Evaluating the safety profile of defibrotide in sickle cell disease: an *in vitro* study

Pre-existing vasculopathy, liver impairment and increased adhesion of sickle red blood cells (RBC) and platelets to endothelial cells (EC) may increase the risk of sinusoidal obstructive syndrome in patients with sickle cell disease (SCD) exposed to conditioning regimens. Defibrotide, which is the only specific treatment for sinusoidal obstructive syndrome, activates adenosine receptors, which could be harmful in SCD by reducing hemoglobin affinity for oxygen, thereby promoting RBC sickling under hypoxic conditions. We evaluated the safety of defibrotide in SCD patients by examining its effects on RBC sickling, hemoglobin affinity for oxygen and RBC adhesion to EC under flow. Incubation of whole blood from SCD patients with defibrotide did not affect RBC maximal deformability and point of sickling or alter the levels of p50 (the oxygen tension at which hemoglobin is 50% saturated). Defibrotide also reduced RBC adhesion to EC exposed to hemolysate or tumor necrosis factor-alpha (TNF-α), with decreased release of interleukin-6 (IL-6), interleukin-8 (IL-8) and monocyte chemotactic protein 1 (MCP-1) following hemolysate conditioning. These findings indicate that defibrotide use is safe for treating sinusoidal obstructive syndrome in SCD patients and emphasize its potential in improving vasculopathy in this population.

SCD is the most prevalent genetic disorder, affecting over 500,000 newborns annually. SCD results from a single-nucleotide mutation in the β-globin gene, causing the production of hemoglobin S, which polymerizes and forms fibers under hypoxic conditions. The introduction of gene therapy and hematopoietic stem cell transplantation in this population may increase patients' exposure to conditioning regimens, which can lead to the development of sinusoidal obstructive syndrome in up to 32% of SCD patients.[1] This severe complication of hematopoietic stem cell transplantation arises from sinusoidal EC injury induced by chemotherapy or radiation, leading to the obstruction of sinusoids.[2]

Defibrotide, a complex mixture of oligonucleotides, is the only specific treatment available for sinusoidal obstructive syndrome.[3] Defibrotide can act as an agonist of adenosine receptors, to exert, at least in part, antithrombotic effects.[4] Additionally, defibrotide reduces EC activation and oxidative stress,[5] although the receptor involved in this process remains unknown. Defibrotide was also shown to reduce neutrophil extracellular trap formation and thrombosis through adenosine A2A receptors.[6] Interestingly, a detrimental effect of adenosine signaling has been identified in SCD[7] and attributed to the activation of A2B receptors in RBC. Signaling through A2B receptors increases the concentration of 2,3-diphosphoglycerate, which reduces hemoglobin affinity for oxygen, thereby promoting RBC sickling under hypoxic conditions. This mechanism has been demonstrated in a mouse model of SCD, and in human sickle cells studied *in vitro*.[7] A previous study involving 11 SCD patients undergoing myeloablative conditioning before haploidentical hematopoietic stem cell transplantation found that prophylactic use of defibrotide was well tolerated,[8] although it has not been proven effective in patients at high risk of sinusoidal obstructive syndrome;[9] nonetheless, some teams still use defibrotide as prophylaxis due to the lack of alternative preventive options. Preliminary results from the same research group, based on 20 patients treated with defibrotide for acute chest syndrome, also suggested that defibrotide use is relatively safe in SCD.[10,11] However, studies investigating defibrotide use in SCD patients[8,10,11] are only available as conference abstracts, highlighting the need for additional investigations to further assess its safety in this population.

