Disruption of Rosetting in *Plasmodium falciparum* Malaria with Chemically Modified Heparin and Low Molecular Weight Derivatives Possessing Reduced Anticoagulant and Other Serine Protease Inhibition Activities

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Received October 24, 2007

Severe malaria has been, in part, associated with the ability of parasite infected red blood cells to aggregate together with uninfected erythrocytes to form rosettes via the parasite protein PfEMP-1. In this study, inhibitors of rosetting by the *Plasmodium falciparum* strain R-29, based on chemically modified heparin polysaccharides ($IC_{50} = 1.97 \times 10^{-2}$ and 3.05×10^{-3} mg·mL^{-1}) and their depolymerized, low molecular weight derivatives were identified with reduced anticoagulant and protease (renin, pepsin, and cathepsin-D) activities. Low molecular weight derivatives of the two most effective inhibitors were shown to have distinct minimum size and strain-specific structural requirements for rosette disruption. These also formed distinct complexes in solution when bound to platelet-factor IV.

Introduction

Each year, between 350 and 500 million people are infected by species of the protozoan parasite *Plasmodium*, the causative agent of malaria. The female anopheles mosquito, which carries the parasite, is prevalent in geographical regions that place 40% of the world's population at risk. Severe malaria, due to infection by *Plasmodium falciparum*, is characterized by metabolic malfunctions, blood abnormalities, and/or major organ failures. These complications manifest themselves in the form of hemolysis, pulmonary edema, respiratory distress, blood coagulation abnormalities, thrombocytopenia, cardiovascular collapse, and cerebral malaria (CM[a]). The treatment of *P. falciparum* has been hampered by the emergence of resistant strains concurrently in both Southeast Asia and South America with further cases identified in New Guinea and East Africa. Resistance to conventional treatments is common, and combinatorial therapies based on the compound artemisinin are currently being used, although resistance to this drug has already been observed.[1,2] The onset of multidrug resistance places a high priority on the development of novel therapeutic agents to treat the symptoms of CM, as well as adjunct therapies targeted at severe disease.

Cerebral malaria is thought to be caused by the sequestration of parasite-infected red blood cells (RBCs) to venules in the brain, and the incidence of this complication is directly related to the level of sequestration.[3] The underlying mechanism governing how parasitized erythrocytes induce coma is controversial, but is thought to involve pro-inflammatory cytokines stimulated by the presence of malaria toxins and localized hypoxia caused by the mechanical blockage of blood vessels. The mechanism of occlusion relies on cell−cell adhesion, facilitated by interactions between parasite-expressed proteins and a range of carbohydrate and protein–ligands, which include hyaluronic acid (HA), chondroitin sulfate (CS), heparin, heparan sulfate (HS), CD-36, E-selectin, PECAM-1, ICAM-1, VCAM-1, and thrombospondin. The binding of parasitized RBCs to these receptors causes cyto-adherance, autoagglutination, and rosetting (adhesion of a parasitized RBC to one or more uninfected RBCs). Correlations between rosette formation and CM have been reported in a variety of geographical locations including The Gambia, Kenya, Thailand, and Madagascar, and the immunogenic response may be distinct in different geographical regions. The parasite expressed and RBC membrane-bound protein, *P. falciparum* erythrocyte membrane protein I (PfEMP-1), has been implicated as the major protein involved in these interactions.[4,5]

The exploitation of carbohydrates in host−cell recognition and the attachment of pathogenic micro-organisms has been demonstrated in a wide range of cases,[6] and glycosaminoglycans (GAGs) (including heparin and HS) are involved in the invasion of host cells by a plethora of micro-organisms. Intact heparin is known to disrupt rosettes, which are formed between infected and uninfected RBCs,[7] but its potent anticoagulant and other activities preclude its clinical use for this purpose. The rosette receptor–ligand, PfEMP-1, possesses a Duffy binding-like domain (DBL), which binds heparin polysaccharides and low molecular weight (LMW) fractions generated by depolymerization.[8] Antibodies to the Duffy domain also ablate rosette formation and prevent sequestration, suggesting the involvement of heparin and HS in rosette formation.

