

Low Anticoagulant Heparin Disrupts *Plasmodium falciparum* Rosettes in Fresh Clinical Isolates

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Abstract. The binding of *Plasmodium falciparum* parasitized erythrocytes to uninfected erythrocytes (rosetting) is associated with severe malaria. The glycosaminoglycan heparan sulfate is an important receptor for rosetting. The related glycosaminoglycan heparin was previously used in treatment of severe malaria, although abandoned because of the occurrence of severe bleedings. Instead, low anticoagulant heparin (LAH) has been suggested for treatment. LAH has successfully been evaluated in safety studies and found to disrupt rosettes and cytoadherence *in vitro* and *in vivo* in animal models, but the effect of LAH on fresh parasite isolates has not been studied. Herein, we report that two different LAHs (DFX232 and *Sevuparin*) disrupt rosettes in the majority of fresh isolates from Cameroonian children with malaria. The rosette disruption effect was more pronounced in isolates from complicated cases than from mild cases. The data support LAH as adjunct therapy in severe malaria.

INTRODUCTION

Malaria is a major health problem in sub-Saharan Africa but also in parts of Asia and Latin America, and it is estimated that about 1 million children die every year because of *Plasmodium falciparum* malaria.¹ Despite major efforts, there is still no vaccine for malaria, and there is a great need for new effective therapies, especially against severe malaria. A striking difference between *P. falciparum* and other human *Plasmodium* species is the capacity of the *P. falciparum* parasitized red blood cells (pRBCs) to adhere to host cells, both through binding to the endothelium and other cells in the microvasculature (cytoadherence) and through binding to non-pRBCs (rosetting).² Cytoadherence and rosetting lead to sequestration of pRBCs and RBCs, cause obstruction of the blood flow in the microvasculature, and may result in severe disease symptoms if binding is pronounced.^{3,4} A number of previous studies have shown a correlation between rosetting and the severity of malaria^{5–10} and also between parasitemia and rosetting.¹¹

The binding of pRBCs to host cells is mediated by the variant multidomain protein *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) encoded by the *var* gene family (with around 60 copies per genome). The N-terminus of PfEMP1 has a number of cysteine-rich domains.^{8,12–15} One such domain is the Duffy binding-like domain- α (DBL1 α), which has been shown to mediate rosetting and endothelial binding of pRBCs through heparan sulfate (HS), blood group A antigen, and complement receptor 1 (CR1).^{13,16–19} Immunization of rats and macaques with one DBL1 α domain from a highly rosetting strain (FCR3S1.2) has been shown to diminish sequestration of pRBC in the circulation of these animals.²⁰

HS as well as heparin belong to a family of glycosaminoglycans (GAGs), and heparin is a highly sulfated form of HS. GAGs are composed of the same building blocks, glucosamine and glucuronic or iduronic acid, and most are negatively charged based on sulfate modifications. It has been shown that both HS and heparin bind directly to DBL1 α of

PfEMP1,^{13,16} and avid interaction requires at least a 12-mer (3.6-kDa) fragment of heparin as well as N-sulfatation and 6-*O*- and 2-*O*-sulfatations.²¹