The objective of this study was to evaluate the safety of defibrotide in the treatment of SCD patients, by assessing its effects on RBC sickling and hemoglobin affinity for oxygen. Additionally, we investigated the impact of defibrotide on RBC adhesion to EC using an *in vitro* flow-based model. To evaluate the effect of defibrotide on RBC sickling, whole blood from 20 SCD patients was incubated with various doses of defibrotide (0, 50, 150, 300 and 1,000 μg/mL), for 1 hour at 37°C. RBC deformability was then measured under an oxygen gradient by ektacytometry (LORRCA, RR Mechatronics). Hemoglobin affinity for oxygen was assessed on a Hemox analyzer (TCS Scientific), following incubation for 1 hour with 300 µg/mL defibrotide (N=7). Defibrotide doses were chosen based on concentrations measured in plasma of healthy donors (60-200 μg/mL[12-14]). To investigate the effect of defibrotide on EC-RBC interactions, we utilized a previously published[15] flow-based cell culture model of human umbilical vein endothelial cells (HUVEC) in μ-slides (IBIDI) coated with fibronectin. Cells were perfused for 4 hours with or without 300 μg/mL of defibrotide, in medium containing 20 ng/mL TNF-α or 10% hemolysate (prepared from sonicated RBC). Culture supernatant was then collected and incubated (10% final concentration) for 30 minutes with whole blood from SCD patients (5 at steady state and 5 hospitalized for a vaso-occlusive crisis). Whole blood was then perfused over HUVEC for 10 minutes at 1 dyn/cm^2 (OB-1 pump, Elveflow), and, following a wash with medium, adherent RBC were quantified by microscopy (Axio Observer, Zeiss). The levels of IL-6, IL-8, MCP-1, soluble E-selectin, P-selectin, intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and

interleukin-1 beta (IL-1β) were measured in the HUVEC supernatant (collected after 4 hours of activation by TNF-α or hemolysate) using Luminex (MagPix, Thermofisher), while von Willebrand factor (VWF) levels were quantified by enzyme-linked immunosorbent assay (Thermofisher). Written informed consent was obtained from all patients, in accordance with the Declaration of Helsinki, and the research was approved by the local Institutional Review Board (CPP). Maximal deformability, measured via ektacytometry on oxygenated RBC from 20 SCD patients, was not altered by incubation with defibrotide (50-1,000 μg/mL) (Figure 1A). The point of sickling, defined as the partial oxygen pressure (pO_2) required to reduce RBC deformability by 5%, showed no significant changes, although a trend was noted at the highest dose tested (5 times the plasma concentration measured *in vivo*, *P*=0.07 with the Friedman test) (Figure 1B). This suggests that defibrotide exposure is unlikely to increase RBC sickling in treated patients (*P*=0.2 at the therapeutic dose).

To further verify that defibrotide does not activate adenosine signaling in RBC, we measured hemoglobin affinity for oxygen in whole blood samples from seven patients; the blood was incubated with 300 μg/mL defibrotide for 1 hour (1.5 times the highest plasma concentration observed *in vivo*). Defibrotide treatment did not affect the p50 measured during either the deoxygenation or reoxygenation cycles (Figure 1C). A representative set of curves in Figure 1D illustrates that the dissociation (full lines) and association curves (dotted lines) obtained after defibrotide incubation (in red) were comparable to those from untreated samples.

To confirm the safety of defibrotide use in SCD, we evaluated its effect on EC-RBC interactions using a flow-based

