Sevuparin binds to multiple adhesive ligands and reduces sickle red blood cell-induced vaso-occlusion

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Summary

Sevuparin is a novel drug candidate in phase II development as a treatment for vaso-occlusive crises (VOC) in patients with sickle cell disease (SCD). As a heparin-derived polysaccharide, sevuparin has been designed to retain anti-adhesive properties, while the antithrombin-binding domains have been eliminated, substantially diminishing its anticoagulant activity. Here, we demonstrate that sevuparin inhibits the adhesion of human sickle red blood cells (SS-RBCs) to stimulated cultured endothelial cells *in vitro*. Importantly, sevuparin prevents vaso-occlusion and normalizes blood flow in an *in vivo* mouse model of SCD vaso-occlusion. Analyses by surface plasmon resonance (SPR) and fluorescence correlation spectroscopy (FCS) demonstrate that sevuparin binds to P- and L-selectins, thrombospondin, fibronectin and von Willebrand factor, all of which are thought to contribute to vaso-occlusion in SCD. Despite low anticoagulation activity, sevuparin has anti-adhesive efficacy similar to the low molecular weight heparin tinzaparin both *in vitro* and *in vivo*. These results suggest that the anti-adhesive properties rather than the anticoagulant effects of heparinoids are critical for the treatment of vaso-occlusion in SCD. Therefore, sevuparin is now being evaluated in SCD patients hospitalized for treatment of VOC.

Keywords: sickle cell disease, cell adhesion, adhesion receptors, vaso-occlusion, heparinoids.

Sickle cell disease (SCD) is the most common haemoglobinopathy, affecting millions of people worldwide (Weatherall, 2010). A single point mutation in the sixth codon of the β-globin gene (*HBB*) results in the synthesis of abnormal sickle haemoglobin (HbS), which polymerizes and causes red blood cell (RBC) deformation ('sickling') under hypoxic conditions. RBCs containing predominantly HbS (SS-RBCs) also demonstrate an adhesive phenotype, (Hebbel *et al*, 1980a) and the degree of adhesiveness is correlated with vaso-occlusive crisis (VOC) frequency (Hebbel *et al*, 1980b). Vaso-occlusion, the pathognomonic finding of SCD, involves binding of blood cells to the endothelium. SS-RBCs adhere to and activate endothelial cells and leucocytes, and also stimulate coagulation (Kaul & Hebbel, 2000; Brown *et al*, 2001; Zennadi *et al*, 2007, 2008; Ataga *et al*, 2012). Hypoxia/reperfusion injury also results in leucocyte activation, chronic and acute inflammation, and endothelial activation and damage (Frenette, 2002). All of these events, particularly the adhesiveness of both leucocytes and SS-RBCs to endothelial receptors, contribute to VOC (Manwani & Frenette, 2013), the most common clinical consequence of SCD with the exception of chronic haemolytic anaemia.

Pain is a hallmark of SCD (de Montalembert, 2008; Steinberg, 2011; Ballas & Darbari, 2013), can be acute or chronic, and is most often located in the back, chest, joints and the long bones of the extremities. VOC presents as acute pain that can last for several days to a week or more (de Montalembert, 2008; Steinberg, 2011). The frequency and severity of such painful episodes vary among patients (de Montalembert, 2008; Steinberg, 2011).

The initial attachment of SS-RBCs to the vessel wall has previously been demonstrated to be mediated by very short-lived interactions via selectins (Matsui *et al*, 2001, 2002; Burnette *et al*, 2011; Gutsaeva *et al*, 2011; Kutlar *et al*, 2012). Following further cascade activation, firm attachment of SS-RBCs and leucocytes occurs mainly via interactions with integrins, causing subsequent vessel obstruction (Kaul *et al*, 2000, 2006; Zennadi *et al*, 2004, 2007, 2008; Finnegan *et al*, 2007a). P-selectin contributes to SS-RBC adhesion and vaso-occlusion both *in vitro* and *in vivo* (Matsui *et al*, 2001, 2002;

Embury *et al*, 2004; Burnette *et al*, 2011). P-selectin blockade can improve blood flow and can decrease serum markers of disease in patients with SCD (Kutlar *et al*, 2012). In addition, E-selectin is upregulated during inflammatory processes (Ebnet & Vestweber, 1999; Parent & Eichacker, 1999; Langer & Chavakis, 2009) and participates in white blood cell adhesion in SCD (Turhan *et al*, 2002; Zennadi *et al*, 2008). However, as E-selectin is also an important immune component in response to infections, blocking E-selectin could theoretically be detrimental in infection-induced VOC. L-selectin contributes to lymphocyte and neutrophil trafficking and is cleaved from leucocytes during activation. Children with SCD and a history of silent cerebral infarct (SCI) have significantly higher soluble L-selectin levels than non-SCI patients, (Faulcon *et al*, 2013) suggesting that increased inflammatory responses may be associated with disease severity (Kinney *et al*, 1999; Faulcon *et al*, 2013). Although not yet demonstrated, blockade of adhesive interactions via L-selectin could be beneficial in SCD, especially in this group.

While cell-cell interactions between SS-RBCs and the endothelium are necessary for VOC to occur, VOC pathogenesis also involves endothelial cell activation and changes in the levels of various plasma proteins (Pathare *et al*, 2003; Finnegan *et al*, 2007b; Sparkenbaugh & Pawlinski, 2013). Several non-membrane bound proteins, such as von Willebrand factor (VWF) (Krishnan *et al*, 2008; Zhou *et al*, 2009; Chen *et al*, 2011) and thrombospondin (TSP) (Brittain *et al*, 1993, 2001a, b; Novelli *et al*, 2012), may contribute by serving as anchoring or bridging proteins, and several of these proteins have heparin-binding sites. Furthermore, both heparin and low molecular weight heparins (LMWHs) can bind to several adhesion proteins (Koenig *et al*, 1998; Gupta *et al*, 1999; Polanowska-Grabowska *et al*, 2010; Burnette *et al*, 2011) to inhibit SS-RBC adhesion (Barabino *et al*, 1999; Matsui *et al*, 2001, 2002; Gayen Betal & Setty, 2008) and to restore blood flow in animal models of SCD (Embury *et al*, 2004).

In a clinical study of patients with SCD who were experiencing acute VOC, treatment with anti-thrombotic doses of the LMWH, tinzaparin, resulted in significant reductions in VOC duration, the number of days with severe pain and hospitalization duration (Qari *et al*, 2007). However, bleeding events required treatment cessation in some patients (Qari *et al*, 2007). Thus, while heparins and LMWHs have anti-adhesive and anti-inflammatory properties, as well as anticoagulant effects, all of which are potentially beneficial in VOC treatment, the associated risk of bleeding has slowed further clinical testing of heparinoids in SCD patients (Lever & Page, 2002; Qari *et al*, 2007; Shah *et al*, 2013).

Sevuparin (DF02) is a novel drug candidate currently in early phase development for VOC treatment in patients with SCD and parsite caused RBC adhesion in malaria (NCT02515838 and NCT01442168, respectively). Sevuparin is a negatively charged polysaccharide derived from heparin through chemical depolymerization, in which the specific pentasaccharide that is involved in high-affinity binding to antithrombin III is deleted. As a result, sevuparin has no direct effect on factor Xa nor on thrombin, and the effect on the activated partial thromboplastin time (aPTT) is markedly reduced in comparison with heparin and LMWH (Lindgren *et al*, 2015). Here, we demonstrate the binding characteristics of sevuparin with proteins involved in SCD vaso-occlusion and provide evidence of the pronounced anti-adhesive properties of this new potential treatment. Finally, we demonstrate that sevuparin improves blood flow in an *in vivo* model of VOC, likely through a multi-factorial mechanism of action involving several adhesion proteins.