Previous work has shown that both HS and heparin have the ability to disrupt rosettes.^{19,22–24} Importantly, binding of heparin and HS to pRBC is more pronounced in isolates from children with severe malaria, thus suggesting that HS is one of the receptors involved in the causation of the pathology of severe malaria.⁶ Heparin was previously used in the treatment of severe malaria, especially when the malaria was accompanied by disseminated intravascular coagulation,^{25–29} but it was withdrawn because of its strong anticoagulant action, with side effects such as intracranial bleeding.³⁰ The binding of heparin to antithrombin III (AT), which accounts for its major anticoagulant activity, is mediated by a pentameric sequence.^{31,32} Heparin devoid of its anticoagulant activity can be obtained through oxidation of this pentameric sequence, leading to depolymerization of the GAG and generating a low anticoagulant heparin (LAH).^{23,31,33} The molecular weight distribution and chemical composition of LAH are similar to that of the low molecular weight heparin (LMWH) frequently used in the clinic as an anticoagulant. However, the anticoagulant activity of LAH is strongly reduced compared with standard heparin and LMWH. The pentamer responsible for anticoagulant activity has been shown to not be essential for binding to the DBL1 α domain,^{21,23,34} and the oxidation does not affect the rosette disruptive capacity or the inhibition of endothelial binding mediated by DBL1 α .²³ Experiments with laboratory isolates and *in vitro* adapted isolates from malaria patients in Uganda have shown that LAH can disrupt rosettes and reduce cytoadherence both *in vitro* and in rat and macaque monkey models of severe malaria *in vivo*.^{23,35} An intravenous (i.v.) injection of LAH blocked up to 80% of infected erythrocytes from binding in the microvasculature of the rat and also released previously sequestered pRBCs into the circulation. In the corresponding monkey model, 55% of the sequestration was reduced after i.v. treatment of the animal.²³ Furthermore, LAH inhibited merozoite invasion of erythrocytes *in vitro*.²³

It is important to investigate rosettes of fresh clinical isolates, because *var* gene expression and therefore, rosetting may change early after *in vitro* adaptation of *P. falciparum*.^{36–38} Thus, to fortify the pre-clinical data, we have here studied the capacity of LAHs to disrupt rosettes formed by *P. falciparum*-infected erythrocytes obtained directly from

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children with malaria. We report that LAH disrupts rosettes of 42 of 47 fresh isolates obtained from Cameroonian children with mild or complicated malaria, and we state that rosettes from children with complicated malaria are more sensitive to the LAH than are rosettes of children with mild malaria.

MATERIALS AND METHODS

Sample collection in Cameroon. The study was conducted in Buea, a city with 150 000 inhabitants in the southwestern part of Cameroon. Malaria transmission takes place throughout the year but peaks during the rainy season that lasts from April to October.³⁹ The entomological inoculation rate (EIR) has been shown to be 0.56 infected *Anopheles* mosquito bites per person per night in this area during the rainy season.⁴⁰ The fieldwork was performed between March and May 2007 at three different health centers and four different hospitals in the Buea area. The study included malaria-infected children between 6 months and 14 years of age. Informed consent was given by the guardian. The diagnosis was based on laboratory tests (Giemsa-stained blood smears) and clinical examination. Hemoglobin levels in whole blood were measured using HemoCue Hb 201+ (Hemocue, Ängelholm, Sweden). Complicated *P. falciparum* malaria was defined as a patient requiring hospital admission and quinine or artemether infusion because of anemia, hyperparasitemia (parasitemia > 5%), or severe symptoms including hyperpyrexia, seizures, prostration, and/or vomiting. Cases with a positive blood smear for *P. falciparum* without complicating manifestations were classified as mild malaria and treated as outpatients with treatment *per os*. Ethical permissions for the study were obtained both in Cameroon and Sweden (numbers G379/900 and 2006/1323-31, respectively).

***P. falciparum* clinical isolates used in the study.** Blood was withdrawn from patients with parasitemia above 10,000 pRBC/ μ L blood and collected in ethylene diamine tetraacetic acid (EDTA) tubes. The blood samples were depleted of leukocytes by treatment with polymorph preparation (Axis-Shield, Oslo, Norway) according to the manufacturer's instructions within 2 hours of collection. Briefly, 2–5 mL whole blood were carefully layered over 5 mL polymorph preparation and centrifuged at $500 \times g$ for 30 min. Alternatively, if polymorph preparation could not be used because of the 2-hour time limit after collection, the fresh blood samples were centrifuged at $400 \times g$ for 5 min to separate RBCs, leukocytes, and plasma. The plasma and leukocyte band were removed, and separated packed RBCs were washed three times with RPMI (Roswell Park Memorial Institute) 1640 medium (Sigma Aldrich, St. Louis, MO). A total of 200 μ L packed RBCs was transferred to 4.0 mL malaria culture medium supplemented with 10% inactivated human AB⁺ non-immune Swedish serum and placed at 37°C for maturation of ring-stage parasites to trophozoites using standard methods.^{41,42} The parasitemia was counted, and the rosetting rate was determined by calculating the number of trophozoite pRBCs within rosettes relative to the total number of trophozoite pRBCs present in the culture. A rosette was defined as at least two uninfected RBCs bound to one pRBC.⁴¹

