Interactions with heparin-like molecules during erythrocyte invasion by *P. falciparum* merozoites

Michelle J. Boyle¹,², Jack S. Richards¹,², Paul R. Gilson³, Wengang Chai⁴, James G. Beeson¹

¹The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050, Australia,
²Department of Medical Biology, University of Melbourne, Victoria, Australia ³Macfarlane Burnet Institute for Medical Research and Public Health, Melbourne, Victoria 3004, Australia, and
⁴Glycosciences Laboratory, Imperial College London, Northwick Park Hospital & St Marks Campus, Harrow, Middlesex, UK,

Corresponding author:

James Beeson

The Walter and Eliza Hall Institute of Medical Research

1G Royal Parade, Parkville, Victoria 3050, Australia

Ph: +61-3-9345-2555

Fax +61-3-9347-0842

beeson@wehi.edu.au

Short title: Heparin interactions in *P. falciparum* invasion

Category: Red Cells, Iron and Erythropoiesis
ABSTRACT:

During erythrocyte invasion Plasmodium falciparum merozoites use multiple receptor-ligand interactions in a series of coordinated events, but current knowledge of these interactions is limited. Using real-time imaging of invasion, we established that heparin-like molecules block early, and essential, events in erythrocyte invasion by merozoites. All P. falciparum isolates tested, and parasites using different invasion pathways, were inhibited to comparable levels. Furthermore, it was not possible to select for heparin-resistant parasites. Heparin-like molecules occur naturally on the surface of human erythrocytes, where they may act as receptors for binding of merozoite surface proteins. Consistent with this, we demonstrated that MSP1-42, a processed form of merozoite surface protein 1 (MSP1) involved in invasion, bound heparin in a specific manner; furthermore, binding was observed with the secondary processing fragment MSP1-33, but not MSP1-19. We defined key structural requirements of heparin-like molecules for invasion inhibition and interactions with MSP1-42. Optimal activity required a degree of sulfation ≥2, disulfation of the N-acetylglucosamine or hexuronic acid residue, and a minimum chain length of 6 monosaccharides. These findings have significant implications for understanding P. falciparum invasion of erythrocytes, and the development of novel therapeutics and vaccines.
ABBREVIATIONS:

MSP1 – merozoite surface protein 1

AMA1 – apical membrane antigen 1

PfRh – *P. falciparum* reticulocyte binding homologues

HS – heparan sulfate

GlcA – glucuronic acid

GlcNAc – N-acetyl glucosamine

IdoA – iduronic acid

K5-NS – N-sulfated K5 polysaccharide

K5-OS-L – low level O-sulfated K5 polysaccharide

K5-OS-H - high level O-sulfated K5 polysaccharide

K5-NSOS-L - low level N- and O-sulfated K5 polysaccharide

K5-NSOS-H – high level N- and O-sulfated K5 polysaccharide

EK5-NSOS-L - low level N- and O-sulfated epimerized K5 polysaccharide

EK5-NSOS-H - high level N- and O-sulfated epimerized K5 polysaccharide

EBA – erythrocyte binding antigen

CSC - chondroitin sulfate C

HexA – hexuronic acid
INTRODUCTION:

*Plasmodium falciparum* causes the majority of malaria morbidity and mortality world-wide and is a leading cause of childhood deaths\(^1\). The pathogenic processes of malaria occur during blood-stage infection when merozoites invade erythrocytes using multiple receptor-ligand interactions and replicate inside them. Antigens on the merozoite surface, such as merozoite surface protein 1 (MSP1)\(^2\), are thought to mediate initial attachment, followed by apical reorientation involving apical membrane antigen 1 (AMA1)\(^3\) and possibly other antigens. Proteins contained within apical organelles, such as erythrocyte binding antigens (EBAs) and *P. falciparum* reticulocyte-binding homologues (PfRh), are also released to form a tight junction between the merozoite and erythrocyte membrane. Following this the merozoite is propelled into the erythrocyte by an actin-myosin-like motor\(^2\). Very few receptor-ligand interactions involved in invasion events, particularly early events, have been defined. Furthermore, specific functions have been determined for only a small number of merozoite antigens. This limited knowledge has restricted the development of approaches to block essential interactions with vaccine-induced antibodies or molecules that could be used therapeutically.

MSP1 appears to be essential for erythrocyte invasion and involved in initial attachment of merozoites to erythrocytes. MSP1 exists as a high molecular mass protein (Mr ~180 kDa) and is proteolytically processed into 83kDa-, 30kDa-, 38kDa- and C-terminal 42kDa- (MSP1-42) fragments\(^4\) just prior to egress from the schizont by protease PfSUB1\(^5\). These remain non-covalently associated on the merozoite surface, together with MSP6 and MSP7\(^6,7\). During invasion, PfSUB2 further processes MSP1-42 into MSP1-19 and MSP1-33 fragments\(^8\). The latter protein along with its non-covalently linked binding partners is shed during invasion. The short C-terminal MSP1-19 fragment is anchored to the merozoite surface via a surface-expressed glycosylphosphatidyl inositol anchor and is carried inside the erythrocyte\(^9\). While the reason for MSP1 processing remains poorly understood, primary and secondary processing is thought to be essential for MSP1 function and invasion because MSP1-42 and
MSP1-19 specific antibodies and inhibitors that block processing also inhibit invasion\textsuperscript{10,11}. MSP1-19 may function by binding to erythrocyte membrane protein band 3\textsuperscript{12}. Heparin and other sulfated polysaccharides such as pentosan polysulfate, dextran sulfate, curdlan sulfate, and fucoidan can inhibit blood-stage growth of \textit{P. falciparum in vitro} and fucoidan and dextran sulfate have anti-malarial activity in mouse models of malaria\textsuperscript{13-17}. Heparan sulfate (HS) proteoglycan (structurally very similar to heparin) has been identified on the surface of erythrocytes\textsuperscript{18}. This suggests a potential role for heparin-like molecules in invasion by \textit{P. falciparum} merozoites; however, specific interactions have not been demonstrated and the mechanism of action of heparin remains unknown. Other stages of the \textit{P. falciparum} life-cycle, such as sporozoites, utilize heparin-like molecules as cell surface receptors for invasion\textsuperscript{19}. Heparin and HS, are members of the glycosaminoglycan family and consist of repeating disaccharide units of $\beta$-glucuronic acid (GlcA) and $\alpha$-N-acetylglucosamine (GlcNAc). During biosynthesis, linear polysaccharide chains are modified with N-deacetylation/N-sulfation, epimerization of GlcA to iduronic acid (IdoA) and O-sulfation at different positions (reviewed\textsuperscript{20}). There is considerable heterogeneity and structural diversity in polysaccharides generated by varying levels and patterns of sulfation and degree of epimerization. Heparin and HS differ in level of sulfation and proportion of GlcA and IdoA. K5 polysaccharides are heparin-like molecules isolated from the capsule of \textit{Escherichia coli} K5\textsuperscript{21}. They have the same backbone structure as heparin and HS [\(\rightarrow\text{4-D-GlcA-(1}\rightarrow\text{4)-D-GlcNAc-}\text{1(1}\rightarrow\text{]}\text{n}\) (reviewed\textsuperscript{22}) and can be used for the generation of semi-synthetic heparin-like polysaccharides\textsuperscript{23}. Modified K5 polysaccharides have defined levels and patterns of sulfation that facilitate studies to characterize the structural requirements of heparin/HS for specific biological activities.