Materials and methods

Sevuparin

Sevuparin, like other members of the chemical class of heparins, is polydisperse, encompassing a range of polysaccharide chain lengths. Sevuparin chains range in molecular weight from 1·2 to 15 kDa. The predominant size is 6–16 disaccharide units of 2-N-sulfo-6-O-sulfo-glucosamine and iduronic-2-O-sulfate acid, which corresponds to molecular weights of 3·6–9·6 kDa. The average molecular weight is approximately 8 kDa. The predominant chemical structure of sevuparin is illustrated in Fig 1. Sevuparin does not contain the specific binding sequence for antithrombin; as a consequence it has minimal anti-Xa and anti-IIa activity compared to other heparinoids (Table SI in Data S1).

Synthesis of fluorescein-labelled sevuparin

For direct interaction measurements with target proteins by fluorescence correlation spectroscopy (FCS), fluorescein-labelled sevuparin was purchased from EMC Microcollections GmbH (Tübingen, Germany), which performed sevuparin labelling via its free carboxy groups using standard ethylene-di-carbodiimide coupling.

Surface plasmon resonance measurements

Surface plasmon resonance biosensor technology (SPR, Biacore, GE Healthcare, Diegem, Belgium) was used to compare the interactions of sevuparin, LMWH (tinzaparin) and full-length heparin. P-, E-, and L-selectin Fc chimeras (R&D Systems, Minneapolis, MN, USA) were attached via anti-Fc antibodies to the sensor chip, and binding of sevuparin from solution to the immobilized selectins was recorded in real time. The data were subjected to both one-site and two-site binding non-linear analysis in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA).

Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy autocorrelation measurements employed the FCS unit of a Leica TCS SP5

Fig 1. Chemical structure of sevuparin. The diagram represents the predominant chemical structure of sevuparin with the main monosaccharide constituents. Sevuparin is a polysaccharide-based drug that is designed to retain the anti-adhesive properties of heparin. The mean molecular weight of sevuparin is approximately 8 kDa.
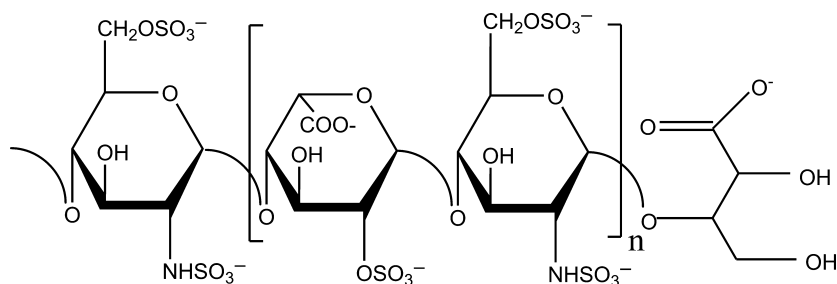
confocal microscope equipped with an HCX PL APO 63 × N.A. 1·2 water immersion lens (Leica Microsystems, Mannheim, Germany). Autocorrelation functions were analysed using ISS VISTA software (http://www.iss.com/microscopy/software/vistavision.html). Fitting was performed according to a 3D-Gaussian diffusion model, assuming two diffusing components and a triplet component. The fractions of bound and free ligand were obtained from the fractional contributions of the slow and fast diffusing components of the autocorrelation functions (Mader et al, 2004). The particle number obtained from the fit was corrected for uncorrelated background (Eq. 1), (Koppel, 1974)

$$G_{tot}(0) = 1 + 1/N_{app} = 1 + (1 - CPS_b/CPS_t)^2/N_{corr} \quad (1)$$

where CPS_t is the total signal (counts per second), CPS_b is the background signal, N_{app} is the apparent particle number obtained from the fit to the autocorrelation function and N_{corr} is the corrected particle number. The ratio of total fluorescence divided by the number of particles gives the counts per molecule (CPM), which is the measure of the average brightness of each particle. The CPM as a measure of molecular brightness was then determined according to (Eq. 2).

$$CPM = (CPS_t - CPS_b)/N_{corr} \quad (2)$$

RBC collection and preparation

Blood samples from patients who were homozygous for Hb S were collected into citrate tubes according to an Institutional Review Board (IRB)-approved protocol. Blood samples were collected without regard for presence of absence of hydroxycarbamide (also termed hydroxyurea) therapy; donors were excluded if they had experienced vaso-occlusion within the preceding 3 weeks or had been transfused within the last 3 months. RBCs were washed in phosphate-buffered saline (PBS, pH 7·4), and the plasma and buffy coat were removed.

In vitro adhesion assays

RBC adhesion assays were performed in a graduated height flow chamber as described previously (Zennadi et al, 2004). RBCs were labelled with a PKH-26 red fluorescent cell linker kit (Sigma, St. Louis, MO, USA) following the

manufacturer's instructions before adhesion assays. Confluent human umbilical vein endothelial cells (HUVECs) were grown and prepared on glass slides that had been coated with 2% gelatin (Sigma). P-selectin expression was induced by treating HUVECs with 50 ng/ml interleukin (IL) 13 (R&D Systems) for 48 h in culture medium without heparin (EGM-2, Clonetics, Walkersville, MD, USA) at 37°C and 5% CO_2, washing with PBS++ (PBS with Ca^{2+} and Mg^{2+}) and stimulating with histamine (100 µmol/l, Sigma) in PBS++ for 10 min at 37°C (see Data S1). For inhibition experiments, slides were incubated for 20 min at 37°C with sevuparin, antibodies or tinzaparin, washed again, and then mounted in the flow chamber. An RBC suspension (0·05%) was infused at a constant flow rate, determined by the heights measured in the adjustable flow chamber, to achieve the desired range of shear stresses (0·3–5 dynes/cm²). After RBCs had been infused through the chamber for 2 min, adhesion events were counted for 2 min at each of seven different locations along the length of the slide during continuous flow. Events counted included both transient and permanent adhesion, and each field counted was 0·25 mm² in area. The number of adhesion events was then calculated as cells/mm² at various shear stresses, and the shear stress at each point inside the flow chamber was calculated as previously described (Zennadi et al, 2004).

Mouse model of human sickle RBC adhesion and vaso-occlusion

Adhesive events and vaso-occlusion were studied in an adoptive nude mouse (Jackson Laboratories, Bar Harbor, ME, USA) model. In this model, infused human SS-RBCs cause vaso-occlusion, which is directly observed in subcutaneous vessels through implanted window chambers, as previously described in detail (Zennadi et al, 2007, 2012; Zennadi, 2014). This model has the advantage of using human SS-RBCs. Murine SS-RBCs lack several human RBC adhesion molecules, including the laminin receptor basal cell adhesion molecule/Lutheran antigen (BCAM/Lu, CD239) and the multifunctional receptor involved in RBC-leucocyte adhesion, CD44. The role of activation of red cell adhesion receptors may also be different (Trinh-Trang-Tan et al, 2010). In our adoptive model, a minority population of human SS-RBCs causes vaso-occlusion comparable to vaso-occlusion occurring in transfused SCD patients; moreover, we have shown

that these events also include adhesion of (murine) leucocytes, as they are believed to do in human illness (Zennadi *et al*, 2008). Finally, studies of inhibitors of signalling pathways important in vaso-occlusion suggest that findings using this adoptive model are well-replicated using sickle cell mouse models (Zennadi, 2014; Zhao *et al*, 2016).

