

## Sevuparin blocks sickle blood cell adhesion and sickle-leucocyte rolling on immobilized L-selectin in a dose dependent manner

Adhesion of sickle red blood cells (SSRBC) to the vascular endothelium may initiate and propagate vascular obstruction in sickle cell disease (SCD) (Hoover *et al*, 1979; Hebbel *et al*, 1980). Hebbel *et al* (1980) were the first to report a correlation between erythrocyte adherence and disease severity. Subsequent studies demonstrated that the pathological adhesion of SSRBCs involves red cell receptors, adhesive bridging proteins and endothelial receptors (Joneckis *et al*, 1993; Swerlick *et al*, 1993; Udani *et al*, 1998; Hillery *et al*, 2000). These complex and multimodal mechanisms of SSRBC adhesion may require a multi-targeted approach to achieve the best clinical outcome.

Sevuparin is a new drug candidate that is chemically derived from heparin and in which most of the anticoagulant activity has been removed while retaining the anti-adhesive effects. Sevuparin is currently in Phase 2 clinical studies for acute treatment of vaso-occlusive crisis (VOC) in SCD (NCT02515838). Heparins exhibit blockade of P-selectin-mediated adhesion. Selectins contribute to both SSRBC and neutrophil adhesion (PMN) *in vitro* and in SCD mouse models. Sickle mouse models lacking both P- and E-selectins are relatively resistant to tumour necrosis factor-alpha (TNF $\alpha$ )-induced vaso-occlusion. *In vitro* and *in vivo* studies

with sevuparin have shown potent anti-adhesive effects with a multimodal mechanism of action (Telen *et al*, 2016). In this study, a standardized flow adhesion assay was used to evaluate the effects of sevuparin on the adhesion of sickle whole blood to cultured endothelial cells and vascular cell adhesion molecule-1 (VCAM-1), and sickle-leucocyte rolling on L-selectin.

Whole blood samples were drawn in sodium citrate from homozygous SCD patients at steady state ( $n = 12$ , age range 15–25 years). The study protocol was approved by the Institutional Review Board at Wayne State University. A comprehensive assessment of the effect of sevuparin on whole blood adhesive properties during simulated blood flow was assessed using standardized adhesion bioassays (Functional Fluidics, Detroit, MI, USA). Whole blood flow adhesion was measured during physiological flow in a Bioflux 1000 flow system (Fluxion Biosciences, San Francisco, CA, USA) coated with either VCAM-1 (R&D Systems, Minneapolis, MN, USA) or cultured human umbilical vein endothelial cells (HUVECs, Lonza, Walkersville, MD, USA). HUVECs have been used in numerous sickle cell adhesion studies. HUVECs used in this study were only an *in vitro* model and not a surrogate for *in vivo* adhesion. HUVECs were activated by TNF $\alpha$  (25 ng/

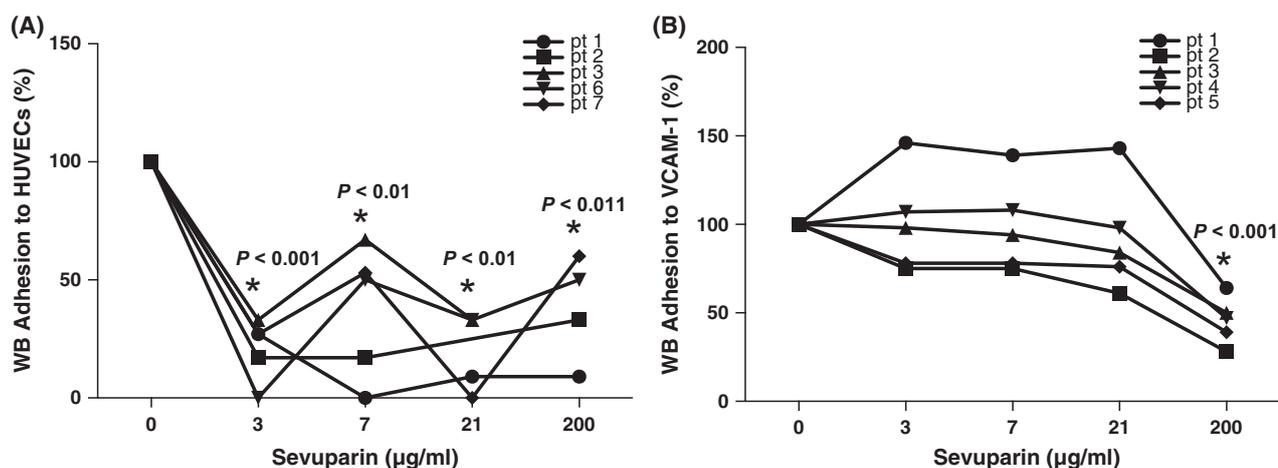


Fig 1. Sevuparin dose response of sickle whole blood adhesion. Whole blood adhesion to VCAM-1 and HUVECs was measured during physiological flow. HUVECs were activated by tumour necrosis factor-alpha and histamine prior to the assay. Whole blood was treated with increasing doses of sevuparin (0, 3, 7, 21, 200 µg/ml). Dose response of whole blood adhesion was measured. Statistically significant comparisons ( $P < 0.05$ ) are indicated by “\*” and P-values. HUVECs, human umbilical vein endothelial cells; pt, patient; VCAM-1, vascular cell adhesion molecule-1; WB, whole blood.