Glycosaminoglycans used in the study. LAHs are heparin derivatives prepared from porcine intestinal heparin. LAH was chemically generated by periodate oxidation of the heparin at the antithrombin (AT) binding sequence.³¹ This process led to splitting of C2–C3 bonds of all non-sulfated hexuronic

acid residues and subsequent cleavage of the heparin chain at these sites. Because the AT binding pentasaccharide sequence contains a non-sulfated glucuronic acid unit, this structure and hence, the anticoagulant activity were eliminated. A first LAH batch, DFX232, was prepared by oxidizing heparin followed by a reduction. The second LAH batch, *Sevuparin*, was prepared in the same manner but included an acidic hydrolysis step. The preparations were dried, and the anticoagulant activities of the two LAH batches were determined by measuring the anti-IIa and anti-Xa activities. The assays were performed according to the Ph Eur procedure for low molecular mass heparin (monograph 0828) in the European Pharmacopoeia.⁴³ The anticoagulant activity of LAH was strongly reduced compared with heparin and LMWH. The final products DFX232 and *Sevuparin* had molecular weights of 10.1 and 7.4 kDa, respectively.

Rosette disruption assay. Rosette disruption assays were performed on pRBC cultures harboring trophozoites 20–24 h post-invasion. LAH was diluted in RPMI, and samples were analyzed in triplicates as previously described.²⁴ Briefly, parasite cultures were concentrated two times from a hematocrit of 5–10%, and 25 μ L culture were added to 25 μ L LAH in RPMI, giving final concentrations of 1,000 and 100 μ g/mL. After incubation of samples at 37°C for 30 minutes, the parasites were stained with acridine orange, and the rosettes were counted. For each sample, 25 fields, equivalent to approximately 3,500–4,000 RBCs, were counted. In parallel, mock-treated samples were analyzed in the presence of RPMI alone.

Statistical analysis. Before statistical analysis, the rosetting rates for the isolates were converted into relative rosetting rates by calculating the proportion of remaining rosettes after treatment compared with the mock-treated erythrocytes. The statistical analysis was carried out with Prism version 5.0 for Windows (Graphpad Software, San Diego, CA). Student paired *t* test or Wilcoxon signed rank test (when the samples were not normally distributed) was used. For the comparisons between groups with mild and complicated disease, unpaired *t* test and Mann–Whitney *U* test, respectively, were used. Pearson's χ^2 test was used when the data were binary. As estimated in a previous study, rosette disruption of $\geq 15\%$ was regarded as significant.²⁴ Where applicable, data are presented as mean and standard error of mean (SEM).

RESULTS

Characteristics of the study sample. In all, 1,079 children between 6 months and 14 years of age were screened for malaria, and 63% were found to be infected (682 of 1,079). When the parasite density was $\geq 10,000$ pRBC/ μ L blood, a venous blood sample was collected, which resulted in a total of 144 samples (Table 1). Of the 144 samples, 140 samples were cultivated, and 112 isolates (80%) successfully grew into trophozoites. Forty-seven isolates showed a rosetting phenotype, with a rosetting rate that ranged from 2.1% to 79% (Figure 1 and Table 1). Of these, 20 samples were from children diagnosed with complicated malaria, and the other 27 samples were from children with mild malaria. There was a significant difference in the number of isolates that formed rosettes between the mild and complicated malaria groups ($P = 0.0009$) (Table 1), because rosettes were present in 22% of the samples in the mild group and 50% of the samples in the complicated group. There was a significant difference in parasitemia between the