Dil (Molecular Probes, Thermo Fisher Scientific, Waltham, MA USA) dye was used to label SS-RBCs. A window chamber consisting of a double-sided titanium frame was surgically implanted into the dorsal skinfold of each mouse, and animals were allowed to recover for 3-5 days to allow healing. To upregulate endothelial selectin expression and to promote SS-RBC adhesion and vaso-occlusion, 500 ng murine tumour necrosis factor α (TNFα; Peprotech, Rocky Hill, NJ, USA) in 100 μl saline was injected intraperitoneally (i.p.) 4 h before SS-RBC infusion. Generally, an aliquot from each patient sample was infused into three to five mice to allow for meaningful comparisons between treatments (e.g. sevuparin or tinzaparin) and control [sham (saline) treatment], due to the fact that the degree of adhesion is variable among patients. Thus, conclusions about per cent inhibition of adhesion always included a control experiment utilizing the same SS-RBCs as were used for animals receiving an adhesion inhibitor.

The extent of SS-RBC adhesion was determined and expressed as % vessel occupancy. The sum of vessel lengths along which there were adherent SS-RBCs was divided by the total vessel length examined and is presented as % SS-RBC occupancy for each vessel. All measurable venules were analysed, and at least 30 vessels for each mouse were measured. The average of these values represented the overall percentage of SS-RBC vessel occupancy in an individual mouse.

All measured vessels were also classified for blood flow status. Vessels were categorized into one of three categories: normal flowing, non-flowing or slow (or intermittent) flowing. Total vessel lengths in each category were summed for each mouse (from 4–5 movies), and the percentage was calculated by the total length of each category divided by the total length of vessels analysed in all three categories.

The degree of adhesion (% vessel occupancy) and vaso-occlusion (% of vessels with retarded or absent flow) associated with each treatment were first aggregated for multiple observed vessels with the same patient sample and animal. These data were then aggregated among patient samples. The data were compared using GraphPad Prism 4 software using a paired Student's *t*-test, one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. Specific comparisons between two treatments were performed using unpaired *t*-tests. A *P*-value <0·05 was considered to be significant.

Results

Selectin binding by sevuparin

Heparinoids have been associated with inhibition of P-selectin-mediated SS-RBC adhesion in SCD (Matsui *et al*, 2001;

Embury *et al*, 2004; Kutlar *et al*, 2012). Therefore, an initial experiment was performed to investigate sevuparin's selectin binding profile in comparison to LMWH and full-length heparin. The sevuparin-selectin interactions were studied by using surface plasmon resonance (SPR) and selectin chimeras in which the selectin ectodomain was combined with an immunoglobulin Fc domain (Scheffler *et al*, 1995). As sevuparin consists of long carbohydrate chains, it contains several potential binding sites; thus, more than one P-selectin chimera may interact with one sevuparin molecule. Multivalent binding of sevuparin was supported by analysis of the dose-response function of the binding reaction. Assuming a two-site binding model increased the quality of the fit compared with a one-site model. Using a two-site binding model for data analysis, the corresponding dissociation constants (Kd) were calculated as 0·022 and 1·6 μmol/l for heparin, 0·14 and 5·3 μmol/l for sevuparin, and 1·2 and 89 μmol/l for tinzaparin. However, the values from the one-site binding model (0·22 μmol/l for heparin, 0·73 μmol/l for sevuparin and 3·6 μmol/l for tinzaparin) (Fig 2A) ease the comparison to previously published data. These dissociation constants indicated that the P-selectin binding pattern was better conserved in sevuparin than in the LMWH tinzaparin, for which dissociation constants were considerably higher.

Given that E-selectin-mediated adhesion also has been implicated in SCD VOC, (Turhan *et al*, 2002; Hidalgo *et al*, 2009; Chang *et al*, 2010) we then extended our SPR analyses to chimeras of all three selectins. Kd values were determined using SPR measurements in which E-, P-, and L-selectin chimeras were immobilized on a chip. Sevuparin demonstrated high-affinity binding for both P-selectin (38 nmol/l) and L-selectin (0·95 μmol/l) (Fig 2B), while the Kd of sevuparin for E-selectin was 19 μmol/l (Fig 2B), demonstrating that sevuparin bound to E-selectin with low affinity.

Effect of sevuparin on SS-RBC adhesion to HUVECs

Given the promising results of the selectin binding profile, we then measured the anti-adhesive properties of sevuparin by employing a graduated height flow chamber to directly measure RBC adhesion to cultured HUVECs that had been stimulated with IL13 and histamine to upregulate P-selectin expression. Overall, sevuparin inhibited SS-RBC adhesion to these cells with a clear dose-dependent relationship (Fig 3A). An effect became observable when sevuparin was used at concentrations as low as 10 μg/ml, and at 50, 100, 200, 400 and 600 μg/ml, sevuparin had clear and significant effects (*P* = 0·006, 0·006, 0·001, 0·037, 0·001, respectively, using a paired *t*-test in which patient samples were compared to tests done in the absence of sevuparin using the same donor samples, see Fig 3A). Inhibition of adhesion was most effective with higher sevuparin doses and in patients' RBCs with higher baseline adhesion. The differences in inhibition
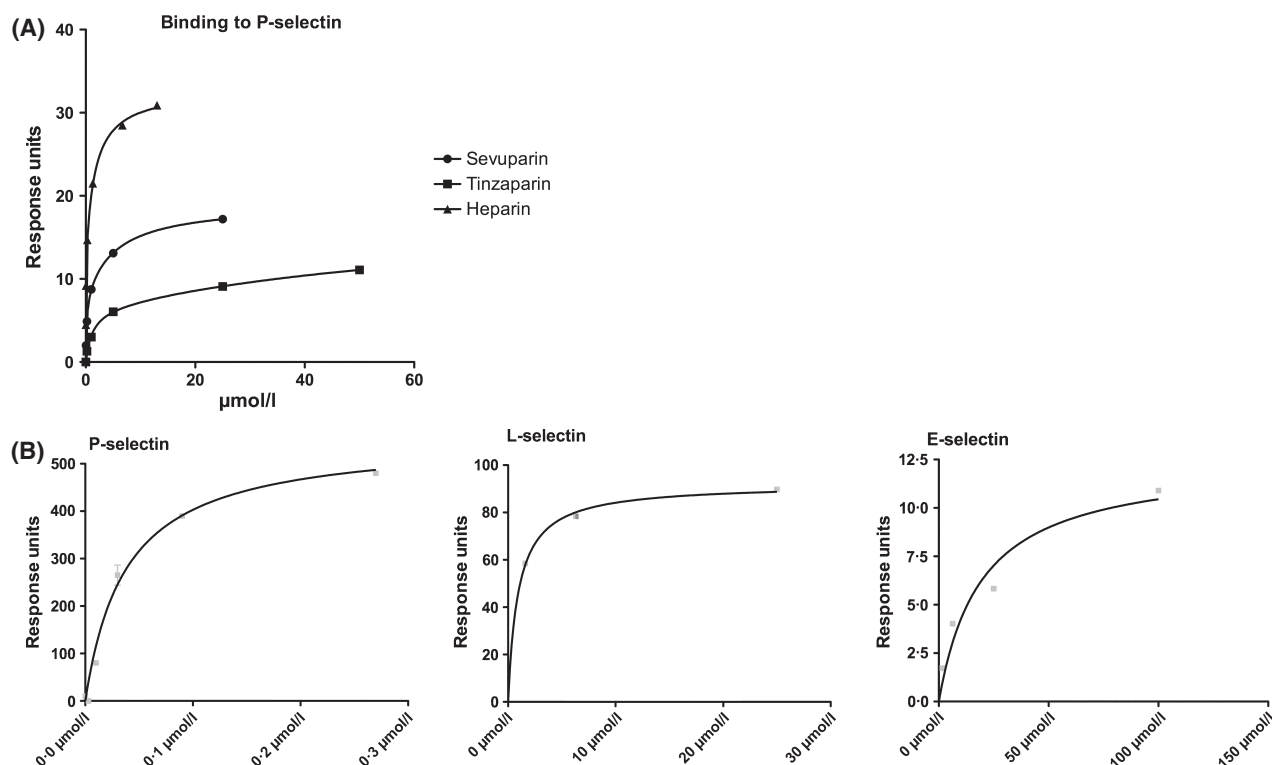
Fig 2. Sevuparin interactions with selectins. (A) Binding of sevuparin, tinzaparin and unfractionated heparin to P-selectin. Binding was recorded by SPR measurements with the heparinoids in solution binding to a P-selectin chimera that was attached to the chip. (B) Representative binding curves of sevuparin binding to E-, P- and L-selectins. P-selectin: The K_d values were calculated based on sevuparin concentrations of 1·6, 6·3, and 25 µmol/l without $CaCl_2$. L-selectin: The K_d values were calculated based on sevuparin concentrations of 1·6, 6·3, 25, and 100 µmol/l without $CaCl_2$. E-selectin: The K_d values were calculated based on sevuparin concentrations of 30–270 µmol/l without $CaCl_2$.