Here, we identify cellular and molecular interactions with heparin-like molecules during erythrocyte invasion by \textit{P. falciparum} merozoites. We found that heparin-like molecules primarily act by inhibiting early and essential events in erythrocyte invasion. MSP1-42 and MSP1-33 can bind heparin,
suggesting the use of heparin-like molecules as receptors for invasion. Furthermore, we determined the
dkey structural requirements of heparin-like molecules for invasion-inhibitory activity and interaction with
MSP1-42.
MATERIALS AND METHODS:

Reagents

Heparin (porcine intestinal mucosa), de-N-sulfated heparin (re-N-acetylated), de-N- and de-O-sulfated heparin (re-N-acetylated), chondroitin sulfate C (CSC), heparin-agarose beads, heparin conjugated to BSA (heparin-BSA), and lactoferrin were obtained from Sigma-Aldrich (Sydney, Australia). Partially de-N-sulfated (re-N-acetylated) heparin, de-6-O-sulfated heparin, de-2-O-sulfated heparin, K5 polysaccharides derivatives with N-sulfation (K5-NS), low and high level of O-sulfation (K5-OS-L and K5-OS-H), low and high level of N- and O-sulfation (K5-NSOS-L and K5-NSOS-H), and epimerized K5 (EK5) polysaccharide derivatives with low and high level of N- and O-sulfation (EK5-NSOS-L and EK5-NSOS-H) were obtained from Iduron (Paterson Institute for Cancer Research, University of Manchester, UK). Sheep anti-rabbit-HRP and sheep anti-mouse-HRP antibodies were purchased from Chemicon (Australia). Rabbit anti-bovine albumin (BSA) was obtained for Sigma-Aldrich (Sydney, Australia).

Antibodies used include rabbit antibodies to MSP1-19 (recombinant *P. falciparum* 3D7 MSP1-19 protein expressed in *E. coli* and purified by nickel affinity chromatography), EBA175 and MSP3, (from Alan Cowman, Walter and Eliza Hall Institute), MSP1-33 (Mike Blackman, National Institute of Medical Research), AMA1 (Kerstin Leykauf and Brendan Crabb, Burnet Insitute), MSP4 (Ross Coppel, Monash University). Recombinant MSP1-42 and AMA1 (expressed as his-tagged proteins in *E. coli*1) were provided by Carole Long, NIH, and, Robin Anders, La Trobe University; neuraminidase (*Vibrio cholerae*) was from Calbiochem (Merck, Australia), and chymotrypsin and trypsin were from Worthington Biochemical Corporation (NJ, USA).
Heparin and chondroitin sulfate C oligosaccharide fragments

Heparin and CSC oligosaccharides were prepared and characterized as described\textsuperscript{27,28}. Heparin was partially digested heparinase I and CSC (200 mg) was partially digested with chondroitinase ABC. Fractionation of heparin oligosaccharides was performed with a Bio-GelP-6 column (1.6 × 90 cm), elution by 0.2 M NH₄Cl (pH 3.5). CSC oligosaccharides were fractionated on a Bio-GelP-4 column (1.6 × 90 cm), elution by 0.2 M ammonium acetate. Oligosaccharide fractions were desalted and quantified by carbazole assay\textsuperscript{29}. Oligosaccharides were re-suspended in phosphate-buffered saline (PBS) and filter sterilized before testing in growth inhibition assays.

Parasite culture and growth inhibition assays

\textit{P. falciparum} isolates were cultured as described\textsuperscript{30}, and genetic identity of isolates was confirmed by sequencing the \textit{ama1} gene. Genetic knock out lines lacking Rh1, Rh2a, EBA140, EBA175, EBA181 and MSP3 were kindly provided by Alan Cowman\textsuperscript{2}. Heparin-like molecules (Table 1) were tested for growth-inhibitory activity against \textit{P. falciparum} parasites using established assays\textsuperscript{30}. Samples were tested for inhibitory activity in duplicate, and cultures were incubated for 40 hours and parasitemia determined by flow cytometry (FACSCalibur; Becton-Dickinson). The inhibitory effects of molecules were expressed as percent of growth compared to controls (PBS) for each experiment. Enzyme treatment of erythrocytes\textsuperscript{31} was performed with neuraminidase (67 µM, 15 minutes), chymotrypsin (1 mg/ml, 45 minutes) or trypsin (100 µg/ml, 45 minutes) at 37°C, then washed, before use in growth inhibition assays. For live filming of erythrocyte invasion, parasites at 5-10% schizont stage were maintained in culture medium at 37°C, 6% CO₂ with humidification\textsuperscript{32}. Only definite merozoite invasion events accompanied by erythrocyte deformation, were counted.
Binding of native parasite proteins to heparin-agarose beads

Proteins were extracted from *P. falciparum* schizonts into 1% Triton X100 (TX100) in PBS as described\textsuperscript{33}. Proteins from culture supernatants were collected by allowing highly synchronous schizonts to rupture into protein free culture medium and cells removed by centrifugation. Heparin–agarose beads were washed twice in PBS, then blocked with 1% casein PBS overnight, 4°C. Schizont protein extracts were incubated overnight at 4°C with beads containing 0.1% casein and 200 µg/ml of test inhibitor, or PBS control (50 µl of packed beads plus 100 µl of protein supernatant). Unbound proteins in the supernatant were collected through Micro Bio-Spin Chromatography Columns (Bio-Rad) and beads washed five times with PBS containing 0.1% casein, 1% Triton X-100 and protease inhibitors. Bound proteins were eluted from beads with 50 µl of warmed reducing SDS-PAGE sample buffer. Bound and unbound proteins were separated by SDS–PAGE under reducing conditions and Western blotted onto PVDF membranes for probing with antibodies to detect MSP1, MSP3, MSP4, AMA1 or EBA175.