TABLE 1
Details of field isolates

Field isolate	Measurement
No. of children tested for <i>Plasmodium falciparum</i>	1,079
No. of children infected with <i>P. falciparum</i>	682 (63%)
No. of children included in the study (> 10,000 parasites/ μ L blood)	144 (12%)
No. of cases of complicated malaria	54 (38%)
No. of cases of mild malaria	90 (62%)
No. of isolates successfully matured to trophozoites	112 (80%)*
No. of rosetting isolates (mild/complicated)	47; 42% [†] (27/20)
Percent rosetting isolates (mild/complicated)	22%/50% [‡]
Mean rosetting rate (range)	20.42% (2–79%)
Mean rosetting rate (mild/complicated)	20.9%/19.7%
Mean parasitemia (mild/complicated)	3.6% (2.7%/5.3%) [‡]
Mean parasitemia in rosetting isolates (mild/complicated)	4.5% (3.4%/6.1%) [‡]
Mean hemoglobin level (mild/complicated)	90.2 g/L (95/82) [§]
Mean hemoglobin level in rosetting isolates (mild/complicated)	90.9 g/L (92/89)

* One hundred twelve of one hundred forty isolates grew to the mature trophozoites stage in culture.

[†] Forty-seven of one hundred twelve isolates showed a rosetting phenotype.

[‡] $P < 0.05$.

[§] $P < 0.0001$.

groups, with a mean parasitemia of 3.4% and 6.1% for the mild and complicated groups, respectively ($P = 0.04$). However, there were no significant differences in rosetting rates or hemoglobin levels between the mild and complicated groups.

LAH disrupt rosettes from fresh isolates. To examine the activity of LAH against a large sample size of fresh patient samples collected from malaria-infected children, 47 rosetting isolates were tested for sensitivity to two different batches of LAH (DFX232 and *Sevuparin*). Two concentrations, 100 and 1,000 μ g/mL, were used, and results were compared with mock-treated samples prepared in RPMI. Rosette disruption of $\geq 15\%$ (defined as significant²⁴) was shown in 42 of 47 (89%) samples with DFX232 at a concentration of 1,000 μ g/mL, 39 of 47 (83%) samples with *Sevuparin* at a concentration of 1,000 μ g/mL, 34 of 47 samples with DFX232 at 100 μ g/mL, and 36 of 47 samples with *Sevuparin* at 100 μ g/mL (Figures 1 and 2). Both DFX232 and *Sevuparin* caused disruption of $\geq 50\%$ of the rosettes in 32 of 47 (68%) samples at the higher concentration. At the lower concentration, the number of highly reacting samples was 18 of 47 for DFX232 and 25 of 47 for *Sevuparin* (Figure 2A and B). Only three samples showed no significant effect with either of the two substrates used at any of the concentrations. Relative rosetting rates (RR; the proportion of remaining rosettes after treatment compared with the control-treated erythrocytes) ranged from 36% (*Sevuparin*, higher concentration) to 61% (DFX232, lower concentration) (Figure 3A). Statistically significant rosette disruption compared with the control was seen in all groups, with a $P < 0.0001$ (Figure 3A). There was no significant difference in rosette disruption effect between the two batches.

Rosettes in complicated malaria isolates are more sensitive to LAH than rosettes in mild malaria isolates. When comparing samples from mild and complicated malaria, a