between highly adherent and only moderately adherent SS-RBCs were statistically significantly different in the group studied using 100 µg/ml sevuparin ($P = 0·02$, data not shown). The capacity of sevuparin to inhibit neutrophil adhesion to HUVECs was also assessed in the flow chamber system; however, higher concentrations of sevuparin were required to achieve significant inhibition of neutrophil adhesion (Figure S1, Data S1). The inhibitory effect of sevuparin on neutrophil adhesion was statistically significant at 400 µg/ml, and the effect size continued to increase up to 800 µg/ml *in vitro*.

P-selectin-mediated adhesion of SS-RBCs

Heparinoids have been previously shown to block P-selectin (Matsui *et al*, 2002; Embury *et al*, 2004). Therefore, to confirm that P-selectin was involved in the adhesion events observed in this study, monoclonal antibody (mAb) clone 9E1 (R&D Systems) was used to inhibit SS-RBC adhesion to IL13+histamine-stimulated HUVECs. The mAb markedly inhibited SS-RBC adhesion when concentrations of 7·5 µg/ml and 10 µg/ml were used, with 10 µg/ml 9E1 mAb producing a similar reduction in adhesion as was observed with 200 µg/ml sevuparin (Fig 3B).

Effect of sevuparin on SS-RBC adhesion and vaso-occlusion *in vivo*

After confirming the anti-adhesive effect of sevuparin in the *in vitro* assay, we sought to also assess its effect *in vivo*. Therefore, sevuparin was studied in a mouse model with both an anti-adhesion read-out and the crucial functional read-out of restoring normal blood flow. The results show that sevuparin markedly decreased the degree to which SS-RBCs adhered to blood vessel walls in animals that had been pretreated with TNFα. (Fig 4A, global analysis of variance $P < 0·0001$). Although the mean % occupancy observed with a 40 mg/kg sevuparin dose was only slightly lower than that observed with the 20 mg/kg dose, the difference between the doses was statistically significant ($P = 0·0459$). When microvascular functionality (blood flow) was investigated, sevuparin clearly demonstrated a dose-dependent capacity to prevent vaso-occlusion, with 20 mg/kg given subcutaneously demonstrating maximal or near-maximal effect. The read-out is given as the % of vessels with normal flow, and here a much larger proportion of vessels had normal blood flow after 20 mg/kg sevuparin than after saline infusion as control ($P = 0·0003$, Fig 4B). For this parameter, 40 mg/kg sevuparin led to a small and
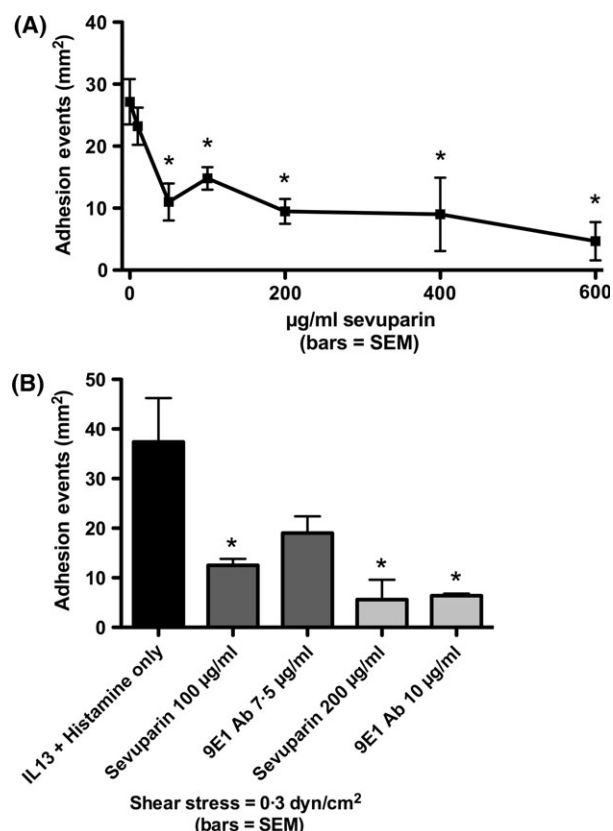
Fig 3. Effect of sevuparin and anti P-selectin monoclonal antibodies on SS-RBC adhesion to HUVECs. (A) Dose response of sevuparin concentration (μg/ml) *versus* number of adhesive events per mm^2 between human SS-RBCs and activated HUVECs, during continuous flow at 0·3 dynes/cm^2. * indicates $P < 0.05$ compared to adhesion in the absence of sevuparin. (N: IL13 and histamine only, 20. Sevuparin 10 μg/ml, 2; 50 μg/ml, 4; 100 μg/ml, 7; 200 μg, 6; 400 μg, 4; 600 μg, 3.) HUVECs were activated to increase P-selectin expression by IL13 and histamine treatment as described in Materials and methods. (B) Comparison of the effect of sevuparin and anti-P-selectin antibodies on SS-RBC adhesion to activated HUVECs. * indicates $P < 0.05$ compared to adhesion in the absence of sevuparin or mAb 9E1. (N: IL13 and histamine only, 6. Sevuparin 100 μg/ml, 3; 200 μg/ml, 2. 9E1 mAb 7·5 μg/ml, 4; 10 μg/ml, 2.)

statistically non-significant improvement of blood flow compared with 20 mg/kg sevuparin.