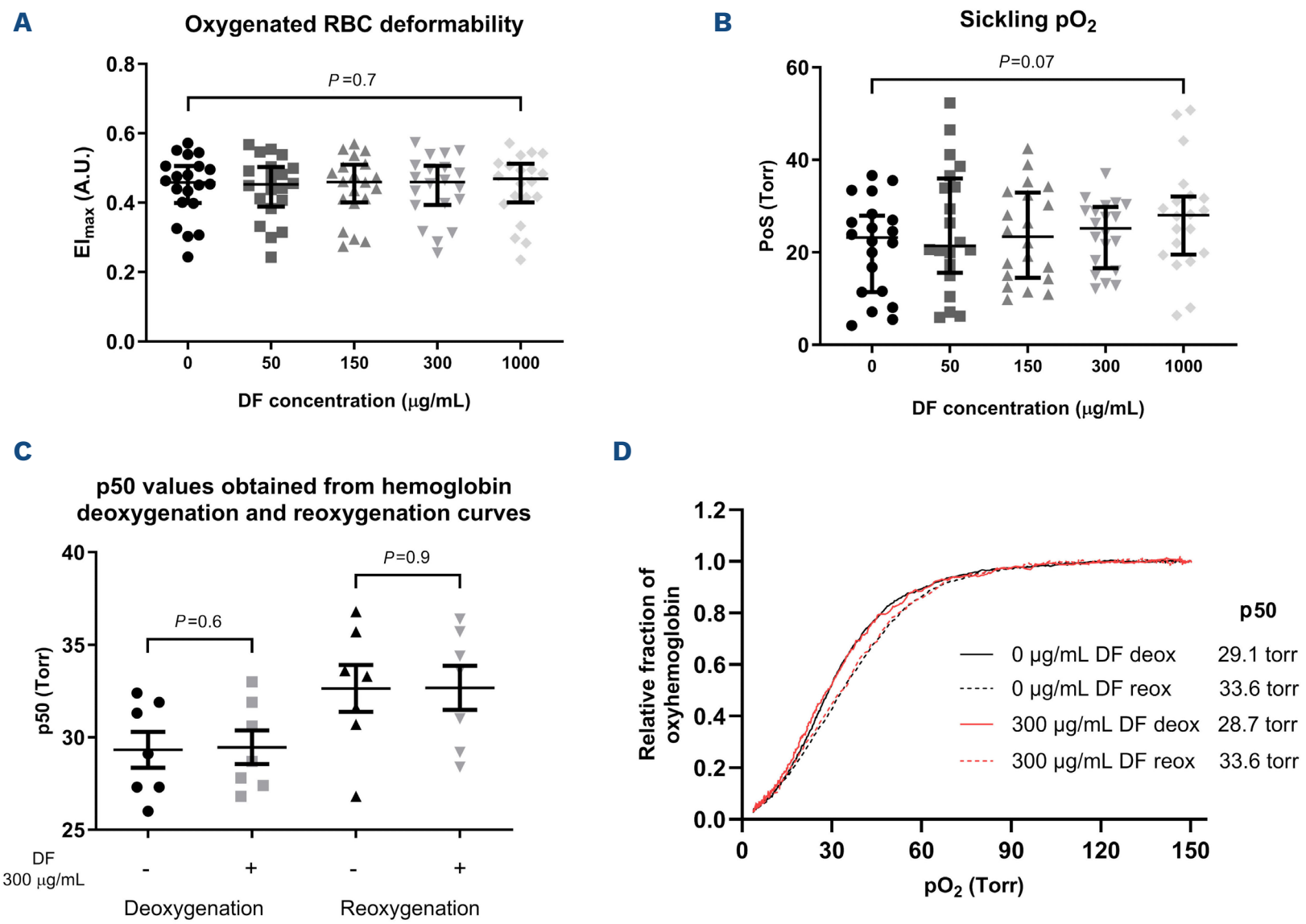
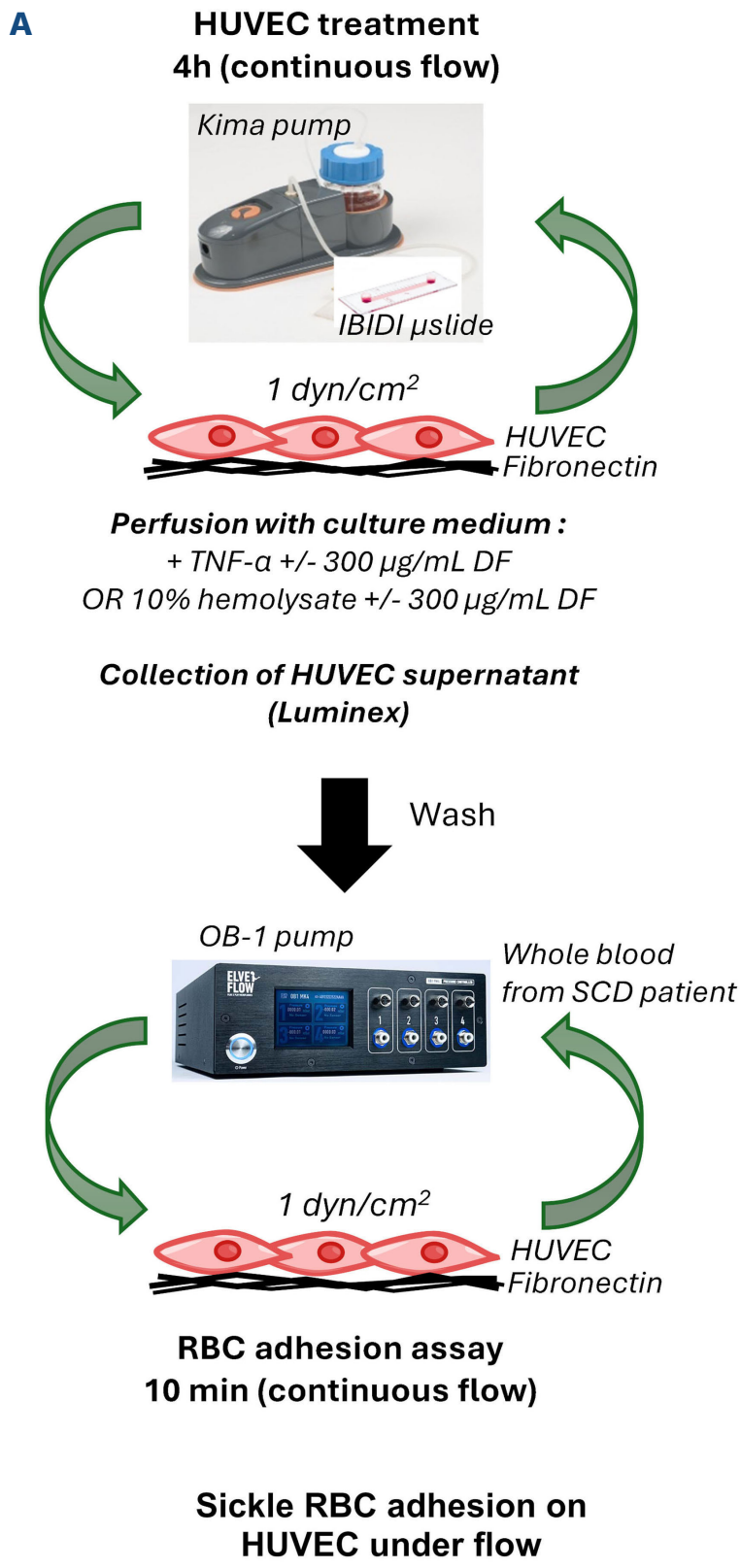
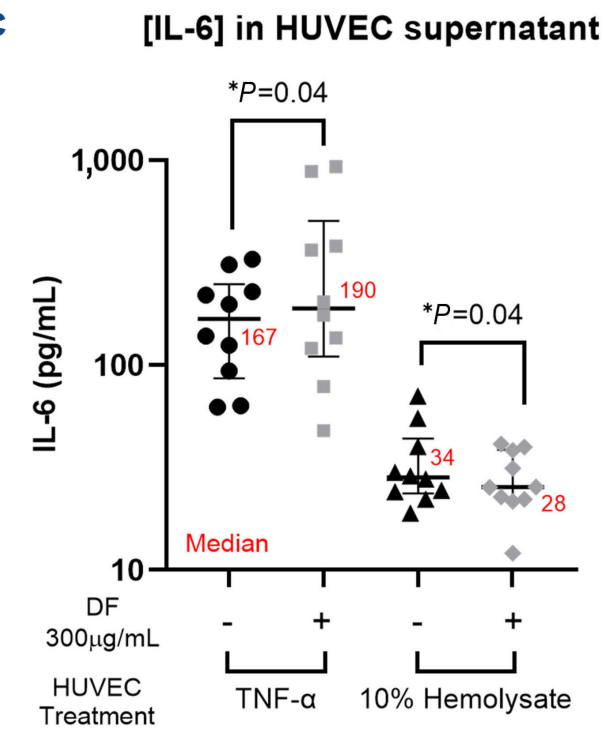
