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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Abstract

Heparin is a widely used anticoagulant. Due to its negative charge it forms complexes with positively charged platelet factor 4 (PF4). This can induce anti-PF4/heparin IgG antibodies. 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 two 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 antibodies with platelets. Neither dabigatran nor rivaroxaban had any effect on the interaction of PF4 or anti-PF4/heparin antibodies 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, inhibited anti-PF4/heparin antibody binding to PF4/heparin complexes and subsequent platelet activation. Dabigatran and rivaroxaban seem to be options for alternative anticoagulation in patients with a history of HIT. ODSH prevents formation of immunogenic PF4/heparin antigens, and, when given together with heparin, may have the potential to reduce the risk for HIT during treatment with heparin.
**Introduction**

Heparin remains a widely used parenteral anticoagulant for prevention and treatment of thrombotic events due to a number of favorable pharmacologic properties, including 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 antibody production,\(^3-5\) which trigger platelet activation through immune-complex engagement of Fc\(\gamma\)IIa receptors.\(^6-8\) This results in thrombocytopenia and a heightened prothrombotic state with an increased risk for new thrombosis\(^9\) that necessitates treatment with alternative anticoagulants.

Dabigatran and rivaroxaban are two 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. Due to their molecular structure, neither drug would be expected to interact with PF4 or PF4 binding to platelets. While rivaroxaban did not induce platelet activation in the presence of anti-PF4/heparin antibodies 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.\textsuperscript{13} It has a low affinity for antithrombin, and therefore low anti-Xa and anti-IIa activities and does not activate factor XII.\textsuperscript{13} ODSH was initially developed to separate the anticoagulant and the anti-inflammatory effects of heparin.\textsuperscript{14} The latter are dependent on 6-O-sulfation\textsuperscript{15} 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.\textsuperscript{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 antibodies in the presence of heparin. We found that dabigatran and rivaroxaban did not interact with PF4 or anti-PF4/heparin antibodies, 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 antibodies in the presence of heparin. If administered in combination with heparin, it might prevent the adverse effect of HIT.
Methods

Dabigatran, rivaroxaban, 2-O, 3-O desulfated heparin (ODSH)

Dabigatran, the active form of dabigatran etexilate (Pradaxa™), was provided by Boehringer Ingelheim (Biberach, Germany) and dissolved in 95% DMSO + 50 mM HCl to obtain a stock solution with a concentration of 5 mg/mL. Rivaroxaban (Xarelto™) was provided by Bayer HealthCare (Wuppertal, Germany) and dissolved in 100% DMSO (0.5 mg/mL); ODSH was provided as a solution (50 mg/mL) by ParinGenix Inc. (Weston, FL, USA). 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, Hamburg, Germany) whole blood of healthy volunteers by centrifugation (120 g, 20 min, 30°C). Gel-filtered platelets (GFP) were obtained by adding PRP onto a Sepharose CL-2B liquid chromatography column (30 mL; Sigma-Aldrich, Taufkirchen, Germany) preequilibrated with buffer (137 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂ x 6 H₂O, 2 mM CaCl₂ x 2 H₂O, 12 mM NaHCO₃, 0.4 mM NaH₂PO₄, 0.4% BSA, 0.1% glucose, pH 7.2).

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

GFPs (40,000/µL) were incubated (30 min, 37°C) with 25 µg/mL PF4 (ChromaTec, Greifswald, Germany) in the presence of increasing concentrations of anticoagulant or buffer: unfractionated heparin (UFH, 150 IU/mg; Braun, Melsungen, Germany), dabigatran, rivaroxaban, ODSH (all use at 0.07, 0.13, 0.26, 0.52, 1.04, 2.08, 4.17, 8.33, 16.67, 33.33 µg/mL). Then GFPs were fixed (1% paraformaldehyde, Merck, Darmstadt, Germany; 20 min, 4°C), washed twice with buffer.
(600 g, 7 min, 4°C), incubated with rabbit anti-human PF4 (Dianova, Marl, Germany) FITC-labeled with FluoReporter FITC Protein Labeling Kit (Molecular Probes, Eugene, OR) or mouse anti-human CD62P-PE Cy5 (BD Biosciences, San Jose, CA), and mouse anti-human CD42a-PE (BD Biosciences, San Jose, CA), or isotype controls (30 min, 4°C), washed, and analyzed by flow cytometry (Cytomics FC 500; Beckman Coulter, Krefeld, Germany). Antibody binding was quantified by geometric mean fluorescent intensity (MFI).