Comparison of the anti-adhesive effects of sevuparin and tinzaparin

Considering the clinically interesting effects on VOC in SCD observed with the LMWH tinzaparin, (Qari *et al*, 2007) a direct comparison between the anti-adhesive activities of sevuparin and tinzaparin was performed. When the capacity of sevuparin and tinzaparin to inhibit SS-RBC adhesion to endothelial cells in flow chambers *in vitro* was compared, sevuparin and tinzaparin were equally effective in reducing SS-RBC adhesion at 200 and 400 μg/ml compared with the control (Fig 5A), although due to variability, the differences

for control *versus* sevuparin 400 μg/ml ($P = 0.0593$), did not quite reach statistical significance.

Sevuparin and tinzaparin were also compared in the *in vivo* model. Each drug reduced vessel occupancy by adherent SS-RBCs to a similar degree at similar mg/kg doses (Fig 5B); there were no significant differences between the effects of sevuparin and tinzaparin at any dose. Indeed, the effects exerted by the two compounds are not statistically significantly different (vessel occupancy and blood flow, Fig 5B, C), while the difference between control and drug treatment was significant for all doses of both sevuparin and tinzaparin. The dose-responses were compared with matched controls, and a global statistical data analysis demonstrated that both sevuparin and tinzaparin were able to prevent vaso-occlusion in a dose-dependent manner (Fig 5C).

Target protein profiling

Taking together the initial selectin binding data and the anti-adhesive effects demonstrated both *in vitro* and *in vivo*, it could be hypothesized that sevuparin is also blocking additional adhesive interactions through more firm adhesion events, possibly via other adhesion receptors. To address this possibility, a second round of binding interaction studies was performed. FCS was employed to further define the target spectrum of sevuparin in a homogeneous format in solution. The selectins were included in order to to relate these new results to the previously generated data. For direct interaction measurements with target proteins, fluorescein-labelled sevuparin (fluo-sevuparin) was used, which had a diffusional autocorrelation time of 78 μs, as expected for molecules of this size. A degree of labelling of 0·2 per molecule was calculated by ultraviolet/Vis spectroscopy. At this low level of labelling, a sufficient number of molecules carry a label to allow detection, while the risk of interference with molecular interactions due to multiple labels per molecule should be minimal.

We investigated binding of sevuparin to selected cellular adhesion mediators, including, P-, E- and L-selectins, VWF, TSP and fibronectin (FN). For 1–2 different concentrations of each potential target protein, fluo-sevuparin was titrated over a concentration range of 5–350 nmol/l, and autocorrelation functions were acquired for each sample. To assess whether interactions changed over time, measurements were conducted both directly after mixing and after a 3-h incubation period (Figures S2 and S3, Data S1). Binding of sevuparin to a target protein slows down diffusion, leading to the appearance of a second component in the autocorrelation function. Fitting of the autocorrelation functions with a two-component model, in which one component corresponded to the free and one to the bound sevuparin, yielded the fraction of bound sevuparin. Based on the known concentrations of binding partners in the sample, this was translated into dissociation constants (Table SII, Data S1). E-selectin and FN demonstrated the lowest capacity to bind sevuparin,
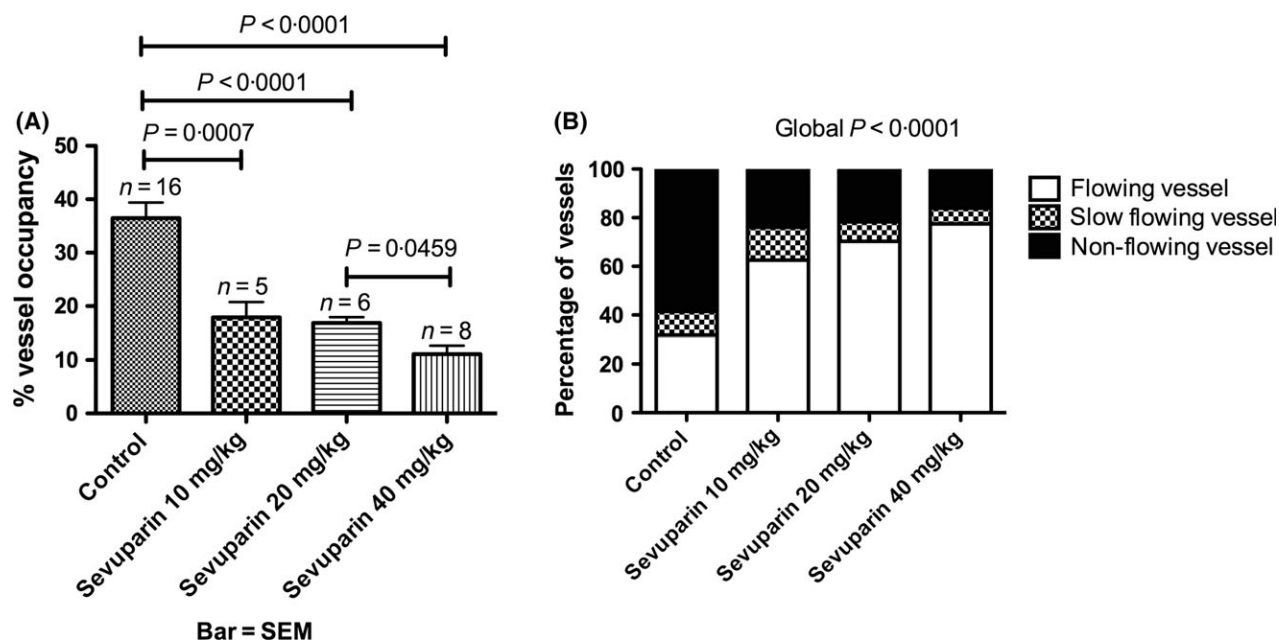
Fig 4. Effect of sevuparin *in vivo* on SS-RBC adhesion to vessel walls. Microscopic observations of postcapillary venules were conducted through implanted window chambers after infusion of SS-RBCs into the tail veins of nude mice using 20× magnification. (A) Dose response of sevuparin concentration on vessel wall SS-RBC occupancy reflecting the anti-adhesive properties of sevuparin. The percentage of vessel length with diameters up to 25 μm occupied by SS-RBCs was quantified as follows: % venular length occupied by SS-RBCs × (length of vessel wall with adherent cells/total length of the vessel segments analysed) × 100. Values are means of >30 vessels analysed for each mouse. Error bars show SEM of at least 3 different experiments for each experimental condition. (B) Dose-dependent restoration of vascular function (blood flow) by sevuparin.

although as expected, the fraction of bound ligand increased with sevuparin concentration in both cases. Also as expected, the dissociation constant, as determined from binding equilibria, was independent of the concentration of the labelled sevuparin and the interactor concentration, except for VWF (Figure S2). For VWF, lower dissociation constants were calculated for higher labelled sevuparin concentrations. The reason for this concentration dependence is not clear.

Dissociation constants were also determined by competitive titration of fluorescently labelled sevuparin with unlabelled sevuparin. Two different approaches were used to determine dissociation constants in the presence of unlabelled ligand. The first approach determined dissociation constants for each individual sample as before for the total sevuparin concentration (labelled and unlabelled in the sample, Figure S4, Data S1). The second approach employed the Cheng–Prusoff equation to derive dissociation constants from inhibitory constant (K_i) values for those ligands to which binding of fluorescently labelled ligand could be fully competed (Figures S5, S6 and S7, Data S1).

