown in 1991 that this fraction of danaparoid fully cross-reacts with anti-PF4/heparin Abs, but that this cross-reactivity is inhibited by the 95% of low sulfated polysaccharides, such as heparan sulfate, chondroitin sulfate, and dermatan sulfate.

Taken together, the present study suggests that ODSH may have the potential to prevent induction of HIT in patients requiring heparin. It is an attractive concept to mix UFH or LMWH with ODSH in a 1:2 or 1:4 ratio to inhibit PF4/heparin complex formation mainly in situations where UFH is given in prophylactic dosing (~ 0.05-0.2 IU/mL = 0.3-1.3 μg/mL). The necessary concentrations of ODSH (~ 0.6-5.2 μg/mL) might probably not influence hemostasis by itself as these concentrations had only a minor effect on the PTT or anti-Xa assay alone or in combination with UFH (Table 1). This concept could be tested in patients receiving heparin for thromboprophylaxis after major surgery. If the concomitant application of UFH/ODSH reduces formation of anti-PF4/heparin Abs by preventing PF4/heparin complex formation, it is likely that it would also reduce the risk for developing HIT. ODSH has also the potential to inhibit platelet activation by anti-PF4/heparin Abs in acute HIT, but therapeutic dose anticoagulation of heparin (~ 0.5-0.8 IU/mL = 3.3-5.3 μg/mL), as well as the released PF4 from strongly activated platelets, would likely require higher concentrations of ODSH (~ 6.6-21.2 μg/mL) for disrupting PF4/heparin complexes. At this concentration, ODSH might already have some anticoagulant potency. In addition, our in vitro data cannot take into account the effect of ODSH on UFH interacting with cell surfaces or plasma proteins in vivo. ODSH may change the pharmacokinetics of UFH by influencing absorption, plasma protein binding, and clearance. Thus, the effect of the combination of UFH and ODSH in vivo on clotting parameters and potential other biologic effects of ODSH requires further testing in appropriately designed clinical studies.
A composite drug of ODSH and heparin which might reduce the risk of HIT might be especially attractive for developing countries. In these rapidly changing health care systems, thrombosis prophylaxis and treatment are increasingly important, but for reasons of availability and costs primarily UFH remains a mainstay of anticoagulant therapy. In regard to HIT, UFH has a much higher risk for causing HIT than LMWH, and testing for anti-PF4/heparin Abs might be very demanding and costly in these countries. Even if HIT is suspected, often no alternative treatments are available. In these clinical settings, combined ODSH and UFH may be beneficial for not only reducing immune complications but also averting Ab-mediated platelet activation.

In summary, we provide in vitro evidence for new concepts of treatment and prevention of HIT. Dabigatran and rivaroxaban may present alternative treatment options especially for patients with a history of HIT who require anticoagulation as they do not interact with PF4. ODSH prevents formation of immunogenic PF4/heparin Ags. A “blended” UFH-ODSH anticoagulant may have the potential to prevent one of the most dangerous adverse effects of heparin, HIT. These concepts should be assessed by appropriately designed clinical studies.

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Authorship
Contribution: K.K. designed and supervised the experiments, reviewed and analyzed the results, and wrote the manuscript; C.H. performed the experiments and analyzed the data; B.F. performed the experiment with the PF4-expressing HEK-293 cell line; and A.G. developed the concept, designed the experiments, reviewed and analyzed the results, and wrote the manuscript.

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Heparin-induced thrombocytopenia: in vitro studies on the interaction of dabigatran, rivaroxaban, and low-sulfated heparin, with platelet factor 4 and anti-PF4/heparin antibodies

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