Heparin and HS are highly charged, 1–4-linked linear sulfated polysaccharides, sharing a common biosynthetic pathway, and

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[a] Abbreviations: CD, circular dichroism; CM, cerebral malaria; CR1, complement receptor I; CS, chondroitin sulphate; DBL, Duffy binding-like domain; dp, degree of polymerization; GAG, glycosaminoglycan; HA, hyaluronic acid; HIT, heparin-induced thrombocytopaenia; HPAEC, high-performance anion-exchange chromatography; HS, heparan sulfate; LMW, low molecular weight; PfEMP-1, *Plasmodium falciparum* erythrocyte membrane protein I; PF4, platelet factor IV; PG, proteoglycan; PIMH, porcine intestine mucosal heparin; RBC, red blood cell; RD, rosette disruption; SRCD, synchrotron radiation circular dichroism; TR, therapeutic ratio.

10.1021/jm701337t CCC: $40.75
Published on Web 02/09/2008

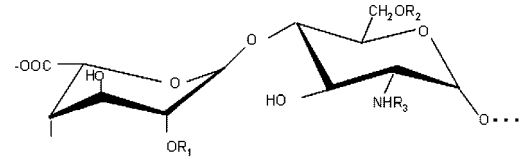
are covalently attached to protein cores to form proteoglycans (PGs). While heparin is generally more homogeneous and highly sulfated than HS, it lacks the latter's complex domain structure. Both HS and heparin are composed of repeating disaccharide units consisting of an uronic acid and an α-D-glucosamine residue. The uronic acid residue can exist as one of two epimers: β-D-glucuronic acid or its C-5 epimer α-L-iduronic acid. Esterification with sulfate groups to form *O*-sulfates can occur at position-6 of the glucosamine residue and/or position-2 of the uronic acid residue as well as, more rarely, at position-3 of the glucosamine. The glucosamine residue may also be *N*-sulfated or *N*-acetylated and reportedly can exist in nature as a free amine.[9]

There have been previous reports of malaria rosette disruption in the literature employing chemically modified heparin derivatives. Barragan et al. concluded that the *N*-sulfate group is of particular importance in maintaining high rosette disruption potential.[7] Their data also showed that an *N*-acetylated heparin derivative possessed only minimal ability to disrupt rosetting,[7] while paradoxically, the constituent disaccharide of this polymer was surprisingly more active when assayed against other component disaccharides present within chemically modified heparin derivatives. The size requirement for disruption by depolymerized heparin fractions was found to be dp12 (reported to be ∼4 kDa).[10]

One important question is the level of strain specificity that exists for these interactions. The present study investigates whether a different parasite strain to that previously studied (R-29, derived from the ItG lineage) possesses distinct requirements for rosette disruption with a range of structurally well-defined chemically modified heparin polysaccharides and their low molecular weight (LMW) derivatives. The R-29 strain has previously been associated with rosetting mediated by complement receptor I (CR1)[5] and has been shown to be disrupted by unmodified heparin.[11] Heparin polymers have previously been reported to disrupt complement receptor I binding to other (non-PfEMP-1) ligands,[12,13] and although CR1 has been shown to interact with PfEMP-1 for the R-29 strain,[5] the possibility of GAGs interacting with either PfEMP-1 and/or CR1 directly, or acting as bridging molecules, should not be excluded.

An important consideration when determining potential heparin-based pharmaceuticals is their likely side effects; one of the most important is anticoagulation, arising from the interaction between antithrombin III and a specific pentasaccharide sequence found in heparin. In the present study, a library of chemically modified heparin derivatives, in which selective chemical changes have been made to the underlying substitution pattern of *O*-sulfation, *N*-sulfation, and *N*-acetylation, are employed as potential agents of rosette disruption with the R-29 malaria strain (Table 1 and Figure 1). Their activities against a range of other potential side effects of heparin derivatives including anticoagulation (via inhibition of factor Xa) (Table 1) and inhibition of serine proteases renin, pepsin, and cathepsin-D were also determined. Selected polysaccharides which exhibited potent rosette disruption abilities were also subjected to partial depolymerization and fractionation to yield low molecular weight (LMW) chemically modified heparin derivatives. These were also tested for their ability to disrupt rosetting (Figure 2) and as anticoagulants. The abilities of the polysaccharide inhibitors to induce conformational change when binding platelet-factor IV (PF4) in solution were established and compared to those which occurred when PF4 bound intact heparin (Figure 3). The presence of the complex between intact heparin and PF4 can lead to thrombocytopenia.[14]