**MSP1 processing inhibition assay**

MSP1 processing inhibition assay was performed as described previously for AMA1 processing inhibition\textsuperscript{34}. Schizonts were purified by passing synchronized mature trophozoites (~38 hours post invasion) through MACS columns to remove uninfected erythrocytes, followed by further culture to mature parasites to the schizont stage. Purified schizonts at 10^7/ml were incubated for >12 hours until rupture had completed, with or without the addition of ethylenediaminetetraacetic acid (EDTA) 1 mM or heparin 1 mg/ml. Merozoites were collected by centrifugation at 10,000 g for 10 minutes and washed in cold PBS containing protease inhibitors. Proteins were extracted with non-reducing SDS-PAGE sample buffer, freeze thawed and sonicated, and used in Western blots.
**Heparin-BSA binding assay**

Recombinant merozoite antigens were coated (1 µg/ml) onto 96-well plates (Nunc Maxisorb) in PBS overnight at 4°C. Plates were washed and blocked with 1% casein, then incubated with heparin-BSA or BSA. Plates were washed, and bound heparin-BSA/BSA was detected with anti-BSA antibodies (rabbit, Sigma-Aldrich), followed by anti-rabbit–HRP, and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (Sigma-Aldrich). All incubations were performed in PBS with 0.1% casein and 0.05% Tween20, 1 hour at room temperature.
RESULTS:

**Heparin inhibits initial contact and reorientation of merozoites during erythrocyte invasion**

Previous studies suggested that heparin may act by inhibiting schizont rupture\(^{13,16}\) and/or merozoite invasion\(^{17,35}\). To define the mechanism of action of heparin and heparin-like molecules we performed time-course studies of schizont rupture, merozoite invasion and intra-erythrocytic development of *P. falciparum* in the presence of heparin. Parasite stages were evaluated using flow-cytometry, differentiating between late stage trophozoites, schizonts, and ring-stage parasites with ethidium bromide staining. In the presence of heparin (100 μg/ml), there was evidence of some delay, but not complete inhibition of schizont rupture compared to control (Figure 1A). In contrast, rates of merozoite invasion and ring formation were very low, suggesting heparin acts primarily by blocking merozoite invasion (Figure 1B). No inhibition of intra-erythrocytic growth was observed (data not shown).

The mechanism of action of heparin was further defined using real-time microscopy of invasion. In control parasite cultures, schizont rupture and step-wise invasion of erythrocytes by merozoites was readily observed as reported previously\(^{32,36-38}\) (Figure 1C; Supplementary Figure S1A). Briefly, we observed initial merozoite contact with the erythrocyte surface resulting in its rapid and brief oscillatory deformation, followed by merozoite reorientation and internalization. Subsequently the erythrocyte underwent deformation, or echinocytosis, followed by a gradual recovery of its biconcave shape over several minutes. In the presence of heparin (100 μg/ml) schizont rupture occurred and merozoites appeared to disperse normally, further indicating that heparin does not act by inhibiting schizont rupture. Initial contact between merozoites and the erythrocyte surface was also observed; however, no oscillatory deformation of the erythrocyte was seen and contact between erythrocytes and merozoites was not sustained with merozoites dissociating after various lengths of time (Figure 1D; supplementary figure S1B). No successful invasion events were observed; additionally, reorientation of merozoites and
echinocytosis of erythrocytes were not clearly seen. The total filming time of control parasites was 5442 seconds covering 13 separate schizont rupture events, with 21 confirmed invasion events. Total filming time of cultures with heparin was 4529 seconds over 15 separate events, with no invasion events. These studies clearly established that heparin acts early in invasion by inhibiting the ability of the merozoite to sustain contact with the erythrocyte surface.

**Heparin inhibits essential erythrocyte invasion events**

Heparin inhibited six genetically different lines tested (3D7, W2mef, D10, E8B, XIE, HCS3) (data not shown). *P. falciparum* is known to use different pathways for invasion of erythrocytes, determined by different receptor-ligand interactions. Pathways can be partially defined by their sensitivity to enzyme treatment (e.g., neuraminidase, chymotrypsin, and trypsin) of erythrocyte surface receptors and can be broadly classified into two groups based on the use of sialic acid (SA) on the erythrocyte surface by parasite ligands. Parasite lines W2mef and 3D7 were used as representative of SA-dependent and SA-independent invasion phenotypes respectively, and were equally inhibited by heparin, while the related compound CSC was non-inhibitory (Figure 2A). We next measured inhibition of merozoite invasion by heparin using erythrocytes treated with enzymes to selectively remove subsets of erythrocyte invasion receptors. With 3D7 parasites the inhibitory activity of heparin was equivalent when tested for inhibition of merozoite invasion into neuraminidase-, trypsin-, or chymotrypsin-treated, compared to untreated erythrocytes (Figure 2B), suggesting heparin inhibits essential step(s) common to all invasion pathways. Heparin also gave similar inhibition of invasion of W2mef into chymotrypsin and trypsin treated erythrocytes (Figure 2C). Of note, heparin effectively inhibited invasion of merozoites into neuraminidase-treated erythrocytes in both 3D7wt (Figure 2B) and by W2mefEBA175KO (data not shown) parasites, indicating that heparin does not act by interfering with the binding of parasite ligands to
negatively-charged SA on the erythrocyte surface.

In the presence of 100 µg/ml of heparin, very low parasitaemias were detected after one cycle of replication. It was possible that this represented parasites that were invading utilizing an alternative pathway resistant to heparin inhibition. To test this possibility, we re-cultured parasites (3D7) that were present after a 48-hour incubation with heparin until a high parasitemia was obtained, and these parasite were again treated with heparin for 48 hours and surviving parasites were re-cultured. When the parent and the heparin-selected (1 and 2 cycles of selection) parasites were tested in growth-inhibition assays, the sensitivity to heparin was found to be the same; there was no evidence that we were able to select for increased resistance to heparin reflecting parasites using an alternative invasion pathway (Figure 2D). Furthermore, higher concentrations of heparin completely inhibited invasion (data not shown).