ml  $\times$  24 h) and histamine (100 mmol/l  $\times$  10 min) prior to the assay. TNF $\alpha$  treatment increases the surface expression of multiple adhesion molecules, including VCAM-1. An anti-VLA-4 (very late antigen-4) antibody resulted in potent, dose-dependent inhibition of over 50% of whole blood cell binding to activated HUVEC monolayers under physiological flow (White *et al*, 2016). Histamine, a rapid-acting inflammatory mediator, causes rapid mobilization of P-selectin from Weibel-Palade bodies to the surface of endothelial cells. This mobilization begins in seconds, with maximum expression occurring within ~6–10 min. P-selectin and other adhesion receptors have been shown to be involved in VOC in SCD. Whole blood was treated with increasing doses of sevuparin (0, 3, 7, 21, 200  $\mu$ g/ml) for 30 min (provided by Modus Therapeutics, Stockholm, Sweden) and the dose response of whole blood adhesion to sevuparin was measured. Previously, sevuparin markedly abrogated SSRBC adhesion to blood vessel walls in animals pretreated with TNF $\alpha$ . The effective target plasma concentration in this study

was 20  $\mu$ g/ml (corresponding to about 2.5  $\mu$ mol/l) via pharmacokinetic determination in mice. Clinical phase II studies are ongoing to evaluate the capacity of sevuparin to prevent or reverse VOC and the resultant pain in humans. Rolling adhesion of isolated sickle-leucocytes on L-selectin was measured during physiological flow. Isolated sickle-leucocytes were treated with sevuparin (0, 3, 7, 21, 200  $\mu$ g/ml) for 30 min. Dose response of rolling cell density (cells/mm<sup>2</sup>), rolling cell percentage (%), and average rolling velocity ( $\mu$ m/s) to sevuparin was assessed. Cell identification and tracking of rolling were digitally analysed (Metamorph image analysis software, Nashville, TN, USA).

Patient-to-patient variability in sevuparin response was observed (mean  $\pm$  standard deviation). Statistically significant inhibition of sickle whole blood adhesion to HUVECs was observed at 3.0  $\mu$ g/ml sevuparin (20.8  $\pm$  12.97% inhibition,  $P < 0.001$ ; Fig 1A). In the same manner, statistically significant inhibition of sickle whole blood adhesion to VCAM-1 was observed at 200  $\mu$ g/ml sevuparin