Table 1. Structures of Chemically Modified Heparin Polysaccharide Derivatives **1**−**8** Showing the Predominant Disaccharide Repeat Based on the Repeating Disaccharide −4) L-Iduronic α(1–4)-D-glucosamine α(1−: Rosette Disruption (RD), Anticoagulant (Anti-Xa), and Therapeutic Ratios (TR) (Defined in Two Ways) for **1**−**8** Are Shown



$R_1 = SO_3$ or H $R_2 = SO_3$ or H $R_3 = SO_3$, $COCH_3$ or H

	R_1	R_2	R_3	RD[a]	antiX_a[b]	TR[c]	TR[d]
1	SO_3^-	SO_3^-	SO_3^-	98(1)	100	0.98	0.69
2	SO_3^-	SO_3^-	$COCH_3$	97(1)	0.03	3200	23
3	H	SO_3^-	SO_3^-	26(5)	0.4	65	
4	H	SO_3^-	$COCH_3$	16(2)	0.03	530	
5	SO_3^-	H	SO_3^-	98(1)	0.5	196	244
6	SO_3^-	H	$COCH_3$	17(2)	0.03	570	
7	H	H	SO_3^-	15(1)	0.03	500	
8	H	H	$COCH_3$	16(2)	0.03	530	

[a] RD: rosette disruption (defined as the percentage of parasite infected red blood cells that are nonrosetting) at final polysaccharide concentrations of 0.4 mg·mL^{-1} (intact heparin (**1**) achieved 98%) with SD ($n = 3$) in parentheses. The IC_{50} for heparin (**1**) was 2.16 μg·mL^{-1} (70 nM). [b] Anticoagulant (antifactor Xa) activity expressed as a percentage of intact heparin (1) (100%). [c] TR, therapeutic ratio defined as the ratio of RD to Anti Xa activity. [d] TR (for selected compounds): therapeutic ratio defined as IC_{50} for rosette disruption divided by the antifactor Xa activity relative to intact heparin (**1**).

Disruption of Rosetting by Modified Heparin Polysaccharides

The results in Figure 1 indicate that there is no simple correlation between the overall charge density or predominant substitution pattern of the polysaccharides and their ability to disrupt rosetting for the R-29 strain. This finding is entirely in keeping with most of the biochemical activities reported for modified heparin derivatives and reflects the subtle relationship between substitution pattern and conformation in these molecules.[15–17] The conformation of sugar rings, glycosidic bonds, and hence the disposition of appended groups, such as hydroxyl, carboxylic, and sulfate groups, which interact with proteins, are known to vary in a complex manner with substitution pattern.[16,17] The most potent polysaccharide disruptors of rosetting (RD in Table 1), excluding intact heparin **1**, were the *N*-acetyl derivative **2** and the 6-desulfated derivative **5**, and these also exhibited the best therapeutic ratios (as defined by the IC_{50} for disruption divided by the anti-Xa activity relative to heparin; TR^d in Table 1).

Anticoagulation Activities

The anticoagulant activities of derivatives **2**−**8** are all highly attenuated compared to unmodified heparin **1** (Table 1). The anticoagulation activity of heparin/HS is due to the interaction between antithrombin III and the specific pentasaccharide sequence −4) GlcNAc(6S) α(1–4) GlcA β(1–4) GlcNS(3,6S) α(1–4) IdoA(2S) α(1–4)GlcNS(6S) α (1−,[18] causing a drastic change in the affinity of antithrombin III for factor Xa. The presence of a 3-*O*-sulfate group in the glucosamine residue at the center of this pentasaccharide sequence is crucial for high activity. Its absence resulted in the virtual abolition of activity,[18] while removal of other *N*- or *O*-sulfate groups had a less dramatic, but still significant, effect.