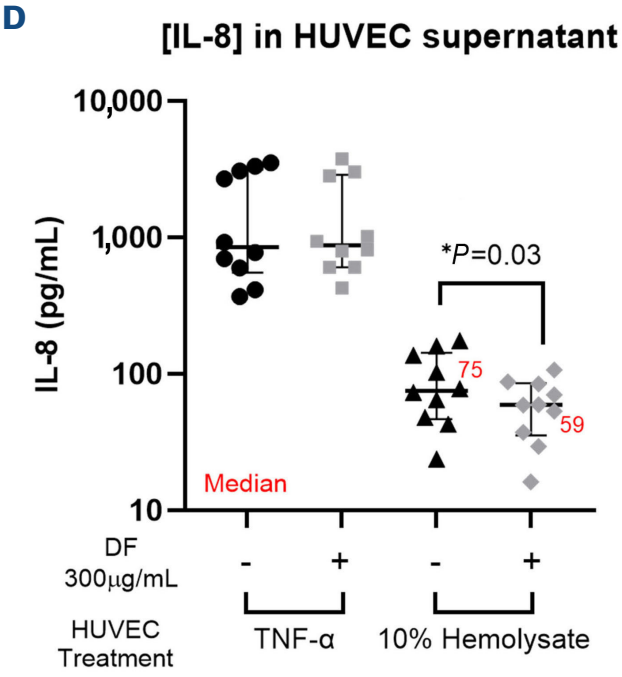
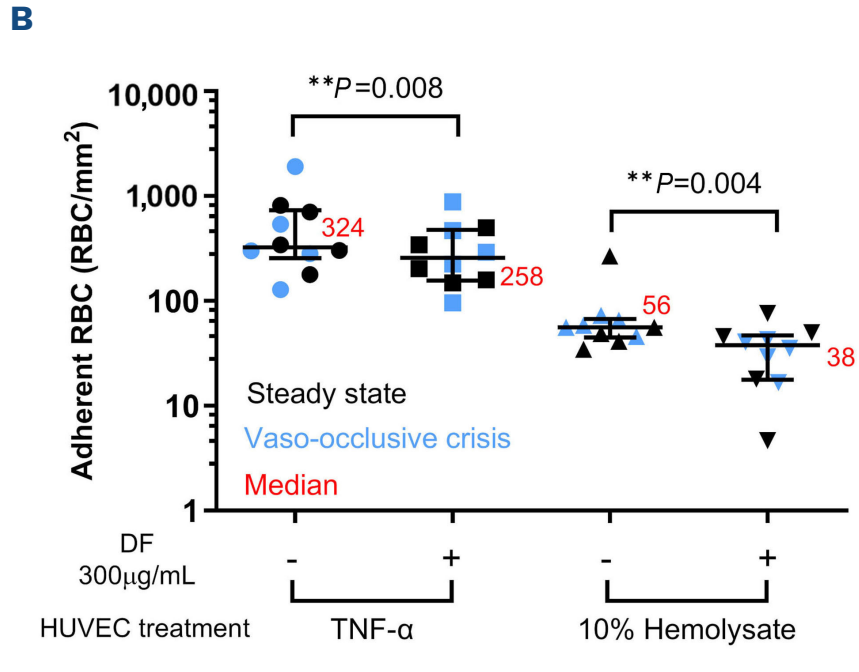