Overall, there was good accordance for dissociation constants as determined in the absence (first approach, direct titration) and presence of competitive inhibitor and derived from the fraction of bound fluorescent sevuparin (Table I). Also, the dissociation constants for the selectins were in the same range as those determined with the one-site binding model. FN had the highest dissociation constant, followed by E-selectin. L- and P-selectins and VWF displayed low and

very similar dissociation constants. Thrombospondin A had the lowest dissociation constant. Similar Kd values for heparinoids have been documented elsewhere (Koenig *et al*, 1998; Barabino *et al*, 1999; Wang *et al*, 2002; Wang & Geng, 2003; Brittain *et al*, 2004; Simonis *et al*, 2007; Gayen Betal & Setty, 2008).

Discussion

The results presented here support the hypothesis that sevuparin can improve vascular blood flow in SCD by binding to several key cellular adhesion receptors and plasma components that contribute to vaso-occlusion. Thus, our observations with sevuparin are both of potential clinical importance as well as interesting from the point of view of understanding SCD pathophysiology, as they support the role of anti-adhesion therapy in counteracting the causative processes of sickle cell adhesion and vaso-occlusion. Abnormal adhesion between blood cells and the endothelium in SCD has been linked to disease severity (Hebbel *et al*, 1980b; Setty & Stuart, 1996; Dworkis *et al*, 2011). Although the pathophysiology of acute pain episodes is complex and not fully understood, a cascade of cell adhesion events leading to vaso-occlusion and ischemia has been convincingly demonstrated (Manwani & Frenette, 2013). Therefore, inhibition of abnormal adhesion should restore normal blood flow and thereby result in faster pain relief and reduced tissue injury. Preclinical and early phase clinical studies of various compounds that inhibit

(A) Adhesion events (mm^2)

Shear stress = 0·5 dyn/cm²
(*n* = 5, bars = SEM)

(B) % vessel occupancy (mean ± SEM)

(C) Global *P* < 0·0001

Percentage of vessels

□ Flowing vessel
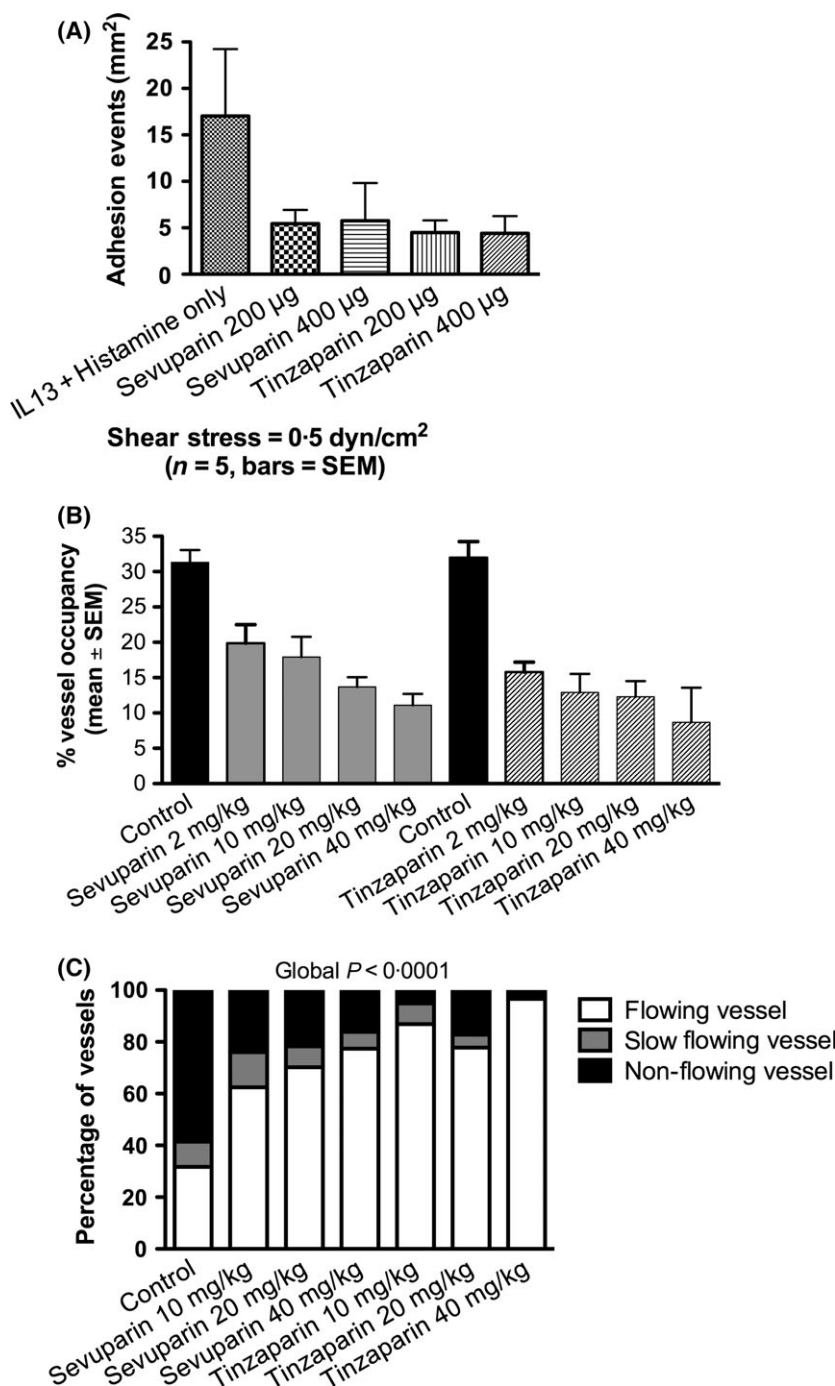▨ Slow flowing vessel
■ Non-flowing vessel

Fig 5. Comparison of the anti-adhesive effects of sevuparin and tinzaparin. (A) Effect of sevuparin compared with tinzaparin on SS-RBC adhesion to HUVECs *in vitro*. Adhesive events per mm^2 were measured as previously described. The relatively large differences in the numbers of adhesive events between controls (IL13 and histamine only) and samples in which sevuparin or tinzaparin were used were not uniformly statistically significant, and the most significant difference was observed for control *versus* 400 µg/ml sevuparin. Lack of statistically significant differences between control and heparinoids was probably due to variability in adhesivity among patient samples. (B) Effects of equivalent mg/kg sevuparin and tinzaparin doses on vessel wall occupancy by adherent SS-RBCs *in vivo*. Vessel occupancy is defined as the % of the vessel occupied by adherent, labelled human SS-RBCs. Global statistical analysis of all data by ANOVA demonstrates that both sevuparin and tinzaparin can prevent vaso-occlusion ($P < 0·0001$). There was no statistically significant difference in the effects of the two drugs. (For Panels B and C, N = 4–11 for each dose of sevuparin and tinzaparin except for tinzaparin 40 mg/kg, for which N = 2.) (C) The effect of sevuparin in mice *in vivo*, measured as vessel function. All of the tested sevuparin and tinzaparin doses were effective in improving vessel function (number of flowing vessels) ($P < 0·0001$). There were no statistically significant differences when results using equivalent doses of sevuparin and tinzaparin were compared.

diverse adhesive interactions further support this hypothesis (Embury *et al*, 2004; Chang *et al*, 2008, 2010; Gutsaeva *et al*, 2011; Manwani *et al*, 2015; Telen *et al*, 2015).