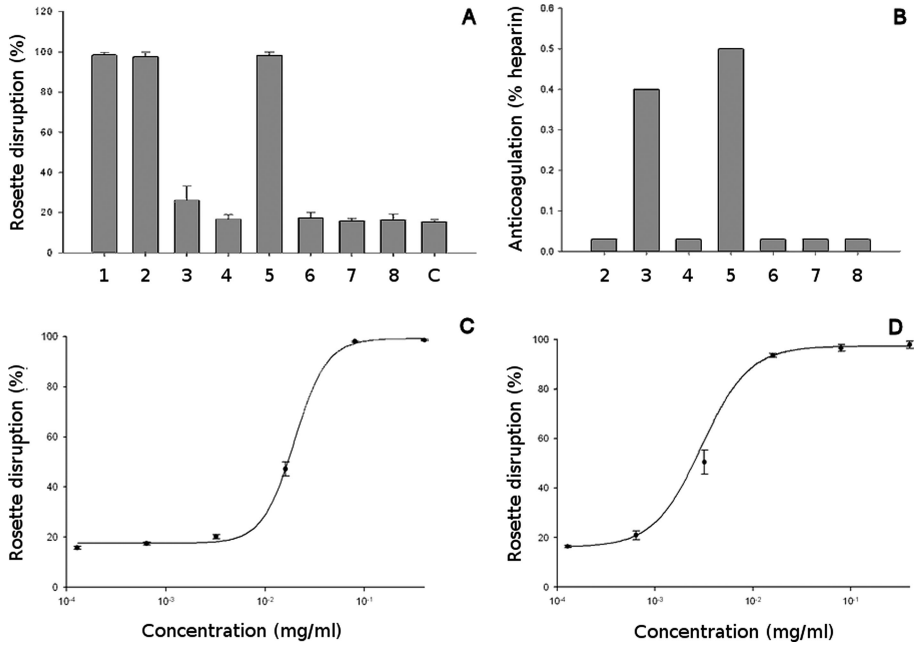


Figure 1. (A) Rosette disruption of RBCs infected with *P. falciparum* strain R-29 by chemically modified heparin polysaccharides at 0.4 mg·mL^{-1}. (B) Anticoagulation activity of chemically modified heparins at 0.4 mg·mL^{-1}. (C) Dose−response curve for rosette disruption by **2**, $IC_{50} \approx 1.97 \times 10^{-2}$ mg·mL^{-1}. (D) Dose−response curve for rosette disruption by **5**, $IC_{50} \approx 3.05 \times 10^{-3}$ mg·mL^{-1}. Polysaccharide structural features are as follows: (**1**) heparin; (**2**) *N*-acetylheparin; (**3**) de-2-sulfated heparin; (**4**) de-2-sulfated *N*-acetylheparin; (**5**) de-6-sulfated heparin; (**6**) de-6-sulfated *N*-acetylheparin; (**7**) de-2-de-6-sulfated heparin; (**8**) de-2,de-6-sulfated *N*-acetylheparin.

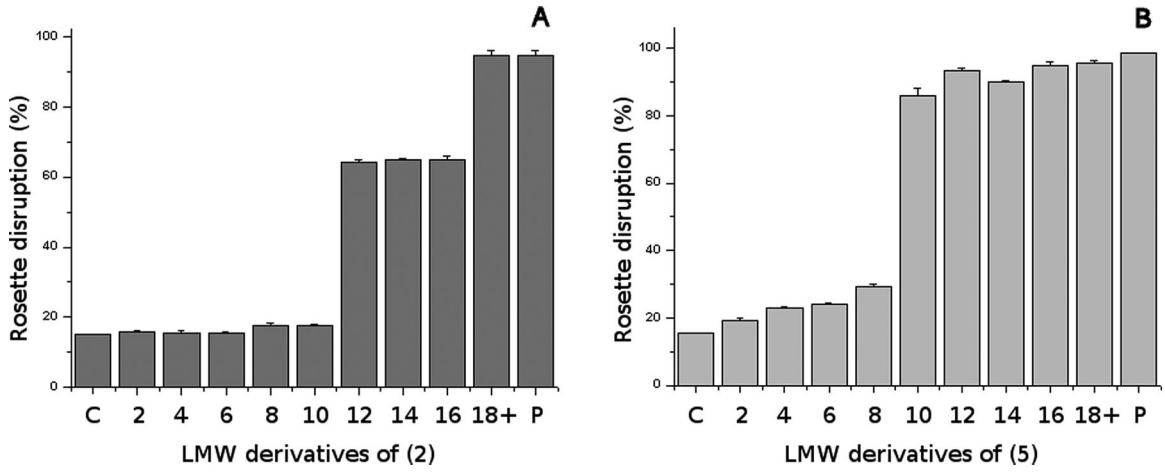


Figure 2. Rosette disruption of RBCs infected with *P. falciparum* strain R-29 by LMW heparin fragments of sample **2** (A) and **5** (B). LMW heparin fragment sizes are given as degree of polymerization (dp) at 0.4 mg·mL^{-1}. P is intact polysaccharide (**2**) and (**5**), respectively, and C is a control (water). Polysaccharide structural features are (**2**) *N*-acetylheparin (A) and (**5**) de-6-sulfated heparin (B).