Figure 1. Defibrotide does not affect sickle red blood cell deformability or hemoglobin affinity for oxygen. (A, B) Oxygenscans (LORRCA, RR Mechatronics) were performed on blood samples from 20 patients with sickle cell disease (SCD), treated for 1 hour at 37°C with various doses of defibrotide (0 in black, 50, 150, 300 and 1,000 μg/mL, in gray), to obtain (A) the maximal elongation index (EImax), measured on oxygenated red blood cells, and (B) the point of sickling (corresponding to the partial oxygen pressure required to achieve 95% of EImax). (C) Oxygen-hemoglobin dissociation and association curves were obtained for blood samples, from seven SCD patients, treated for 1 hour at 37°C with 300 μg/mL of defibrotide (in gray) or without defibrotide (0 μg/mL, Hemox analyzer, TCS Scientific), to determine the p50. (D) Example of oxygen-hemoglobin dissociation (deox, full lines) and association curves (reox, dotted lines) from a single patient (samples treated with defibrotide are shown in red). Data are presented as the median with interquartile range and were analyzed using the Friedman test (*P* values on graphs) and the Wilcoxon test (comparisons to the 0 μg/mL condition) using GraphPad Prism 10. EImax: maximum elongation index; A.U.: arbitrary units; RBC: red blood cells; DF: defibrotide; pO_2: partial oxygen pressure; PoS: point of sickling; p50: oxygen tension at which hemoglobin is 50% saturated.