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

A HEK (human embryonal kidney)-293 cell line transfected with, and expressing, human PF4 and the PF4 receptor,16 kindly provided by Dr. Romagnani (Florence, Italy), was cultured in DMEM (Invitrogen, Karlsruhe, Germany) 10% fetal calf serum until reaching confluence, harvested, adjusted to 3x10^6/mL with buffer (PBS + Ca^{2+}/Mg^{2+}, 0.2% BSA, pH 7.2), incubated with UFH, dabigatran, rivaroxaban, or ODSH (per concentrations described above), or buffer (30 min, 37°C), washed with buffer (190 g, 10 min, RT), incubated with rabbit anti-human PF4-FITC (30 min, 37°C), and washed, before flow cytometric analysis.

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

GFPs (40,000/µL) were preloaded with PF4/heparin complexes (25 µg/mL PF4 and 0.52 µg/mL UFH, 30 min, 37°C), allowing binding of PF4/heparin complexes at experimentally-determined optimal concentrations. Then, platelets with bound PF4/heparin complexes were washed (137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.4 mM NaH₂PO₄, 0.4% BSA, 0.1% glucose, 2.5 U/mL apyrase, 0.1 µg/mL hirudin, pH 6.3; 600 g, 7 min, 37°C) to remove unbound PF4 and heparin, incubated with UFH, dabigatran, rivaroxaban, or ODSH (per concentrations described
above) 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 (per concentrations described above) or buffer concurrently before fixation. Additionally, GFPs were preincubated with the monoclonal GPIIb/IIIa-specific antibody abciximab (4 µg/mL; ReoPro, Centocor, Leiden, Netherlands) to block platelet aggregation before they were incubated with PF4 (25 µg/mL), UFH (0.52 µg/mL) and ODSH (per concentrations described above) or buffer and convulxin (100 ng/mL; kindly provided by Dr. Clemetson, Bern, Switzerland), concurrently before fixation. Convulxin was used to activate platelets.

**PF4/heparin antibody assays**

An in-house enzyme-linked immunosorbent assay (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 prior to incubation with PF4/heparin coated onto a microtiter plate. Then the plate was washed and incubated with peroxidase-conjugated goat anti-human IgG, washed again, tetramethylbenzidine was added, and the reaction was stopped with 1 M H₂SO₄. 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 antibodies
compared with UFH)\textsuperscript{19}, 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 antibodies, 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, Marburg, Germany) 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 less than .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 (four-fold, P=0.0009), 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=0.0068). Dabigatran had no effect on PF4 binding to platelets at all concentrations tested. Increased PF4 binding was seen with rivaroxaban only at concentrations higher than 8.33 µg/mL. However, when we tested the solution in which rivaroxaban is dissolved and which contains dymethylsulfoxide (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 µg/mL (P=0.0024) but only by two-fold and significantly lower than observed with UFH (P=0.0005). Compared to UFH, ODSH was more effective in inhibiting PF4 binding to platelets, showing strong inhibition already at 2.08 µg/mL (P=0.0039).

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 non-activated 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\(^20\) and monocytes\(^21\). 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 HEK-293 cells. As seen with platelets, ODSH was more effective than UFH in displacing PF4 from the cell surface beginning at 0.13 µg/mL and reaching significance at a concentration of 1.04 µg/mL (P=0.0253) (Figure 1B). UFH reduced PF4 binding at higher concentrations, reaching significance at a concentration of 2.08 µg/mL (P=0.0248).

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 antibody-induced platelet activation. Dabigatran and rivaroxaban had no effect on PF4 or PF4/heparin complex binding to platelets (except the above described DMSO induced artefact). In contrast, ODSH was not only effective in displacing PF4 but also displaced PF4/heparin complexes from the platelet surface starting at 0.07 µg/mL and reaching significance at a concentration of 1.04 µg/mL (P=0.0496), similar to the effects seen with UFH (Figure 1C).