Replacement of *N*-sulfate with *N*-acetyl groups in heparin derivatives (e.g., modifying **1** to **2**) explains the loss of anticoagulant activity in **2**. De-O-sulfation in iduronate residues, under highly basic conditions,[19] also resulted in substantial reduction in antifactor Xa activity and has two potential causes. The first is the removal of the 2-*O*-sulfate group itself, but another modification also occurs in the small proportion of glucosamine residues bearing both 3-*O* sulfate and *N*-sulfate groups (including those in the pentasaccharide sequence). This involves the formation of an *N*-sulfated aziridine group with loss of the 3-*O*-sulfate group.[20] Heparin derivatives **5** and **6** that are 6-*O*-desulfated were prepared under different conditions. They do not contain this modification and the reduction in their activities can only be attributed to the loss of other relevant groups (i.e., 6-*O*-sulfates and *N*-sulfates) within the pentasaccharide sequence because the 3-*O*-sulfate group is stable under mild de 6-*O*-sulfation conditions.[21]

Disruption of Rosetting by Fractions from Depolymerized Heparin Derivatives

Low molecular weight (LMW) fractions generated from **2** and **5** by partial enzymatic cleavage (heparitinase II) and gel permeation chromatography were also assayed (at 0.4 mg·mL^{-1}) for their ability to disrupt rosetting, anticoagulation activity, and several serine protease activities.

LMW fractions from **2** of decasaccharide (dp 10) fractions or smaller were not able to significantly disrupt rosetting. Fragments corresponding to dodecasaccharides (dp 12) and larger achieved significant disruption, and this increased up to octadecasaccharides (dp 18), which had the same capacity as

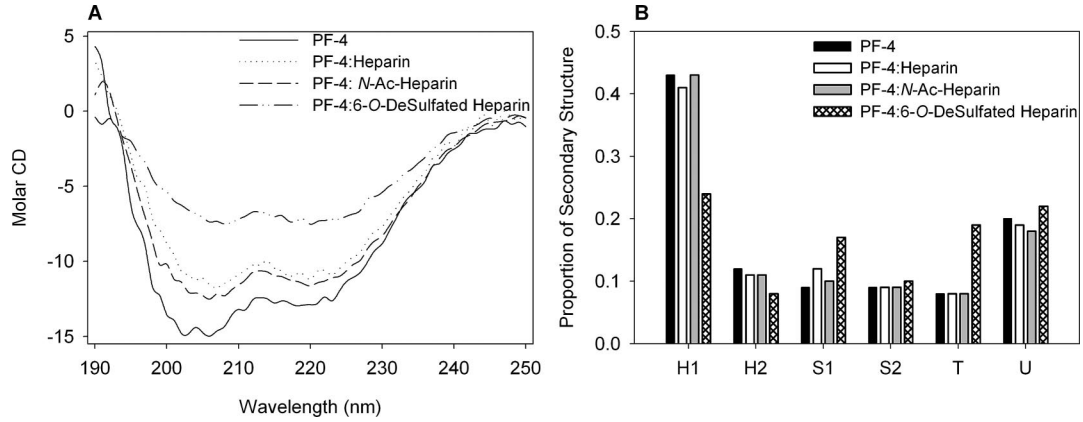


Figure 3. (A) SRCD spectra of PF4 between 190 and 250 nm: PF4 (**1**) (—); *N*-acetyl (**2**) (— —); 6-desulfated heparin (**5**) (— - - —; unmodified heparin (**1**) (- - -). (B) Secondary structural analysis in solution of PF4 (solid black) and complexes formed with unmodified heparin (**1**) (white); *N*-acetylheparin (**2**) (gray); 6-desulfated heparin (**5**) (hatched) (H1, regular α-helices; H2, distorted α-helices; S1, regular β-strands; S2, distorted β-strands; T, β-turns; and U, unordered).

the intact parental polysaccharide. Depolymerized fragments from **5** achieved potent rosette disruption potential at dp 10 and larger and did not exhibit the more gradual increase in potency shown by LMW derivatives of **2**.

LMW fractions all possess very weak anticoagulant activities (on an equivalent weight basis); for **2**, these ranged from 0.7 to 0.9% of that of heparin **1** and for **5**, from 1.7–2.2% of that of **1** for dp 8–14. These contrasting size requirements suggest that effective disruption of rosettes may depend on interfering with several interactions at the cell surface and that the interaction may involve a degree of cooperativity.