**MSP1-42 and MSP1-33 bind to heparin**

Having shown that heparin inhibited essential and early interactions of merozoite invasion, we next sought to identify merozoite antigens that bind to heparin during invasion. The major merozoite surface protein, MSP1 was considered a likely target as this protein is thought to be essential for invasion and involved in initial invasion events. Protein extracts of *P. falciparum* schizonts (3D7 line) containing MSP1 were incubated with heparin-agarose beads and bound proteins were eluted from beads and identified by Western blotting. Using this approach we consistently identified, in repeat experiments, a MSP1 positive band in the bead-eluted fraction that had a *Mr* of ~40kDa; this represents MSP1-42 (Figure 3A, PBS control). The same band was readily detected in protein extracts prior to incubation with beads (data not shown), but was greatly depleted from the supernatant after incubation with heparin-agarose beads. The binding of MSP1-42 to heparin-agarose beads was effectively inhibited by soluble
heparin, but not by the related glycosaminoglycan chondroitin sulfate C (CSC), which does not inhibit erythrocyte invasion; this suggested that binding was specific. In contrast, full-length MSP1 and MSP1-19 were detected in schizont protein extracts, but there was little or no detectable protein in fractions eluted from beads, and little evidence of depletion from the supernatant following incubation with beads, suggesting they do not bind heparin (Figure 3A/B). MSP1 exists as two main allelic forms, 3D7 and FVO. Sequence analysis showed that both contain several putative heparin-binding-like motifs represented by xBBxBx, whereby B is a basic residue and x is a hydrophobic residue (Supplementary Figure S2A). Furthermore, proteins extracted from parasites with the FVO allelic-type (E8B isolate) also bound heparin in a specific manner (Supplementary Figure S2B).

Around the time of invasion into the erythrocyte, MSP1-42 is further processed with shedding of MSP1-33 from the merozoite surface. To investigate whether the MSP1-33 fragment could bind heparin, culture supernatants containing MSP1-33 were incubated with heparin-agarose beads. Like MSP1-42, MSP1-33 was consistently found in the bead-eluted bound fraction, while depleted from the culture supernatant. Binding was inhibited with heparin (Figure 3B). Several other merozoite proteins, extracted from schizonts, did not appear to bind to heparin. EBA175, did not bind heparin-agarose beads and was consistently observed in the supernatant after incubation of schizont protein extracts with beads (Figure 3C). EBA175 binds to SAs, which are also negatively charged like heparin. MSP3 and MSP4 did not bind to heparin-agarose beads and were found only in the supernatant (Figure 3C). Furthermore, heparin inhibited a D10-MSP3-knock-out line to the same extent as parental D10 (data not shown); this line also has a disrupted function of MSP9(ABRA). AMA1 did not bind heparin in a specific manner; although some AMA1 was found eluted from beads, this binding was non-specific as it was not inhibited by soluble heparin (Figure 3C). Parasite isolates with genetic disruption of EBA140, EBA175, EBA181, PfRh1, and PfRh2a were inhibited by heparin at the same level as the parental isolate (data not shown).
To test whether heparin may act by inhibiting secondary processing of MSP1-42 to MSP1-33 and MSP1-19, purified schizonts were incubated with heparin at a concentration in excess of that known to be inhibitory (1mg/ml) and we evaluated levels of MSP1-42 and MSP1-19 in merozoites following schizont rupture compared to controls. Heparin appeared unable to inhibit MSP1-42 processing to MSP1-19; there were comparable amounts of MSP1-42 and MSP1-19 seen in heparin (1mg/ml) and control treated samples (Figure 3D). EDTA (positive control) inhibited processing as expected41.

Binding of MSP1-42 to heparin was further examined by testing binding of recombinant MSP1-42 and MSP1-19 to heparin conjugated to BSA (heparin-BSA). BSA alone was used as a negative control and lactoferrin, a known heparin-binding protein, as a positive control. Recombinant MSP1-42, but not MSP1-19 or AMA1, had significant heparin-binding activity (Figure 4A). The binding of lactoferrin to heparin-BSA was dose-dependent and saturated at concentrations of heparin-BSA above 2.5 µg/ml, and binding was inhibited by heparin, but not CSC (data not shown); this validated the use of this assay in studying heparin-binding interactions. Binding of heparin-BSA to MSP1-42 was similarly dose-dependent and saturated with a heparin-BSA concentration of 1 µg/ml (Figure 4B). MSP1-42 binding was inhibited in a concentration-dependent manner by heparin, but not by CSC (Figure 4C).

The level and pattern of sulfation and chain length of heparin-like molecules determine their invasion inhibitory activity

To further understand the molecular basis of interactions with heparin-like molecules during merozoite invasion of erythrocytes, a range of heparin-like-molecules with defined structural features and oligosaccharides of defined length were tested as inhibitors of invasion (Figure 5/Table 1).

Testing variously desulfated heparins indicated the importance of both N- and O- sulfation for P.
*falciparum* inhibitory activity. Completely de-sulfated (de-N/O-sulfated; degree of sulfation (d.o.s.=0) and de-N-sulfated heparin (d.o.s.=1.8) had no activity, and partially de-N-sulfated heparin (d.o.s.=2.5), de-6-O-sulfated (d.o.s.=1.6) and de-2-O-sulfated (d.o.s.=1.9) had reduced inhibitory activity (IC$_{50}$ 74, 373 and 123µg/ml respectively) (Table 1, Figure 6A). These results show the importance of both N and O sulfation for maximum inhibitory activity. Comparison of fully de-N-sulfated heparin (d.o.s.=1.8; IC$_{50}$ >>> 100 µg/ml) and partially de-N-sulfated heparin (d.o.s.=2.5; IC$_{50}$ 74 µg/ml) further indicated the importance of sulfation level for inhibition, with higher activity observed with an increasing sulfate content. De-2-O-sulfated heparin had greater inhibitory activity than de-6-O-sulfated heparin which may reflect their differences in sulfate content (d.o.s.=1.9 and 1.6, respectively) or a preference for 6-O- rather than 2-O-sulfation for activity.