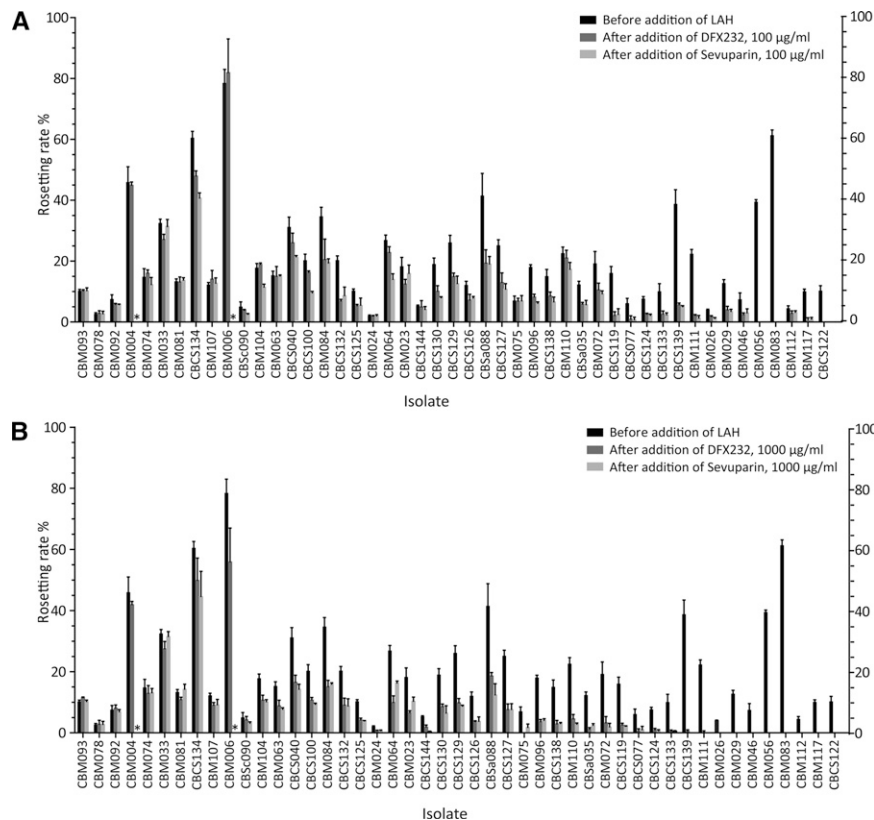


FIGURE 1. Rosetting rates before and after the addition of LAHs in the 47 isolates. The mean rosetting rate \pm SEM is shown. Rosetting rate before addition of LAH (black bars), DFX232 (dark grey bars), or *Sevuparin* (light gray bars). *Not done. (A) DFX232 and *Sevuparin* at concentrations of 100 μ g/mL. (B) DFX232 and *Sevuparin* at concentrations of 1,000 μ g/mL.