Activity against Other Proteases

The aspartyl proteases pepsin, cathepsin-D, and renin have functions in digestion, the regulation of blood pressure, and the lysosomal degradation of proteins, respectively. An effective inhibitor of malaria rosetting could potentially also interact with these, causing unwanted side effects when administered pharmaceutically. Activities of **2** and **5** against these proteases were measured in FRET peptide cleavage assays, but no compounds exerted a significant inhibitory effect on renin, even at concentrations up to 1000 $\mu g \cdot mL^{-1}$. Unmodified heparin **1** showed some inhibitory activity against both pepsin and cathepsin-D, with EC_{50}'s of 0.23 and 0.1 $\mu g \cdot mL^{-1}$, respectively, while *N*-acetylated heparin **2** showed a marked decrease in inhibitory activity against both pepsin and renin compared to **1**, and the IC_{50} for **2** against pepsin was 3.27 $\mu g \cdot mL^{-1}$, which was 14-fold less potent than **1**. The IC_{50} for **2** against cathepsin-D was 0.27 $\mu g \cdot mL^{-1}$. Thus, one of the modified forms of heparin exhibiting high levels of rosetting disruption **2** did not significantly inhibit renin and had much lower activities with these proteases than unmodified **1**. Oligosaccharides (dp 8, 10, 12, and 14) derived from **2** and **5** were also substantially weaker inhibitors of cathepsin-D and pepsin than heparin.

Platelet Factor 4 Complex Formation

One of the most serious side effects of heparin therapy is heparin-induced thrombocytopenia (HIT). This involves the formation of antibodies to a complex of heparin and PF4[14] generating an immune reaction that results in platelet activation and increased coagulation, which can lead to venous and arterial thrombosis.[22] The structure of the epitope (or epitopes) formed between PF4 and heparin, the possibility of forming similar complexes between PF4 and various heparin-based structures, and whether such complexes provoke comparable reactions leading to similar side effects were open questions at the outset. To address the first two of these questions, the outcome of interactions between PF4 and the most potent inhibitors of rosetting, **2** and **5**, were compared to those between PF4 and intact heparin **1** in solution using synchrotron radiation circular dichroism spectroscopy (SRCD).[23] This technique, which is many times more sensitive than conventional CD and highly reproducible, can be used to compare the solution conformation of the PF4−heparin complex (which is known to provoke HIT), as well as that formed between PF4 and other effective heparin-derived inhibitors of rosetting, to determine whether structurally similar or distinct complexes were formed.

SRCD spectra were recorded between 190 and 250 nm on the Daresbury CD-12 beamline (Figure 3). The spectrum of PF4 changed following addition of intact heparin **1** forming a complex with different secondary structural characteristics which is known to be immunogenic.[14] The interaction of PF4 with **1** involved a reduction in helices (∼3%) and turns (∼1%), accompanied by increases in strands (∼3%) and unordered structure (∼1%). For the interaction of PF4 with **2** and **5**, the structural changes for **5** were markedly different than those observed with **1**, while for **2** they were still distinct but more similar to **1**. Both structures **2** and **5** were different from each other (Figure 3 and Supporting Information). This suggests that distinct immunogenic responses are likely, especially since the complexes formed between PF4 and either **2** or **5** are structurally different to that formed between PF4 and **1**.

Chemically Modified Heparin Derivatives as Inhibitors of Rosetting

In contrast to one set of findings by Barragan et al.,[10] the rosette formed by the RBCs infected with the R-29 strain were significantly disrupted by **2**, an *N*-acetylated heparin derivative. The size requirement for disruption by heparin in this former work was dp12 (quoted as ∼ 4 kDa), while in the present work, LMW derivatives of **2** (dp 12) and **5** (dp 10) were found to be 68% and 88% effective, respectively, at 0.4 $mg \cdot mL^{-1}$. LMW fragments of **2** and **5** also exhibited only weak anticoagulant activity: dp 8–14 fragments of **2** between 1 and 2% that of intact heparin and of **5** less than 1% that of heparin. The work of Barragan et al.[7,10] employed the strain FCR3S1, which differs from that used in the present work, R-29, highlighting variation between strains regarding the structural requirements for rosette disruption.

The fact that the two sets of oligosaccharides derived by partial enzyme hydrolysis of **2** and **5** showed distinct size requirements and different trends in rosette disruption suggests that they are not inhibiting in exactly the same way and may not involve the same sites or modes of inhibition.