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).
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 (P=0.0474), even if incubated concurrently with UFH (filled symbols, Figure 2). When platelets were additionally activated (open symbols), ODSH was less effective to inhibit PF4/heparin complex binding at 4.17 µg/mL (P=0.0002), 8.33 µg/mL (P=0.0006), 16.67 µg/mL (P=0.0001), 33.33 µg/mL (P=0.0048) compared to resting platelets (Figure 2), although it inhibited most of PF4/heparin complex binding at the very high concentrations.

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

Dabigatran and rivaroxaban had no effect on antibody 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 antibody binding to PF4/heparin complexes coated onto a solid phase using a PF4/heparin ELISA. The signal of anti-PF4/heparin antibody binding without any anticoagulants was set at 100%. ODSH reduced antibody binding starting at 2.08 µg/mL and reaching significance at concentrations greater than 4.17 µg/mL (P=0.0124) with the same efficacy as UFH (Figure 3). This indicates that ODSH directly interferes with the PF4/heparin complexes.
Influence of dabigatran, rivaroxaban, and ODSH on anti-PF4/heparin antibody induced platelet activation

Dabigatran and rivaroxaban had almost no effect on platelet activation by anti-PF4/heparin antibodies (the inhibition of platelet activation with high concentrations of rivaroxaban was again an artefact due to DMSO). ODSH did not induce platelet activation in the presence of anti-PF4/heparin antibodies without LMWH at any concentrations tested (n=3 sera). However, it inhibited platelet activation by anti-PF4/heparin antibodies in the presence of 0.2 IU/mL LMWH starting at about 1.04 µg/mL ODSH (filled squares, Figure 4). The inhibitory effect was even more pronounced in the presence of UFH (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 aXa activity (Table 1).
Discussion

In this in-vitro study on the interaction of new anticoagulants with PF4 or anti-PF4/heparin antibodies 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 two 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 argatroban23, which also do not interfere with the PF4/heparin antigen or anti-PF4/heparin antibody 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 (lepirudin22, argatroban30, or danaparoid31), 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 antibody binding to PF4/heparin complexes. Through one or all of these mechanisms, ODSH inhibits platelet activation by anti-PF4/heparin antibodies 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 since patients expressing high platelet surface PF4 levels may have a higher risk to develop HIT32. 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 1C, transfected HEK-293 cells express more PF4 than PF4/heparin-loaded platelets (906.0 MFI ± 25.5 SD, n=9 vs 382.9 MFI ± 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 (e.g. 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 antibody binding to immobilized PF4/heparin complexes in the ELISA (Figure 3). This inhibition is likely due to 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 antibody 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 antibody 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 antibodies as needed to inhibit anti-PF4/heparin antibody binding to coated PF4/heparin complexes in the ELISA has interesting implications. The inhibition by high heparin/ODSH concentrations in the HIPA 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 antibodies 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 antibodies depending on the ODSH concentration. In the initial phase of in-vitro activation of platelets by PF4/heparin antibodies, 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 antibodies 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 antibodies 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 antibody binding to isolated PF4/heparin complexes in the ELISA and platelet activation by PF4/heparin antibodies in the functional assay.

Our in vitro data indicate that these different effects of ODSH on PF4 and on PF4/heparin complexes depend on i) the concentration of ODSH, ii) the concentration of PF4, iii) the concentration of additional heparin, and iv) the activation status of platelets. Based on the current experiments we propose following model:
- ODSH binds charge dependently to PF4 but due to its lower charge density, it binds less strongly than heparin.

- PF4 binds to the platelet surface via glycosaminoglycans most likely via chondroitin sulfate. However, binding of PF4 to chondroitin sulfate is weaker than binding of PF4 to heparin.

- ODSH can therefore much more easily outcompete chondroitin sulfate from its binding to PF4 than it can outcompete heparin from its binding to PF4.

- At lower concentrations, ODSH likely displaces PF4 or PF4/heparin complexes, respectively, from the platelet surface, however without disrupting the PF4/heparin complexes (Figure 1; Figure 2).

- When the concentration of ODSH is further increased, it disrupts the PF4/heparin complexes, as shown by the ELISA experiment (Figure 3).

- At the same concentration at which ODSH disrupts PF4/heparin complexes, it inhibits platelet activation by anti-PF4/heparin antibodies in the functional assay (Figure 4).