Anticoagulation treatment for SCD has been investigated in several studies (Salvaggio *et al*, 1963; Chaplin *et al*, 1989; Ahmed *et al*, 2004; Qari *et al*, 2007). However, a substantial proportion of patients with SCD are at clinical risk of haemorrhage, often due to intracerebral angiopathy, and especially so-called Moyamoya abnormalities of the central nervous system vasculature. This risk of haemorrhage has contributed

to some reluctance to adopt therapeutic anticoagulation either in a prophylactic setting or during vaso-occlusive episodes (Qari *et al*, 2007). Ruling out the existence of vascular abnormalities that predispose to intracerebral haemorrhage prior to starting treatment would both be cost-prohibitive and delay treatment. Thus, the pharmacological use of anticoagulation treatments remains of undefined risk and benefit in the SCD population.

Heparin also interferes with P-selectin-mediated SS-RBC adhesion to endothelial cells (Matsui *et al*, 2002). The data

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Table I. Comparison of dissociation constants as determined by FCS using three different analytic approaches.

Adhesion protein*	Kd Direct (μmol/l)		Kd Titration (μmol/l)		IC$_{50}$ (μmol/l)	Ki Cheng Prusoff (μmol/l)
	Average	SD	Average	SD		
P-selectin	–†	–	0·485	0·38	0·942	0·622
E-selectin	4·51	1·52	4·48	–	–§	–§
L-selectin 5 μmol/l	0·807	0·309	0·451	0·43	–§	–§
Fibronectin	6·51	2·44	2·25	0·344	3·15	2·97
von Willebrand factor	0·720‡	0·893‡	0·479	0·126	6·45	4·55
Thrombospondin	0·487‡	0·238‡	0·419	0·180	4·97	2·97

Kd direct refers to averages of dissociation constants derived from binding equilibria with labelled ligand, where bound and free ligand were obtained from two-component fits of the autocorrelation function. Kd titration refers to dissociation constants derived for binding equilibria in the presence of unlabelled ligand. IC$_{50}$ refers to the concentration of an inhibitor where the response (or binding) is reduced by half. Ki Cheng Prusoff refers to dissociation constants that were determined for those binders where binding of labelled ligand could be fully competed with unlabelled ligand. For determination of dissociation constants via Cheng Prusoff, an average of the Kd titration and the Kd direct was employed.
*Adhesion proteins were employed at the following concentrations: P-selectin 3 μmol/l, E-selectin 5 μmol/l, L-selectin 5 μmol/l, fibronectin 10 μmol/l, VWF 2 μmol/l, Thrombospondin 1·6 μmol/l.
†Not determined due to limited amount of material available.
‡1 μmol/l concentration was used for the direct Kd measurements.
§Not possible to calculate, due to failure to compete out the labelled ligand.

for sevuparin presented here strongly suggest that blocking abnormal cell adhesion represents a mechanism of action deserving of more attention. In in vitro flow chamber experiments, sevuparin was an effective inhibitor of SS-RBC adhesion to endothelial cells. Interestingly, the degree of RBC adhesion to endothelial cells varied among patients, confirming both our previous observations and those of others (Hebbel et al, 1980b; Zennadi et al, 2004). Variability among patients may be linked to both the expression of ligands on the RBC for various endothelial cell adhesion receptors, including P-selectin and integrin αvβ3, and their activation state. Because the RBC ligand for P-selectin is currently unidentified, it was not possible to test this directly. In addition, the activation state of erythroid adhesion receptors probably varies among patients, as previously described (Zen et al, 1999, 2004). Hydroxycarbamide can also affect both adhesion receptor expression as well as adhesion receptor activation, often in opposite directions (Gambero et al, 2007; Odievre et al, 2008; Bartolucci et al, 2010; Chaar et al, 2014). Nonetheless, vaso-occlusion, when it occurs, still occurs through the same mechanisms, with and without hydroxycarbamide. This has recently been seen in the similar effect of another anti-adhesive drug/selectin inhibitor, GMI-1070 (rivipansel), in the presence or absence of hydroxycarbamide (Telen et al, 2015). In vivo, variability of both RBC and leucocyte adhesion may also arise from genetic variability affecting leucocytes and endothelial cells, including indirect effects via inflammatory pathways. This, however, is less likely to be a factor in syngeneic mice.

Our in vivo studies focused on human SS-RBCs to ensure that the drug effects observed were relevant to human disease, because murine SS-RBCs, although reported to also bind P-selectin, (Matsui et al, 2001, 2002) lack several known RBC adhesion receptors, including BCAM/Lu and CD44, (Sugimoto et al, 1994; Moulson et al, 2001) which are involved in binding to laminin, FN and leucocytes (Verfaillie et al, 1994; Udani et al, 1998; Zennadi et al, 2008). Nonetheless, at least two major murine endothelial receptors for SS-RBC adhesion are similar enough to the human that reagents that block human SS-RBC adhesion to murine endothelium also block adhesion to human endothelial receptors. Thus, heparins block SS-RBC binding to both human and murine P-selectins (Matsui et al, 2001, 2002) despite the lack in the murine P-selectin of one complement-binding domain (Weller et al, 1992). Likewise, reagents that block adhesion via the αVβ3 integrin of endothelial cells do so for both murine and human cells (Kaul & Hebbel, 2000; Zennadi et al, 2004, 2007).

Although the chronic endotheliopathy and baseline leucocyte activation of SCD is absent, this model system employs TNF-induced activation of both endothelial cells and leucocytes, has repeatedly been proven useful for studying SS-RBC interactions with the endothelium and leucocytes in vivo, and has produced results that have subsequently been replicated in sickle mice and human studies (Zennadi et al, 2007, 2008, 2012; De Castro et al, 2012; Zennadi, 2014). As in similar investigations, two assessments were performed: percentage occupancy as a measure of abnormal adhesion of cells to endothelium, as well as blood flow, which best measures the degree of vaso-occlusion. Unless a vessel is quite narrow, it takes more than one layer of adherent red cells to block blood flow.

Sevuparin clearly demonstrated a dose-dependent capacity to prevent vaso-occlusion, with 20 mg/kg given subcutaneously demonstrating maximal or near-maximal effect. This effect was clearly related to inhibition of RBC adhesion to the vessel walls, although inhibition of leucocyte adhesion may also have contributed (Figure S1). Furthermore,

sevuparin has effects similar to tinzaparin at comparable mg/kg doses. Given that sevuparin has a confirmed low anticoagulant effect (Lindgren *et al*, 2015) and because the dose-response curves for sevuparin and tinzaparin are very similar in our animal model of vaso-occlusion, we conclude that it is primarily the anti-adhesive effect exerted by sevuparin and tinzaparin rather than an anticoagulant effect that is most important for prevention of vaso-occlusion by heparinoids. Notably, sevuparin has no appreciable effect on bleeding time, platelet activation or platelet adhesion.

Binding experiments with isolated potential adhesion receptors suggest that multiple interactions contribute to the anti-adhesive effect of sevuparin. Employing a combination of two orthogonal methods to investigate binding interactions, SPR and FCS, sevuparin demonstrated significant binding to P-selectin, L-selectin, VWF, TSP and FN, but only weak binding to E-selectin (Table S2). Compared with P-selectin and L-selectin, there was only very weak binding to E-selectin (Fig 2 and Figure S2). Sevuparin's low or limited affinity to E-selectin is in line with published data on heparin (Koenig *et al*, 1998). Furthermore, SPR data support the notion that sevuparin engages in multivalent binding, i.e., one sevuparin chain interacting with several P-selectin molecules at the same time. In addition, the difference observed in heparinoid-binding affinity to selectins when using μmol/l or nmol/l levels of the ligand may reflect these multivalent interactions; thus, when incubating at only nmol/l concentrations, only the high-affinity site/interactions are detected, while at μmol/l concentrations, the Kd values reflect a combination of two or more interaction sites.