To further understand the relative importance of N- versus O-sulfation, sulfation pattern, and the overall level of sulfation modified K5 polysaccharides with known sulfation levels and patterns were tested for inhibitory activity (Figure 5, Table 1). K5 polysaccharides with N-sulfation only (K5-NS; d.o.s.=1) or low levels of O-sulfation (K5-OS-L; d.o.s.=1.4) had no growth inhibitory activity (Figure 6B). In contrast, highly O-sulfated K5 (K5-OS-H; d.o.s.=3) had significant activity (IC$_{50}$ µg/ml) despite lacking N-sulfation. This indicates that N-sulfation is not an absolute requirement for activity, in contrast to the prior finding of the lack of inhibitory activity of de-N-sulfated heparin (Figure 6A)$^{42}$. K5 polysaccharides with both N- and O-sulfation (K5-NSOS-L; d.o.s.=2, and K5-NSOS-H; d.o.s.=3.7) had substantial activity (IC$_{50}$ 55.7 and 7.4 µg/ml, respectively) (Figure 6C). K5-NSOS-H was the most inhibitory of all molecules tested. The greater inhibitory activity of K5-NSOS-H compared to K5-NSOS-L suggest higher levels of sulfation enhance activity. The greater inhibition by K5-NSOS-H compared to K5-OS-H, which have similar O-sulfation levels, suggests that the presence of N-sulfate groups increases inhibitory activity.
K5 polysaccharide contains GlcA only, whereas heparin contains mainly IdoA; the inhibitory activity of sulfated K5 polysaccharides suggested that IdoA was not essential for activity. To investigate this we tested epimerized K5 (EK5) polysaccharide derivatives. EK5-NSOS-L and EK-NSOS-H were prepared from K5-NSOS-L and K5-NSOS-H respectively, and contain an estimated 50:50 of IdoA to GlcA but maintain the same sulfation level (supplier’s information) (Table 1). Both epimerized K5 derivatives had significantly less inhibitory activity compared to their parent GlcA-containing polysaccharides (Figure 6C, Table 1). This suggests that IdoA is not required for optimal activity and there is a preference for GlcA.

To further understand the importance of sulfation level and pattern for inhibitory activity of heparin-like-molecules we compared the inhibitory activity of molecules to their d.o.s. (Figure 6D, Table 1). This showed that inhibitory activity requires a threshold level of sulfation of d.o.s.≥2, with a correlation between d.o.s. and IC50 (Spearmans rho= -0.87 p=0.0001). However, there was no significant correlation between sulfation level and IC50 if only analyzing inhibitory molecules with IC50<400 (Spearmans rho= -0.63, p=0.1); this suggests that sulfation pattern is important for activity and not just the overall level of sulfation. Comparing the inhibitory activity of different molecules (Table 1) suggests that sulfation at both N and O positions is not an absolute requirement but that sulfate groups must be spatially positioned on the same monosaccharide residue (i.e. HexA or GlcNAc) of each disaccharide for optimal activity. Inhibitory molecules (heparin, K5-OS-H, K5-NSOS-L, and K5-NSOS-H) have this property, whereas less-inhibitory molecules (de-2-O-sulfated heparin, de-N-sulfated heparin, and K5-OS-L) do not.

To investigate the importance of chain length for inhibitory activity, heparin and CSC oligosaccharides (2 to 16 residues in length) were tested for inhibition of P. falciparum growth. A minimum of 6 monosaccharide units of heparin was needed for inhibitory activity (Figure 6E), and 8mer or higher oligosaccharides had greater activity (IC50 79, 67, and 67µg/ml, for 6mer, 8mer, and 10mer, respectively,
data not shown). CSC oligosaccharides had little or no inhibitory activity compared to heparin oligosaccharides of the same length.

**Structural features of heparin-like molecules for binding MSP1-42**

To understand the structural requirements of heparin for binding to MSP1-42, modified heparins and K5 polysaccharides were tested for inhibition of MSP1-42 binding to heparin. Binding of MSP1-42 was not inhibited by de-N-sulfated heparin or de-6-O-sulfated heparin (Figure 7), demonstrating a dependence on sulfation for binding. Binding of MSP1-42 was inhibited by soluble K5-NSOS-H and to a lesser degree by K5-OS-H. There was very little or no inhibition of binding by K5-NSOS-L, EK5-NSOS-L, or EK5-NSOS-H (Figure 7). MSP1-42 binding to heparin is therefore not only dependent on level of sulfation, but also the pattern of sulfation, with specific spatial positions of sulfate groups likely to be involved in binding. The binding-inhibition activity of the polysaccharides closely reflected their activity in cellular inhibition assays further suggesting that heparin binding to MSP1-42 is likely to be, at least in part, mediating the growth-inhibitory effect of heparin and heparin-like molecules.
DISCUSSION:

In this study we have demonstrated that heparin-like molecules inhibit early and essential events in invasion. We have defined their timing and mechanism of action, and established that a processed form of the major merozoite protein MSP1 has heparin-binding activity and appears to be the target of heparin-like molecules in mediating invasion inhibition. Additionally, we have determined key structural features of heparin-like molecules required for inhibitory activity and binding MSP1-42.

We clearly demonstrated that heparin inhibits the early events in invasion using live video microscopy. Over multiple experiments we observed merozoites make contact with erythrocytes in the presence of heparin; however, this contact was reversible and not sustained. We never observed successful invasion or events downstream of initial attachment, such as apical reorientation or erythrocyte deformation. This inhibitory phenotype was clearly distinct to the phenotype recently described for inhibition of AMA1 function, where initial oscillatory deformation of the erythrocyte, merozoite reorientation and echinocytosis were observed without successful invasion. Tracking the effect of heparin on schizont rupture and ring formation further supported the observation that heparin-like molecules act by inhibiting erythrocyte invasion. Previously, it was reported that heparin may act by blocking schizont rupture. Although we showed that heparin can cause some delay in schizont rupture, the modest effect observed was not sufficient to explain inhibition of parasite replication.

Several observations suggest that heparin-like molecules inhibit essential invasion events. The identification of essential interactions during invasion is important when considering potential targets for drug and vaccine development. Of further significance, heparin inhibited parasite isolates using different invasion pathways. The ability of P. falciparum isolates to use alternative invasion pathways appears to be mediated by differential expression and use of EBA and PfRh ligands (reviewed) and is thought to be one mechanism of immune invasion employed by P. falciparum. These findings suggest that the EBA
and PfRh proteins are not the major targets of heparin-like molecules and that the inhibitory activity of heparin is not mediated by blocking the binding of EBAs to negatively charged SA residues on erythrocyte receptors. EBA and PfRh ligands are thought to play a role in tight junction formation (reviewed\(^2\)), an event downstream of initial attachment and reorientation; therefore, the timing and mechanism of action of heparin inhibition is not consistent with it acting by inhibiting the function of EBAs and PfRhs. Furthermore, parasites lacking specific EBA and PfRh genes were inhibited to the same degree as parental parasites.