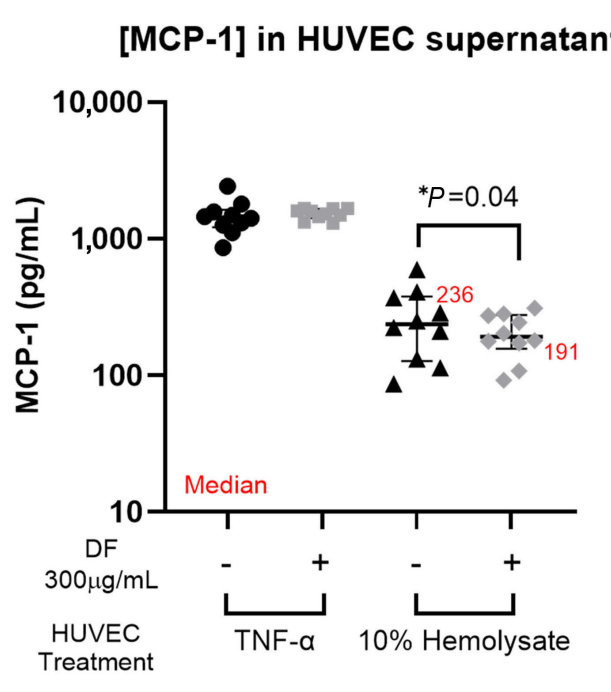


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Figure 2. Defibrotide decreases sickle red blood cell adhesion and cytokine release by endothelial cells. (A) Human umbilical vein endothelial cells (HUVEC) were cultured for 16-40 hours under flow conditions, using Kima pumps (Cellix), on μ-slides (IBIDI) coated with fibronectin. The HUVEC were then perfused with culture medium containing 20 ng/mL tumor necrosis factor-α, with or without 300 μg/mL defibrotide or 10% hemolysate with or without 300 μg/mL defibrotide for 4 hours. Whole blood from sickle cell disease patients (5 in steady state, in black, and 5 undergoing a vaso-occlusive crisis, in blue) was incubated for 30 minutes with 10% HUVEC supernatant obtained after the 4-hour treatment period. The blood was then perfused for 10 minutes over HUVEC (with an OB-1 pump, Elveflow). Slides were washed with medium and (B) red blood cell adhesion was quantified by microscopy (Axio Observer, Zeiss). (C-E) Concentrations of interleukin-6 (C), interleukin-8 (D) and monocyte chemotactic protein 1 (E) in HUVEC supernatant were measured by Luminex (MagPix, Thermofisher) after the 4-hour treatment with tumor necrosis factor-α or hemolysate (samples with defibrotide treatment in gray). Data are presented as the median (numerical values in red) with interquartile range. **P*<0.05; ***P*<0.005 (Wilcoxon test performed on GraphPad Prism 10). TNF-α: tumor necrosis factor-α; DF: defibrotide; SCD: sickle cell disease; RBC: red blood cells; IL-6: interleukin-6; IL-8: interleukin-8; MCP-1: monocyte chemotactic protein 1.