It is well-known that heparin has many pharmacological properties other than its effect on anticoagulation (Mulloy *et al*, 2016). However, less is known about the mechanisms of these other activities. Here, we have used two different methods to investigate the binding of sevuparin to previously identified heparin-binding proteins known to contribute to the abnormal adhesion seen in SCD. FN, VWF and TSP are recognized as complex to study from a biochemical binding perspective, as they can interact in multiple ways (multivalent) and also form oligomers (Barabino *et al*, 1999; Tan & Ginsburg, 2008). There are many similarities between heparin's polypharmacological anti-inflammatory mechanism and sevuparin's mechanism of action as elucidated here. It is reasonable to speculate that sevuparin acts at several steps in the cascade leading to vaso-occlusion, much like the complex anti-inflammatory effects observed for heparin. For example, by binding P- and L-selectin, sevuparin probably blocks the initial rolling of blood cells on the endothelium. Then, firm adhesion may be abrogated by sevuparin binding to the bridging proteins VWF, FN and TSP, thus hindering the interactions between integrins on the activated endothelium and the blood cells.
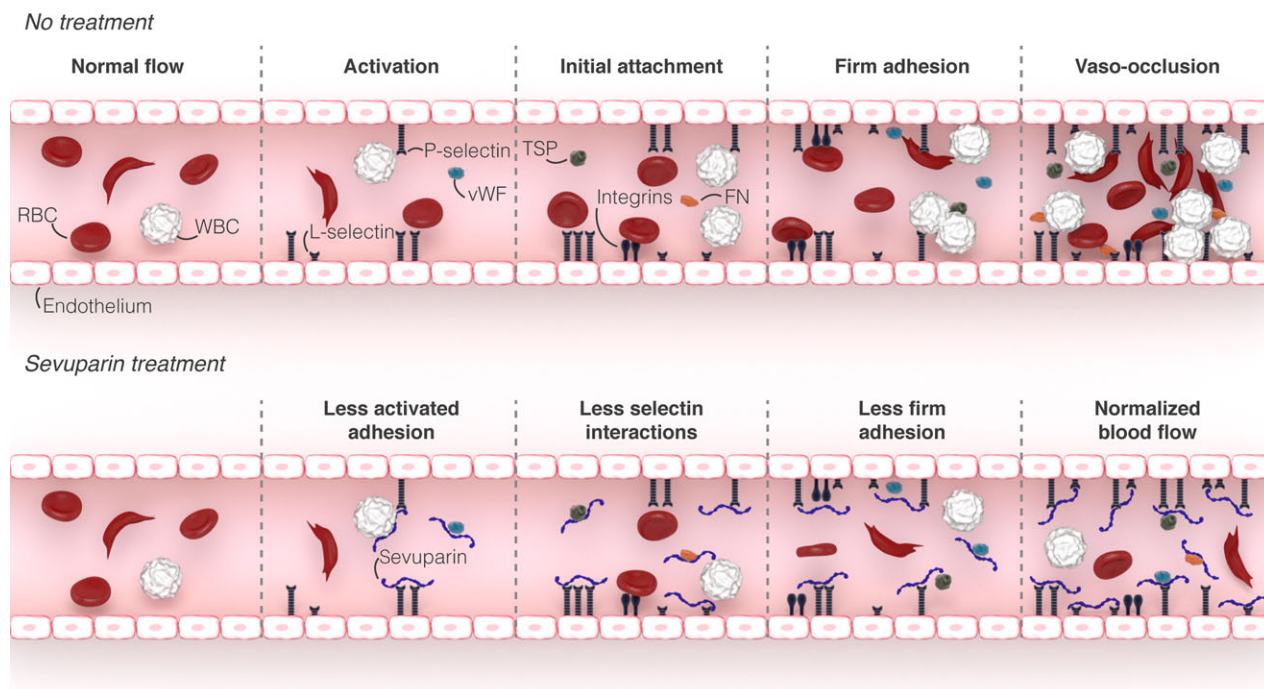

Fig 6. Potential multimodal mechanism of sevuparin action. This figure demonstrates the potential interactions between SS-RBCs and the blood vessel wall endothelium. See figure for explanation of respective symbols. FN, fibronectin; RBC, red blood cell; TSP, thrombospondin; VWF, von Willebrand factor; WBC, white blood cell.

To further understand which of the interactions would be likely to be relevant in the clinical setting, a pharmacokinetic bridge study was performed in mice (Table SIII, Data S1). From this, the effective dose (which is 20 mg/kg in the *in vivo* model) was translated into an effective target plasma concentration of 20 µg/ml (corresponding to about 2·5 µmol/l). At this concentration, sevuparin would bind to the adhesion proteins P-selectin, L-selectin, VWF, TSP and (potentially) FN, while the interaction with E-selectin would be limited (illustrated in Fig 6). The capability of sevuparin to interact with several potential targets of SS-RBC adhesion could be of critical importance in VOC, as blockade of multiple interactions is likely to be beneficial in interrupting abnormal adhesion.

The capacity of sevuparin to prevent or reverse VOC and the resulting pain in clinical use remains to be confirmed. Clinical phase II studies have now been initiated (https://clinicaltrials.gov/ct2/show/NCT02515838; https://www.clinicaltrialsregister.eu/ctr-search/trial/2014-004416-11/NL). Based on the proposed mechanism of action of sevuparin and the activity we have observed, we expect sevuparin to be an effective new treatment for SCD patients who are experiencing vaso-occlusive events.

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Author contributions

M.J.T. designed and supervised the research study, analysed and interpreted all the data, and wrote the manuscript; M.B. performed all *in vitro* adhesion experiments and analysed *in vitro* data; S.S. performed all *in vivo* experiments and analysed *in vivo* data; P.H. B-G. performed and analysed the FCS experiments; R.Z. helped design and analyse *in vivo* experiments; A.L. helped design and analyse *in vitro* experiments; R.B. planned the FCS experiments, analysed the data, and helped write and edit the manuscript; M.L. helped analyse the data and write and edit the manuscript.

Conflict of interest

M.J.T. and R.B. received support for travel to study-related meetings, and their institutions, including other authors at their institutions, received research support through contract research agreements. A.L. and M.L. are employees of Dilaforette SA. M.J. T. reports other research funding (NIH, Doris Duke Charitable Foundation, Pfizer, Inc.).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1: Supplemental Material.

Table SI. The specific anticoagulant activity of sevuparin measured *in vitro* with standard European Pharmacopoeia methods.

Table SII. Dissociation constants (K_D) by direct binding.

Table SIII. Pharmacokinetic parameters of sevuparin.

Fig S1. Effect of sevuparin on adhesion of sickle and normal polymorphonuclear leukocytes (PMNs).

Fig S2. Example graphs of direct binding of fluo-sevuparin as summarised in Table S1.

Fig S3. Examples of control parameters and time dependence of interactions.

Fig S4. Example for the time dependence of binding equilibria.

Fig S5. Competitive titrations of fluo-sevuparin complexes with unlabeled sevuparin.

Fig S6. Competitions for selectins.

Fig S7. Concentration dependence of dissociation constants as determined from competition experiments.

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