Consistent with the timing and mechanism of action of heparin-mediated inhibition of invasion, we found that MSP1-42, a processed form of MSP1, bound to heparin in a specific manner. Other merozoite antigens MSP3, MSP4, AMA1 and EBA175 did not bind. Only MSP1-42 and not full length MSP1 or MSP1-19 bound heparin, suggesting that primary processing of MSP1 is required for binding activity. MSP1-33 also bound heparin suggesting that the binding of MSP1-42 to heparin is at least partly mediated through this region. MSP1 is known to exist in complex with other antigens MSP6\(^44\) and MSP7\(^45\). Although this study has not formally excluded the role of these antigens in the binding of MSP1-42 to heparin, if MSP6 or MSP7 was predominately mediating the binding of MSP1 to heparin it would be expected that full-length MSP1 would bind to heparin to similar degrees, which it did not. Furthermore, the binding of MSP1-42 was confirmed using recombinant MSP1-42. Further studies testing inhibition of heparin binding using antibodies to different MSP1 constructs and alleles may aid the identification of specific interactions and could be valuable in informing vaccine development. Heparin-like molecules are known to be present on the surface of reticulocytes and erythrocytes\(^18\) where they may be bound by MSP1-42 during invasion. Our findings suggests a model in which the primary processing of full length MSP1 to generate MSP1-42 allows the correct conformation for binding to heparin-like molecules on erythrocytes. Upon invasion, secondary processing would disrupt binding from this receptor by shedding MSP1-33, and MSP1-19 would be carried into the erythrocyte possibly bound to band 3. Multiple studies
suggest MSP1 is essential for invasion, and is consistent with our findings that heparin-like molecules inhibit essential interactions during invasion. MSP1-42 remains an important target of vaccine development. Further characterization of the interaction between heparin and MSP1-42 to identify a specific binding site may be valuable in vaccine approaches to generate antibodies with potent inhibitory activity. There may be other interactions between merozoite proteins and heparin-like molecules that are also important during invasion. It was recently reported that PfRh5 and BAEBL/EBA140 have some heparin-binding activity. However, further studies are needed to confirm the specificity and relevance of these interactions, and EBA140 appears to function predominantly by binding glycophorin C. Live imaging revealed that inhibition of invasion by heparin occurred prior to tight-junction formation when the EBAs and PfRh proteins are thought to act. Furthermore, EBA140-knock-out parasite lines showed comparable and complete inhibition by heparin in our studies. Binding of surface antigens may help to establish initial contact, and then binding of other ligands, possibly PfRh5 or EBA140, may be involved in subsequent invasion steps. While these ligands may play some role in the inhibitory activity of heparin, it seems likely that the major inhibitory activity occurs at initial contact steps.

Our studies clearly show that the overall level of sulfation is crucial for the inhibitory activity of heparin-like molecules and a d.o.s. ≥ 2 (i.e. 2 or more sulfate groups per disaccharide unit) is required for substantial activity. The pattern of sulfation is also important and comparing inhibitory and non-inhibitory molecules suggests that sulfate groups need to be present on the same monosaccharide residue of each disaccharide unit for optimal activity. Furthermore, a chain length of ≥ 6 monosaccharide units was the minimum length required for substantial activity and there is a preference for GlcA rather than IdoA. GlcA is thought to convey greater rigidity to the polysaccharide structure than IdoA.

The identification of highly inhibitory molecules may be useful in the development of therapeutic inhibitors; glycosaminoglycans and sulfated polysaccharides (e.g. heparin, chondroitin sulfate, pentosan
polysulfate) are used clinically in other diseases. Whereas polysaccharides are highly heterogeneous, it is technically possible to prepare oligosaccharides with defined compositions that may have much greater activity. This approach using structure/function relationships as the basis for drug design was successful in the development of a pentasaccharide anticoagulant fondaparinux, based on the antithrombin-binding motif of heparin. Heparin is used clinically as an anticoagulant; however, the structural requirements for anticoagulant activity differ from those for optimal antimalarial activity identified here. The most inhibitory molecule we identified, K5-NSOS-H, has little anticoagulant activity. Additionally, IdoA is required for optimal anticoagulant activity, whereas antimalarial activity was greater with GlcA.

Presently, all licensed antimalarials act by inhibiting the intra-erythrocytic development of parasites. Our studies demonstrate the potential for therapeutic approaches that target merozoite invasion.

In conclusion, we have clearly identified the mechanism and timing of inhibitory action of heparin-like molecules, determined key structural features for activity and identified MSP1-42 as a heparin-binding protein and the likely target of inhibitory molecules. These findings have significant implications for understanding the mechanisms of P. falciparum invasion of erythrocytes, identifying parasite proteins that could be targets of novel therapies or vaccines and developing novel antimalarials inhibitors based on the structure and activity of heparin-like molecules.
ACKNOWLEDGEMENTS: We wish to thank Dr. Carole Long for providing recombinant MSP1-42 protein, Dr. Robin Anders for recombinant AMA1, Dr. Alan Cowman for providing antibodies to EBA175 and MSP3 and knockout parasite lines, Dr. Mike Blackman for providing antibodies to MSP1-33, Ross Coppel for antibodies to MSP4 and Kerstin Leykauf and Brendan Crabb for antibodies to AMA1. We also wish to thank Dr. Brendan Crabb for helpful discussions and suggestions and Dr. Mirja Hommel and Dr. Freya Fowkes for comments on the manuscript. Erythrocytes and serum for parasite culture were kindly provided by the Red Cross Blood Bank (Melbourne and Brisbane). Funding was provided by the National Health and Medical Research Council of Australia (Project grant and Career Development Award to J. Beeson; Postgraduate Research Fellowships to J. Richards; Independent Research Institutes Infrastructure Support Scheme Grant to the Walter and Eliza Hall Institute), the Australian Government (Australian Postgraduate Award to M. Boyle), the Victorian State Government (Operational Infrastructure Support Grant to the Walter and Eliza Hall Institute), and the Wellcome Trust (W. Chai).