Heparin polysaccharides and oligosaccharides are known to have complex conformational characteristics, in which sulfation and acetylation pattern influence the conformation of uronic acid residues, particularly IdoA2S,[15] as well as altering the geometry of the glycosidic linkages.[16] These factors result in complex overall conformational changes in which appended groups such as hydroxyl, *N*-acetyl, *N*-, *O*-sulfate, and carboxylic acid groups are presented in distinct spatial orientations. The discussion of structure–activity relationships for these molecules in terms of required sulfate groups attached to a supposedly rigid heparin backbone is therefore probably an oversimplification.

The relevance of developing high cost inhibitor compounds to the areas most affected by severe malaria, whose populations are largely unable to afford them, can be questioned on economic as well as ethical grounds. Heparin derivatives as potential disruptors of malaria rosetting, while not fulfilling the requirements of classic small molecule pharmaceutical agents, are attractive in this context for a number of reasons. Heparin derivatives, especially if their production is comparatively straightforward as here, may offer a more accessible alternative. Their size may, in fact, be advantageous because it could allow them to disrupt multiple molecular interactions, which could explain the trend to higher efficacy with increased size. Indeed, it has been shown, for example, that monosaccharides can only disrupt these interactions at very high concentrations.[7]

The most effective modified heparin polysaccharide derivatives of rosetting in this study, **2** and **5**, both exhibited severely attenuated anticoagulant activities, one of the most serious potential side effects of heparin-based pharmaceutical agents. They also had reduced activities with other proteases and formed distinct complexes with PF4, indicating a possible difference in immunogenicity and consequent thrombocytopenia between LMW heparin derivatives and intact heparin. Given the otherwise generally well-tolerated nature of heparin, its wide availability, and its moderate cost, combined with the simplicity of generating these effective inhibitors of rosetting, their application in severe malaria, most likely as an adjunct to established therapies, is an attractive prospect.

Experimental Section

1. Preparation of Modified Heparins. Chemically modified heparin compounds **1**−**8** were prepared by the following combinations of reactions a−f below: (**1**) PIMH starting material (Celsus Laboratories, Cincinnati, OH); (**2**) *N*-acetylheparin (d) (f); (**3**) ido de-2-sulfated heparin (a); (**4**) ido de-2-sulfated, *N*-acetyl heparin (a) (d) (f); (**5**) de-6-sulfated heparin (b) (e); (**6**) 6-*O*-desulfated, *N*-acetylated heparin (b) (f); (**7**) de-6-sulfated, de-2-sulfated heparin (c) (e); (**8**) de-6-sulfated, de-2-sulfated, *N*-acetylheparin (c) (f). Compounds were characterized by ^{1}H and ^{13}C NMR as previously described[24] and in terms of disaccharide compositional analysis by exhaustive digestion with heparitinase I, II, and III followed by separation using HPAEC (Propac PA-1; 4 × 250 mm) with identification against known standards (Supporting Information). The products were desalted, lyophilized, and resuspended in the appropriate buffer prior to assay.

Chemical Reactions. (a) Selective Removal of Iduronate 2-*O*-Sulfate. Selective removal of iduronate 2-*O*-sulfate was achieved as described by Jaseja and Perlin.[25] Note that there is concomitant modification in the small number of *N*- and 3-*O*-sulfated glucosamine units.[20] **(b) Selective Removal of Glucosamine 6-*O*-Sulfate.** Selective removal of glucosamine 6-*O*-sulfate was carried out according to a modification[24] of the method described.[26] **(c) Exhaustive Removal of *O*- and *N*-Sulfates.** Complete removal of *O*- and *N*-sulfates was achieved using solvolytic desulfation by the method described.[26] **(d) Selective de-*N*-sulfation** was carried out employing controlled solvolytic desulfation under kinetic control as described.[27] **(e) Re-*N*-sulfation** was achieved by use of trimethylamine−sulfur trioxide complex as described.[28] **(f) Re-*N*-acetylation** employed acetic anhydride in saturated sodium bicarbonate.

2. NMR Spectroscopy. The effectiveness of chemical treatments was monitored by ^{1}H and ^{13}C NMR at 500 and 125 MHz, respectively (D_2O, 27 °C). Chemical shifts, δ/ppm (external standard), were in full agreement with well-defined model compounds[27] (Supporting Information).