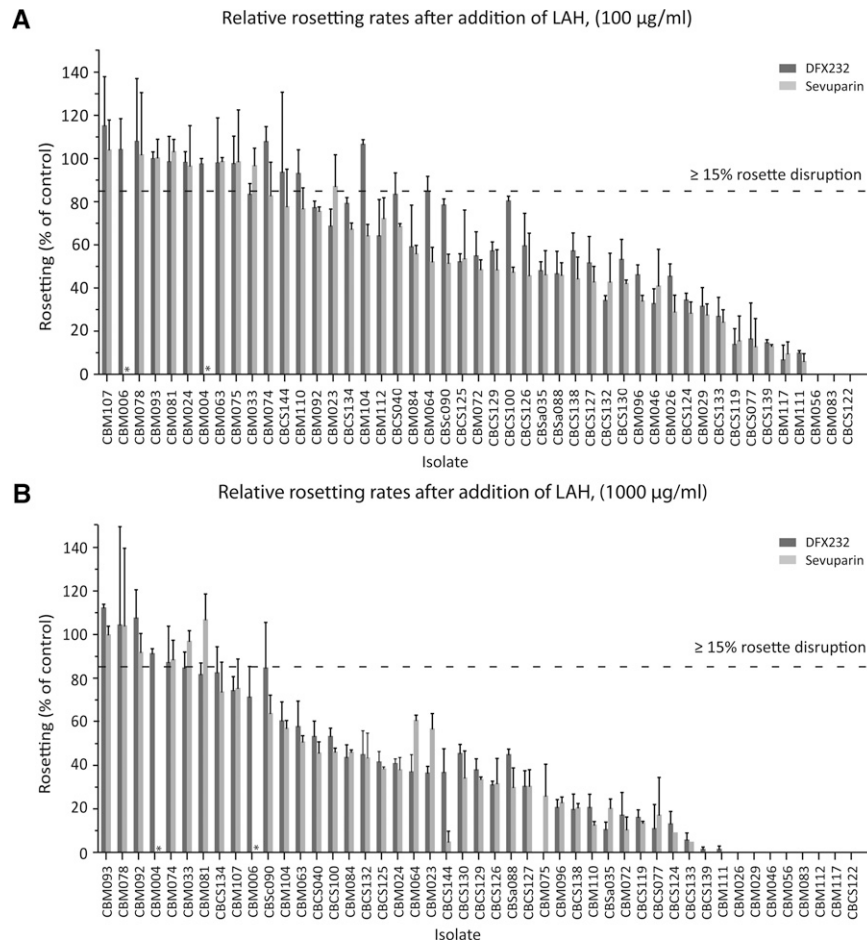


FIGURE 2. Relative rosetting rate after the addition of LAH compared with control. The mean relative rosetting rate (the proportion of remaining rosettes after treatment compared with the control-treated erythrocytes) \pm SEM is shown. Significant rosette disruption $\geq 15\%$ is indicated by the horizontal line. Dark grey bars = DFX232; light grey bars = *Sevuparin*. *Not done. (A) DFX232 and *Sevuparin* at concentrations of 100 $\mu\text{g}/\text{mL}$. Significant disruption was shown in 42 of 47 samples with DFX232 and 39 of 47 samples with *Sevuparin*. (B) DFX232 and *Sevuparin* at concentrations of 1,000 $\mu\text{g}/\text{mL}$. Significant disruption was shown in 34 of 47 samples with DFX232 and 36 of 47 samples with *Sevuparin*.

difference in rosette disruption effect by the LAHs was revealed (Figure 3B and C). Both DFX232 and *Sevuparin* had a greater rosette disruption effect on the isolates from the complicated malaria cases compared with the mild cases, with an LAH concentration of 100 $\mu\text{g}/\text{mL}$. Thus, after treatment with 100 $\mu\text{g}/\text{mL}$ DFX232, the remaining relative RR was 70% (range = 0–115%) in the mild malaria isolates and 49% (range = 14–94%) in the complicated malaria isolates ($P = 0.03$) (Figure 3B). With *Sevuparin* at 100 $\mu\text{g}/\text{mL}$, the figures were 62% (range = 0–104%) in the mild cases and 41% (range = 13–78%) in the complicated cases ($P = 0.02$) (Figure 3B). A similar trend but no statistically significant difference was seen between the groups when the higher concentrations of both compounds were used (Figure 3C). No correlation was found between sensitivity to the LAH and parasitemia or RR (data not shown).

DISCUSSION

The present investigation shows that LAH effectively disrupts rosettes in fresh clinical isolates. We compared two different preparations of LAH (DFX232 and *Sevuparin*) at two different concentrations, 100 and 1000 $\mu\text{g}/\text{mL}$, on 47 fresh isolates from Cameroonian children with mild or complicated

malaria. The concentrations were selected based on previously reported studies performed *in vitro*.^{13,16,22,24} The chosen concentrations are in the range of the doses of LAH evaluated in a finalized Phase I study. In the Phase I study, no severe adverse events were reported, and the drug candidate was proven to be safe and well-tolerated (unpublished data).

A majority, 68% (32/47), of the samples showed a rosette disruption effect $\geq 50\%$ when the compounds were analyzed at the higher concentration. In all, 89% (42/47) of the isolates were sensitive ($\geq 15\%$) to rosette disruption by any of the LAH studied (Figures 1 and 2). We further found a difference in rosette disruption effect by LAH depending on the severity of the malaria infection. Both LAH compounds, when analyzed at the lower concentration, had a significantly stronger rosette disruption effect ($P < 0.05$) in samples from complicated malaria cases than in samples from mild cases (Figure 3B). The difference in rosette disruption effect was about 40% for both compounds between the mild and complicated isolates (Figure 3B). For the higher concentration, the effect was also more pronounced in the complicated cases than in the mild cases, although not significant (Figure 3C). These results are in line with previous findings showing that binding of soluble heparin to the surface of pRBC is significantly associated with severe disease.⁶ The parasites from the mild and