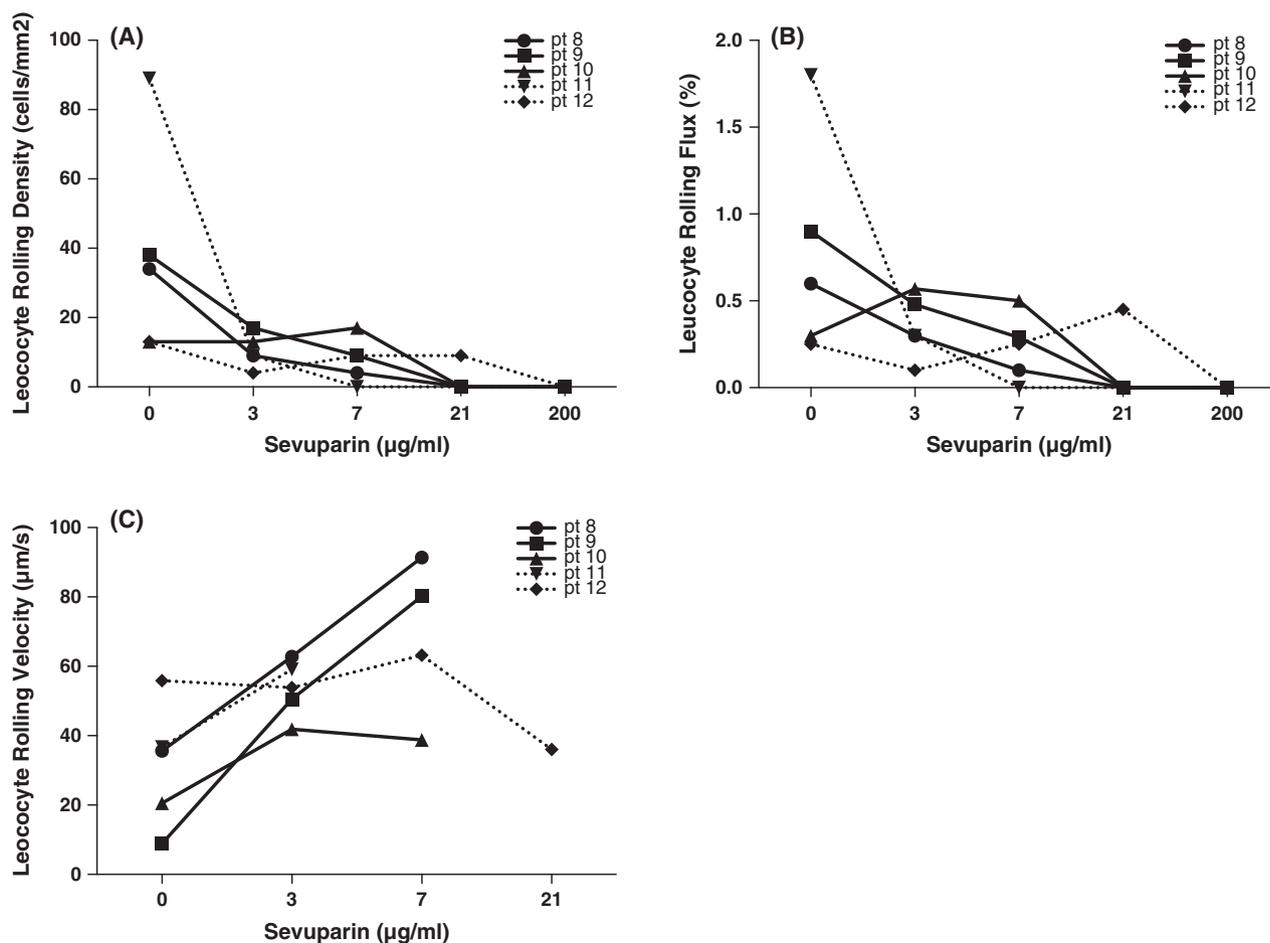


Fig 2. Sevuparin dose response of sickle leucocyte dynamic adhesion to L-selectin. Isolated sickle-leucocytes were treated with increasing doses of sevuparin (0, 3, 7, 21, 200  $\mu$ g/ml) for 30 min. Rolling adhesion of isolated sickle-leucocytes on an L-selectin coated microfluidic channel was measured during physiological flow. Dose response of rolling cell density (Fig 2A; cells/mm<sup>2</sup>), rolling cell percentage (Fig 2B; %), and average rolling velocity (Fig 2C;  $\mu$ m/s) to sevuparin was assessed. Cell identification and tracking of rolling were digitally analysed. pt, patient.

(45.6 ± 13.35% inhibition,  $P = 0.001$ ; Fig 1B). Each patient sample demonstrated a reduction in adhesion. Sevuparin also demonstrated a statistically significant reduction of sickle leucocyte rolling cell density (from 37.4 to 10.4 cells/mm<sup>2</sup> at 3 µg/ml of sevuparin,  $P = 0.048$ ; Fig 2A), rolling cell percentage (from 0.77 to 0.09% at 21 µg/ml of sevuparin,  $P = 0.024$ ; Fig 2B), and a statistically significant increase in average rolling velocity (from 31.52 to 68.4 µm/s at 7 µg/ml of sevuparin,  $P = 0.032$ ; Fig 2C) on L-selectin.

Here we show that sevuparin blocks both sickle whole blood and isolated sickle-leucocyte adhesive interactions under physiological flow at clinically relevant concentrations. The blocking of adhesion to VCAM-1 indicates that sevuparin acts in the same manner as other heparinoids *in vitro*, and block the interaction with VLA-4 (Lancelot *et al*, 2017). L-selectin is another potential target for sevuparin therapy. L-selectin expression by monocytes is increased during VOC, compared to steady state, and both mononuclear cell and neutrophil L-selectin expression is also higher in patients with certain complications of SCD (Okpala *et al*, 2002). Here we show that the multi-model mechanism of action of sevuparin includes inhibition of SSRBC and sickle leucocyte adhesion to VCAM-1 and L-selectin-mediated dynamic adhesion of sickle-leucocytes. Sevuparin's multimodal mechanism of action may result in a broader range of clinical response in patients with vaso-occlusive complications mediated by SSRBC adhesion.

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## Authorship contributions and Conflicts of interest

Jennell White is a shareholder of Functional Fluidics: Paediatric subject recruitment, experimental design and execution, data analysis, and manuscript review. Maria Lindgren was an employee of Modus at time of study. Ke Liu is a shareholder of Functional Fluidics: data analysis and manuscript preparation. Xiufeng Gao is a shareholder of Functional Fluidics: Paediatric subject recruitment, experimental design and execution, data analysis, and manuscript preparation. Lena Jendeborg is an employee of Modus. Patrick Hines is a shareholder of Functional Fluidics: Project conception and funding, paediatric clinical protocol preparation, manuscript review.

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