- As immune complexes in the fluid phase activate platelets by the FcγRIIa without the need to bind to the platelet membrane by a second receptor (other than the FcγRIIa) as shown in functional experiments using aggregated IgG, it is likely that anti-PF4/heparin-IgG complexes in the fluid phase are also able to activate platelets. Therefore, ODSH inhibits platelet activation by anti-PF4/heparin antibodies only when present in concentrations high enough to disrupt the PF4/heparin complexes.

Our experiments provide an additional interesting observation. Lower concentrations of ODSH were needed to inhibit platelet activation by anti-PF4/heparin antibodies in the presence of UFH as compared to LMWH (Figure 4). This indicates that PF4/LMWH complexes differ in
their stability from PF4/UFH complexes which is in line with our empiric observation that the functional assay is more sensitive for platelet activating antibodies when LMWH instead of UFH is used. 19

While ODSH clearly inhibited PF4 binding to platelets at concentrations >>1 µg/mL, at lower concentrations (<1 µg/mL), ODSH enhanced PF4 binding somewhat (Figure 1A). Therefore we tested whether PF4 and ODSH form antigenic complexes using the functional assay. Over a wide range of concentrations, ODSH did not induce platelet activation in the presence of anti-PF4/heparin antibodies indicating that PF4/ODSH complexes might be structurally different from PF4/heparin complexes. This is likely due to desulfation of ODSH at position 2-O, 3-O, which may prevent close approximation of PF4 in optimal distances necessary for antigenicity36. However, the residual negative charge from the remaining sulfate groups still allows interaction with PF4. Thus ODSH actively interferes with the pathogenesis of HIT by changing the structure of the PF4/heparin complexes and by displacing the antigen from the platelet surface. In this regard ODSH has similar characteristics as danaparoid.24

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 about 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 antibodies, but that this cross-reactivity is inhibited by the 95% of low sulfated polysaccharides, such as heparan sulfate, chondroitin sulfate and dermatan sulfate.37

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 were UFH is given in prophylactic dose (~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-Factor 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 antibodies 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 antibodies 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 biological 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 antibodies might be very demanding and costly in these countries. Even if HIT is suspected, often times no alternative treatments are available. In these clinical settings, combined ODSH and UFH may be
beneficial for not only reducing immune complications but avert antibody 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 antigens. 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 EBNA cell line; and A.G. developed the concept, designed the experiments, reviewed and analyzed the results and wrote the manuscript. No company was involved in writing of the manuscript.

**Conflict-of-interest disclosure**

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References


Table 1. ODSH has only minimal anticoagulant activity.

Activated thromboplastin time (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.

Table 2. Concentrations of ODSH needed for inhibition of relevant steps in the pathogenesis of HIT.
### Table 1

<table>
<thead>
<tr>
<th></th>
<th>aPTT (sec)</th>
<th>anti-Xa UFH (IU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ buffer</td>
<td>+ 2 µg/mL UFH</td>
</tr>
<tr>
<td>+ buffer</td>
<td>31.7</td>
<td>49.0</td>
</tr>
<tr>
<td>+ 1 µg/mL ODSH</td>
<td>31.1</td>
<td>48.0</td>
</tr>
<tr>
<td>+ 2 µg/mL ODSH</td>
<td>33.1</td>
<td>48.4</td>
</tr>
<tr>
<td>+ 4 µg/mL ODSH</td>
<td>33.0</td>
<td>51.5</td>
</tr>
<tr>
<td>+ 8 µg/mL ODSH</td>
<td>35.8</td>
<td>55.8</td>
</tr>
<tr>
<td>+ 16 µg/mL ODSH</td>
<td>41.6</td>
<td>64.7</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Relevant reactions in HIT</th>
<th>ODSH concentration with &gt;50% inhibition</th>
<th>See also Figure</th>
</tr>
</thead>
<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 µg/mL</td>
<td>1A</td>
</tr>
<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 µg/mL</td>
<td>1C</td>
</tr>
<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 µg/mL</td>
<td>2</td>
</tr>
<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 µg/mL</td>
<td>2</td>
</tr>
<tr>
<td>Inhibition of antibody binding to PF4/heparin complexes by disruption of the covalently attached complexes</td>
<td>8.33 µg/mL</td>
<td>3</td>
</tr>
<tr>
<td>Inhibition of platelet activation by anti-PF4/heparin antibodies in the presence of platelet-derived PF4 and heparin in the fluid phase</td>
<td>8.33 µg/mL</td>
<td>4</td>
</tr>
</tbody>
</table>
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 µg/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 due to the solvent carrier DMSO. GFPs were incubated with 25 µg/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 antibody using flow cytometry and antibody binding was quantified by geometric mean fluorescent intensity (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 antibody using flow cytometry and antibody binding was quantified by geometric mean fluorescent intensity (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 due to 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
antibody using flow cytometry and antibody binding was quantified by geometric mean fluorescent intensity (MFI). Preincubation of platelets with PF4/heparin complexes was defined as 100% binding. Data are mean ± SD of 4 (DMSO=3) independent experiments.