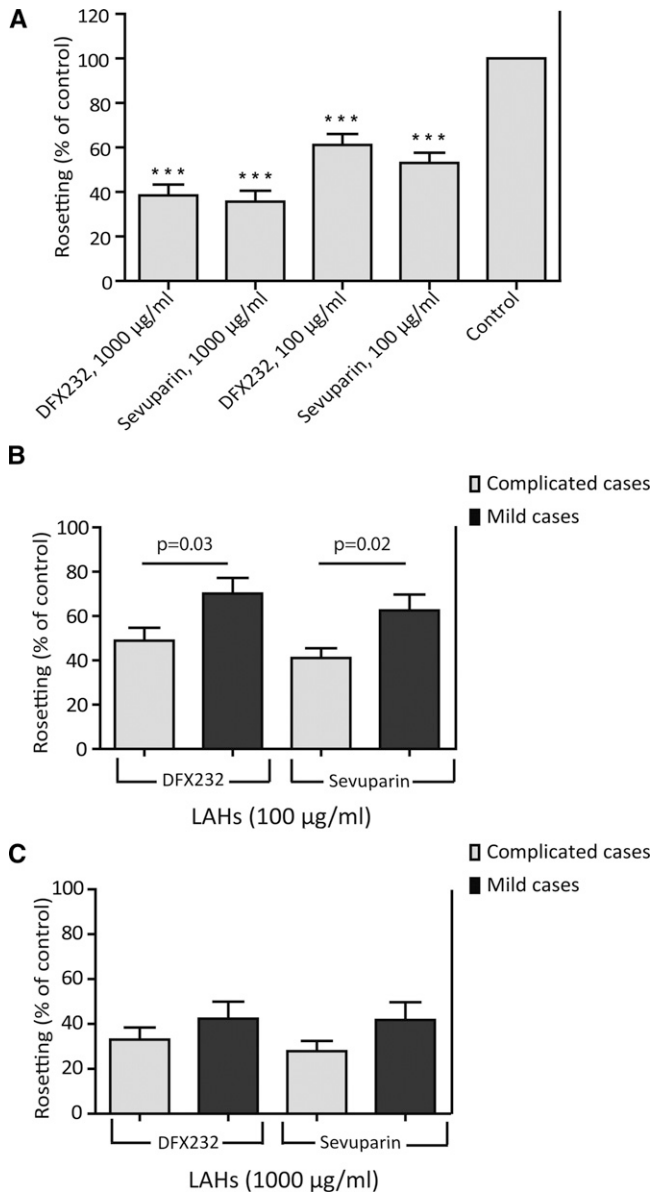


FIGURE 3. (A) Overall rosette disruption effect of the two different LAHs. The mean relative rosetting rate (the proportion of remaining rosettes after treatment compared with the control-treated erythrocytes) \pm SEM is shown. There was a significant disruption effect for DFX232 and *Sevuparin* at both concentrations compared with the mock-treated samples. There was no statistically significant difference between the two LAH preparations. *** $P < 0.001$. (B and C) Rosette disruption effect in isolates from mild and complicate malaria cases. The mean relative rosetting rate (the proportion of remaining rosettes after treatment compared with the control-treated erythrocytes) \pm SEM is shown. Dark grey bars = mild malaria; light grey bars = complicated malaria. The mean relative rosetting rate is shown. (B) DFX232 and *Sevuparin* at concentrations of 100 μ g/mL. (C) DFX232 and *Sevuparin* at concentrations of 1,000 μ g/mL.

complicated groups may use different rosetting receptors. One can speculate that the use of CR1 or blood group A as a rosetting receptor may be more frequent in the mild compared with the complicated group, making these rosettes less sensitive to LAH treatment.

Rosette disruption by heparin, HS, and LAH and the binding of GAGs to the DBL1 α domain of PfEMP1 are well-described in several studies.^{13,16,17,21–23} When comparing rosette

disruption data for heparin or HS^{19,24} with the data for LAH, the present study might indicate a stronger rosette disruption effect by DFX232 and *Sevuparin*. Carlson and others²⁴ reported that 30% (16/54) of the samples collected in Gambia showed $\geq 50\%$ rosetting disruption effect of heparin, and 50% (27/54) of the samples showed $\geq 15\%$ disruption at a concentration of 650 μ g/mL.²⁴ In the present study, we found $\geq 50\%$ rosetting disruption effect in 53% (25/47) of the samples and $\geq 15\%$ disruption effect in 77% (36/47) of the samples at the concentration 100 μ g/mL. Three rosetting isolates (6%) in our study showed no sensitivity to either of the two LAH preparations tested. This finding is not surprising but in concordance with the results of previous studies where it has been shown that some isolates are insensitive to heparin, heparin derivatives, and other rosette-disrupting sulfated glycoconjugates, even at very high concentrations.^{23,24,44}