3. Preparation of Sized-Defined LMW Heparin Derivatives. Porcine mucosal heparin (**1**) and selected chemically modified derivatives **2** and **5** were partially digested with 100 mU of heparatinase II (Ibex Technologies Inc., Montreal, Canada) per 100 mg in 100 mM sodium acetate, 0.1 mM calcium acetate, pH 7.0. The digested fragments were separated (in 100 mM ammonium bicarbonate) using gel filtration chromatography (Superdex-30, 2000 mm × 30 mm, Amersham Pharmacia, UK) and their dimensions identified by reference to size-defined authentic standards. Selected LMW fractions were characterized by exhaustive enzymatic hydrolysis (heparitinase I, II, and III) as described above for the polysaccharides, and their composition is shown in the Supporting Information.

4. Rosette Disruption Assay. Cultured parasites were prescreened prior to use, using Giemsa staining, for parasitemia levels of 5–8%. Erythrocytes were pelleted from the culture by centrifugation (1500 rpm, 5 min at r.t.), and spent medium was removed. The parasites were washed by resuspension in prewarmed serum-free complete medium (RPMI 1640 supplemented with 25 mmol of HEPES, 25 mmol of glucose, 10% pooled human serum, 2 mmol of glutamine, and 25 $\mu g \cdot mL^{-1}$ of gentamicin), and the supernatant was removed after centrifugation (1500 rpm, 5 min at r.t.). The pelleted erythrocytes (O^+) were diluted to yield a parasitaemia of 5% in prewarmed (37 °C) growth medium while maintaining 10% hematocrit. The parasite cultures were incubated at 37 °C and stored under an O_2-depleted environment (96% N_2, 3% CO_2, 1% O_2). Infected erythrocytes (and therefore rosettes) were visualized by the addition of ethidium bromide (2 μL of 10 $mg \cdot mL^{-1}$ per milliliter of cell suspension). A rosette was defined as three or more uninfected RBCs attached to an infected RBC. Samples were agitated for 15 min prior to the addition of the test saccharide. A further 15 min agitation was carried out prior to determination of rosetting level using fluorescence microscopy.

5. Anticoagulant Activity. Anti Factor Xa activity was measured against a porcine mucosal heparin (PIMH) standard of known activity (Celsus, Cincinnati, OH) using a diagnostic grade Coatest Heparin test kit (Chromogenix, MA), adapted to a 96-well plate format, reading absorbance at $\lambda = 405$ nm (Polarstar plate reader (BMG LabTechnologies, UK)).

6. Activity against Other Proteases. The ability of compounds to inhibit the structurally related proteases pepsin and cathepsin-D (Sigma, UK) was measured by FRET cleavage assay (5 pmol of enzyme/well, EnzChek Protease Assay kit (Molecular Probes, UK) according to manufacturer's instructions). Activity against human recombinant renin (Cayman Chemical, Ann Arbor, MI), was measured by FRET peptide cleavage assay (0.08 pmol enzyme/well), using the Renin Substrate 1 (Molecular Probes, Invitrogen, UK) according to manufacturer's instructions.

7. Synchrotron Source Circular Dichroism. SRCD spectra were recorded on the CD-12 beamline at the Daresbury Laboratory, a purpose built SRCD facility, using quartz sample cell (0.02 cm path length, 1 nm resolution), between $\lambda = 260$ and 190 nm. CD spectra of PF4 samples were recorded at 1 $mg \cdot mL^{-1}$ (in the presence or absence of a molar equivalent of heparin/heparin derivatives) and are relative to (+)-10-camphorsulfonic acid (1.0 $mg \cdot mL^{-1}$). CD values are presented as molar circular dichroism

($mol^{-1} \cdot cm^{-1}$) and have been corrected to allow for differences in molecular weight according to composition analysis.[24]

Acknowledgment. We thank the Wellcome Trust (M.A.S., S.E.G., A.G.C., and E.A.Y.) and the Biotechnology and Biological Sciences Research Council for project grants (S.E.G., T.R.R., E.A.E., J.E.T., and E.A.Y.) and a studentship to M.A.S. The Medical Research Council is thanked for a Senior Fellowship to J.E.T. and a studentship to A.D.V. Alan Brown and David Clarke at the Daresbury Laboratory are also thanked for assistance in obtaining SRCD spectra.

Supporting Information Available: NMR characterization, information on the purity of the polysaccharides, and analysis of the secondary structural changes in solution of PF4 and selected polysaccharides used in this study are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM701337T