Figure 2. ODSH inhibits PF4/heparin complex binding to platelets in a mixture with UFH dependent on platelet activation.

ODSH reduced PF4/heparin complex binding even if incubated concurrently with UFH (filled symbols). In case of activated platelets, higher ODSH concentrations are needed for inhibition (open symbols). GFPs were incubated with 25 µg/mL PF4 in the presence of 0.52 µg/mL UFH and increasing concentrations of ODSH concurrently (filled symbols). In addition platelets were first incubated with 4 µg/mL abciximab and then incubated with 25 µg/mL PF4 in the presence of 0.52 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 antibody using flow cytometry and antibody binding was quantified by geometric mean fluorescent intensity (MFI). PF4 binding in the presence of PF4 and UFH alone was defined as 100%. Data are mean ± SD of at least 3 independent experiments.

Figure 3. ODSH but not dabigatran or rivaroxaban reduces anti-PF4/heparin antibody binding to immobilized PF4/heparin complexes.

ODSH and UFH equally reduced antibody binding to PF4/heparin complexes. Dabigatran and rivaroxaban had no effect. Sera from patients with anti-PF4/heparin IgG were tested in an in-house PF4/heparin ELISA in the presence of increasing concentrations of UFH, ODSH, dabigatran, or rivaroxaban. The signal of anti-PF4/heparin antibody binding without anticoagulant was set 100%. Data are mean ± SD of 6 (rivaroxaban=5) independent experiments.
Figure 4. ODSH and UFH but not dabigatran or rivaroxaban prevent anti-PF4/heparin antibody induced platelet activation.

Sera from patients containing anti-PF4/heparin IgG were tested in the HIPA with 0.2 IU/mL LMWH in the presence of increasing concentrations of UFH, ODSH, dabigatran or rivaroxaban. In addition, ODSH was also tested in the presence of 0.2 IU/mL UFH. Fifteen (DMSO, n=14) platelet donors were tested with 3 different patient sera for each anticoagulant. The figure shows the number of donors with platelet activation until 45 minutes. At a concentration of 1.04 µg/mL, ODSH started to inhibit platelet activation by anti-PF4/heparin antibodies in the presence of LMWH (filled squares), while lower concentrations of ODSH were needed in the presence of UFH (open squares). Dabigatran had almost no effect. The inhibition of platelet activation with high concentrations of rivaroxaban was due to its solvent carrier DMSO.
Figure 1

A

B
Figure 1

![Graph showing PF4 binding with different anticoagulants](image1)

Figure 2

![Graph showing PF4 binding with ODSH](image2)
Figure 3

![Graph showing % PF4/heparin antibody binding against anticoagulant concentration (µg/mL). The graph compares different anticoagulants: UFH, ODSH, Dabigatran, and Rivaroxaban. The x-axis represents anticoagulant concentration (µg/mL), ranging from 0 to 33.33. The y-axis shows % PF4/heparin antibody binding, ranging from 0 to 140.](image)

Figure 4

![Graph showing donors with platelet activation against anticoagulant concentration (µg/mL). The graph compares different anticoagulant doses: UFH 0.2 IU/mL + ODSH, LMWH 0.2 IU/mL + UFH, LMWH 0.2 IU/mL + ODSH, LMWH 0.2 IU/mL + Dabigatran, LMWH 0.2 IU/mL + Rivaroxaban, and LMWH 0.2 IU/mL + DMSO. The x-axis represents anticoagulant concentration (µg/mL), ranging from 0 to 33.33. The y-axis shows donors with platelet activation, ranging from 0 to 15.](image)
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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