cell culture model. HUVEC were cultured under flow in μ-slides and activated for 4 hours with either TNF-α or 10% hemolysate, with or without 300 μg/mL defibrotide. After 4 hours of perfusion, supernatant was collected and incubated for 30 minutes with whole blood from SCD patients, collected when the patients were either at steady state or undergoing a vaso-occlusive crisis, to assess the combined effects of defibrotide and EC-released inflammatory factors on RBC adhesion. Whole blood was then perfused over HUVEC, and, after a wash, adherent RBC were quantified using microscopy (experimental setup shown in Figure 2A). Incubation of HUVEC with 300 μg/mL defibrotide reduced RBC adhesion in response to both TNF-α and hemolysate, regardless of whether the blood was collected from SCD patients during a vaso-occlusive crisis or at steady state (Figure 2B). Defibrotide treatment also decreased IL-6, IL-8 and MCP-1 release in response to hemolysate (Figure 2C-E) while increasing IL-6 levels in response to TNF-α. However, defibrotide did not modify the release of soluble E-selectin, P-selectin, ICAM-1, VCAM-1, IL-1β or VWF (*Online Supplementary Figure S1*). Additionally, the surface expression of markers of EC activation (ICAM-1, VCAM-1 and E-selectin), measured by flow cytometry and immunofluorescence, was not modified by defibrotide treatment (*data not shown*).

This study demonstrated that defibrotide treatment, at the estimated therapeutic dose, does not alter RBC deformability and sickling properties *in vitro*. Additionally, the affinity of hemoglobin for oxygen remains unchanged in RBC treated with defibrotide. Moreover, defibrotide reduces RBC adhesion to EC in inflammatory or hemolytic conditions, associated with a decrease in IL-6, IL-8 and MCP-1 release in EC exposed to hemolysate, while IL-6 release is increased in cells treated with TNF-α. This discrepancy between the action of defibrotide on TNF-α and hemolysate exposure might be due to the greater impact of TNF-α on EC activation compared to that of hemolysate in our model. This also suggests that the effects of defibrotide observed on RBC adhesion and inflammatory cytokine release are independent in the context of TNF-α exposure. Additionally, no changes were observed in ICAM-1, VCAM-1 or E-selectin, nor was there an increase in the adhesion of RBC or leukocytes to EC treated with TNF-α and defibrotide. Further studies are needed to fully elucidate the mechanism of action of defibrotide on interactions between EC and RBC in the context of SCD.

Our study supports the potential use of defibrotide in SCD patients undergoing hematopoietic stem cell transplantation or gene therapy, as well as the exploration of its utility in addressing vasculopathy and its complications in SCD.

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Disclosures

LB and MP were employed by Innovhem during the study. PB and MC co-founded Innovhem. PB has received grants from Addmedica, the Fabre Foundation, Novartis and Bluebird; has received consulting fees from Addmedica, Novartis, Roche, GBT, Bluebird, Emmaus, Hemanext and Agios; has received payments for lectures, presentations or educational events from Novartis and Addmedica and has participated in a steering committee for Novartis. AH has been a medical consultant for Novartis, Addmedica, GBT and Vertex. KAN and CN have no conflicts of interest to disclose.

Contributions

LB, KAN and PB designed the research. LB, MP, CN and AH performed the research. LB, MP, KAN and MC analyzed the data. LB and PB wrote the manuscript. MC and PB supervised the research.

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Data-sharing statement

For original data, please contact pablo.bartolucci@aphp.fr

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