AUTHOR CONTRIBUTIONS: MJB – study design, analysis and interpretation of results, performed experiments, wrote the manuscript with JGB; JSR - contributed to study design, interpretation of results, performed experiments and contributed to writing the manuscript; PRG – experimental design and specific experimental expertise, interpretation of results and contributed to writing the manuscript; WC – generated reagents, contributed to study design, interpretation of results and writing the manuscript. JGB – study design, analysis and interpretation of results, performed experiments, co-wrote the manuscript.

CORRESPONDENCE: James G. Beeson, The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3050, Australia, Ph: +61-3-9345-2555, Fax +61-3-9347-0842,

DISCLOSURE OF CONFLICTS OF INTEREST: The authors declare no competing financial interests.
REFERENCES


380.


<table>
<thead>
<tr>
<th>Molecule</th>
<th>Uronate type</th>
<th>Uronate Sulfation(^1) (%)</th>
<th>Glucosamine Sulfation(^1) (%)</th>
<th>d.o.s.(^2)</th>
<th>IC(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>IdoA</td>
<td>2S 84</td>
<td>NS 95; 6S 90</td>
<td>2.6</td>
<td>19</td>
</tr>
<tr>
<td>Heparin, partially de-N-sulfated</td>
<td>IdoA</td>
<td>2S 84</td>
<td>NS 80; 6S 90</td>
<td>2.5</td>
<td>74</td>
</tr>
<tr>
<td>Heparin, de-N-sulfated</td>
<td>IdoA</td>
<td>2S 83</td>
<td>NS 8; 6S 92</td>
<td>1.8</td>
<td>&gt;&gt;&gt;100</td>
</tr>
<tr>
<td>Heparin, de-N/O-sulfated</td>
<td>IdoA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&gt;&gt;&gt;100</td>
</tr>
<tr>
<td>Heparin, de-6-O-sulfated</td>
<td>IdoA</td>
<td>2S 60</td>
<td>NS 95; 6S 9</td>
<td>1.6</td>
<td>373</td>
</tr>
<tr>
<td>Heparin, de-2-O-sulfated</td>
<td>IdoA</td>
<td>2S 5</td>
<td>NS 95; 6S 88</td>
<td>1.9</td>
<td>123</td>
</tr>
<tr>
<td>K5-NS</td>
<td>GlcA</td>
<td>0</td>
<td>NS 100</td>
<td>1</td>
<td>&gt;&gt;&gt;100</td>
</tr>
<tr>
<td>K5-OS-L</td>
<td>GlcA</td>
<td>2S 10</td>
<td>6S 90</td>
<td>1.4</td>
<td>&gt;&gt;&gt;100</td>
</tr>
<tr>
<td>K5-OS-H</td>
<td>GlcA</td>
<td>2,3-diS 100</td>
<td>6S(^3) 100%</td>
<td>3</td>
<td>20.2</td>
</tr>
<tr>
<td>K5-NSOS-L</td>
<td>GlcA</td>
<td>2S 10</td>
<td>NS 100; 6S 90</td>
<td>2</td>
<td>56</td>
</tr>
<tr>
<td>K5-NSOS-H</td>
<td>GlcA</td>
<td>2S 30; 2,3-diS 70</td>
<td>NS 100; 6S 100</td>
<td>3.7</td>
<td>7.4</td>
</tr>
<tr>
<td>EK5-NSOS-L</td>
<td>IdoA/GlcA 1:1</td>
<td>2S 10</td>
<td>NS 100; 6S 90</td>
<td>2</td>
<td>103</td>
</tr>
<tr>
<td>EK5-NSOS-H</td>
<td>IdoA/GlcA 1:1</td>
<td>2S 30; 2,3-diS 70</td>
<td>NS 100; 6S 100</td>
<td>3.7</td>
<td>103</td>
</tr>
</tbody>
</table>

Notes:

\(^1\) Data on sulfation levels are based on information provided by manufacturers and published data\(^23\).
2 d.o.s. is degree of sulfation, which indicates the average number of sulfate groups per disaccharide unit.

3 3-O-sulfation of the glucosamine is also present (level of sulfation is unknown).

Abbreviations: IdoA, iduronic acid; GlcA, glucuronic acid; K5-NS, K5 polysaccharide N-sulfated; K5-OS-L, K5 polysaccharide with low level of O-sulfation; K5-OS-H, K5 polysaccharide with high level of O-sulfation; K5-NSOS-L, K5 polysaccharide with low level of N- and O-sulfation; K5-NSOS-H, K5 polysaccharide with high level of N- and O-sulfation; EK5-NSOS-L, epimerized K5 polysaccharide with low level N- and O-sulfation; EK5-NSOS-H, epimerized K5 polysaccharide with high level N- and O-sulfation.
FIGURE LEGENDS:

**Figure 1:** Heparin inhibits *P. falciparum* merozoite invasion at initial contact events. *P. falciparum* schizonts (3D7 isolate) in culture were monitored over time for schizont rupture (A) and formation of ring-stage (B) parasites in the presence of heparin (100 µg/ml) or PBS control. Data are mean + range of one representative assay (two assays were performed in duplicate). A. There was a small, but significant delay of schizont rupture in the presence of heparin (*p*=0.03 for difference in schizont parasitemia for heparin versus control at 15 hours; Mann-Whitney U test). B. Formation of new ring-stage parasites was greatly inhibited in the presence of heparin showing that heparin acts predominately by inhibition of merozoite invasion. C, D. Real time microscopy of merozoite invasion. C. Merozoite invasion (arrow) in PBS control cultures demonstrated (1) initial contact of merozoite with erythrocyte, (2) reorientation (3) commencement of invasion, (3-5) mechanical invasion and deformation of the erythrocyte, (6) complete invasion and erythrocyte reformation. D. Merozoite invasion with cultures containing heparin (100 µg/ml): (1-5) initial contact of merozoite with erythrocyte without reorientation, (6) initial contact is not sustained and merozoite detached. No invasion, reorientation or erythrocyte deformation was observed in the presence of heparin. Time in seconds is indicated in bottom right corner. Pictures shown are representative selections from real-time microscopy recordings using the 3D7 *P. falciparum* line. Videos can be found as supplementary material. Microscopy was performed on a Zeiss (Germany) Axiovert 200M microscope with 63X/1.4 oil (magnification/numerical aperture) objective lens, at humidified tissue culture conditions (5% CO2, 5% O2, 90% N2), 37°C, standard culture media. Images were collected by Zeiss AxioCam MRm with Zeiss Axio Vision software and processed with Image J.