In the present study, there was a statistically significant difference ($P < 0.05$) between the complicated and mild groups, with a higher parasitemia and more isolates forming rosettes in the complicated malaria cases than in the mild malaria cases. Another observation is that the isolates that showed a rosetting phenotype in the mild group were of higher parasitemia and lower hemoglobin levels compared with the isolates in the same group that did not show any rosetting. There was a trend but no statistical difference in hemoglobin levels between the mild and complicated groups, which may be because the complicated group includes children with malaria with diverse symptoms such as hyperpyrexia, anemia, hyperparasitemia, seizures, prostration, and vomiting. The children with severe anemia, therefore, belong to a subgroup within the complicated group. The rosetting rate did not significantly differ between the mild and complicated groups, although a significantly higher number of isolates formed rosettes in the complicated group than in the mild group. The similarity in rosetting rates in the mild versus complicated group may be a consequence of a different prevalence of hemoglobinopathies, such as sickle cell anemia or thalassemia, in the mild and complicated groups, because these diseases have been shown to impair the ability to form rosettes instead creating small and weak rosettes.¹⁰ Indeed, both sickle cell anemia and thalassemias are common in Cameroon and surrounding areas.⁴⁵

In this report, we only used fresh parasitized blood samples collected from malaria-infected children. The samples were directly analyzed without freezing and thawing. The aim was to obtain parasite samples as close to *in vivo* conditions as possible. In some rosette disruption studies, frozen and thawed parasite samples or laboratory strains have been used.^{19,22,23,44} In a recent study, it was found that the dominant *var* gene coding for PfEMP1 changed before and after cryopreservation in a number of isolates, which was analyzed by quantitative polymerase chain reaction (Q-PCR) using strain and stage-specific primers.³⁶ By using fresh isolates, the risk of changes in antigens presented on the erythrocyte surface, including expression of PfEMP1, is limited. Hence, the use of entirely fresh blood samples may present a more correct picture of the infected cells' surfaces *in vivo* than frozen and thawed blood samples.

Another sulfated glycoconjugate that has been shown to be effective in rosette disruption and inhibition of parasite invasion of RBCs is curdlan sulfate (CRDS).^{44,46} This compound has been reported to be effective *in vitro* at the concentration 50 μ g/mL in the majority of 18 *P. falciparum* isolates from Kenyan children. The mechanism of rosette disruption

by CRDS is unclear; hence, it is not known if the mechanism of action of CRDS is a specific binding to the PfEMP1 protein. Two clinical trials with a small number of severe malaria patients have shown that fever clearance was shortened in the group receiving CRDS, but the study was unable to show any difference in mortality, parasitemia, or coma resolution after treatment with CRDS.⁴⁷

LAH is being documented as adjunct treatment of severe malaria. This includes a recent clinical phase I safety study conducted in healthy male volunteers (unpublished data). No severe adverse effects were reported, and the candidate drug (*Sevuparin*) was proven to be safe and well-tolerated (unpublished data). Here, we show that LAH affects the majority of fresh rosetting clinical isolates analyzed without any manipulation of the parasites. In addition, LAHs have earlier been shown to inhibit cytoadhesion and merozoite invasion *in vitro* and block and reverse sequestration of pRBCs *in vivo* in rats and monkeys.²³ By inhibiting the three processes rosetting, cytoadherence, and merozoite invasion with a receptor analogue like LAH, a change in the sequestered parasite load and parasitemia may also be achieved in humans. One can speculate that this may reestablish the microcirculation and release bound pRBCs into the circulation; this renders the latter more available for an immune attack on passing through the spleen. We conclude that drugs reducing the sequestration of pRBCs, such as LAH, have a potential as adjunct treatment of severe malaria.

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