**Figure 2:** Heparin inhibits essential erythrocyte invasion events. A. Heparin effectively inhibited merozoite invasion of *P. falciparum* lines W2mef (sialic-acid dependent invasion phenotype) and 3D7
(sialic-acid independent invasion phenotype) at comparable levels. CSC was non-inhibitory against all lines tested, data is excluded from following graphs for clarity.  

B. Heparin had similar inhibitory activity against invasion of 3D7 *P. falciparum* into neuraminidase, chymotrypsin, or trypsin-treated erythrocytes versus untreated erythrocytes. C. Heparin efficiently inhibited invasion of W2mef *P. falciparum* into chumotrypsin and trypsin treated erythrocytes compared to untreated.  

D. 3D7 parasites were selected once or twice for invasion of erythrocytes in the presence of heparin 100 µg/ml. The ability of heparin to inhibit erythrocyte invasion by merozoites was comparable between the parent parasites and heparin-selected parasites. All data are mean growth ± s.e.m., expressed as percent of control (PBS) (three assays in duplicate) except for B mean growth range, expressed as percent of control (PBS) (two assays in duplicate).

**Figure 3:** Native MSP1-42 and MSP1-33, but not full length MSP1, MSP1-19 or EBA175, bind to heparin. Merozoite proteins were extracted from whole schizonts (3D7 isolate) and incubated with heparin-agarose beads with and without soluble inhibitors. A. MSP1-42 bound to immobilized heparin in the presence of control (PBS) or soluble CSC, and MSP1-42 was depleted from the supernatant after incubation. In the presence of soluble heparin, binding of MSP1-42 was inhibited, and was not depleted from the supernatant. Full length MSP1 (MSP1-full) did not bind to beads and was only present in the supernatant. B. MSP1-33 from culture supernatant bound heparin, being present in the bead-bound fraction and depleted from the supernatant. In the presence of soluble heparin, MSP1-33 was not able to bind, and was predominately found in the supernatant (upper panel). MSP1-19 did not bind to beads and was only present in the supernatant fraction (lower panel) C. Other merozoite antigens MSP3, MSP4, AMA1 and EBA175 showed little or no binding to heparin-agarose. For MSP3 and MSP4 no protein was seen in the bound fraction. AMA1 showed non-specific binding to heparin-agarose beads that was not inhibited by the addition of soluble heparin. For EBA175 the majority of the protein was seen the
supernatant after incubation with heparin-agarose. Binding of merozoite proteins was tested in the presence of control (PBS), soluble heparin, or CSC. D. Heparin did not inhibit proteolytic processing of MSP1. Purified schizonts were incubated with PBS, EDTA (1mM) or heparin (1mg/ml) until complete rupture of schizonts had occurred, and merozoites were then collected. In the presence of control (PBS) and heparin, processed forms of MSP1 (MSP1-42 and MSP1-19) were observed, in addition to full length MSP1. Heparin did not significantly reduce the appearance of MSP1-42 or MSP1-19 compared to PBS control. EDTA prevented processing of full length MSP1 to MSP1-42 and MSP1-19 fragments, indicating inhibition of processing. For all figures, the antibody used in the Western blot is indicated at bottom of the panel (all antibodies were raised in rabbits). Molecular weight markers are indicated to the left, and proteins are indicated with arrows. Results shown are representative examples taken from multiple assays.

**Figure 4: Recombinant MSP1-42 binds to heparin.** A: Recombinant merozoite antigens were tested for binding to heparin-BSA (5µg/ml) or BSA alone (5µg/ml). Binding to lactoferrin was used as a positive control and to normalize binding between assays. Data are mean binding + s.e.m. expressed at percent binding of lactoferrin (three assays in duplicate). Heparin-BSA had similar growth-inhibitory activity against *P. falciparum in vitro* as heparin, whereas BSA was not inhibitory (data not shown). B. Binding of heparin to MSP1-42 was concentration-dependent and saturable at low concentrations. Data are normalized to percent binding of lactoferrin to heparin-BSA at 5µg/ml. C. Binding of heparin-BSA to MSP1-42 was inhibited with soluble heparin but not CSC. Data are expressed as the proportion of binding observed in the absence of inhibitors. Data are mean ± s.e.m., (three assays in duplicate).

**Figure 5: Structure of heparin and related molecules.** A. Heparin: the major disacharide
unit of heparin is 4-linked $\alpha$-iduronic acid (IdoA) and 4-linked $\alpha$-glucosamine (GlcNAc), in which IdoA is 2-O-sulfated and GlcNAc is 6-O- and de-acetyl-N-sulfated. Heparin can be modified by selective de-N-sulfation and/or de-O-sulfation. B. K5 polysaccharide: this has the same backbone as the precursor to heparin and can be modified with by addition of sulfate groups at indicated positions (arrows) and by epimerization of GlcA to IdoA (indicated in grey). This results in polysaccharides with various sulfation contents and patterns and structural features.

Figure 6: Structural requirements of heparin-like molecules for invasion-inhibitory activity. A panel of modified heparins and K5 polysaccharides was tested for inhibition of invasion. A. Growth-inhibitory activity of selectively de-sulfated heparins against *P. falciparum* blood stage parasites B. Growth-inhibitory activity of K5 polysaccharide derivatives with low (K5-OS-L) and high (K5-OS-H) levels of O-sulfation. C. Inhibitory activity of K5 polysaccharides with low (K5-NSOS-L) and high (K5-NSOS-H) levels of N- and O-sulfation, compared to the same molecules that have GlcA partially epimerized to IdoA (ratio of GlcA:IdoA is 50:50). D. The degree of sulfation (d.o.s.; number of sulfate groups per disaccharide unit) of heparin and heparin-like molecules was compared to the IC$_{50}$ ($\mu$g/ml). A d.o.s. $\geq$2 (dashed line) was required for inhibitory activity. Non-inhibitory molecules were plotted as having an IC$_{50}$ of 500 $\mu$g/ml. Calculated IC$_{50}$ values for all molecules are shown in Table 1. E. Inhibitory activity of heparin and CSC oligosaccharides of different chain lengths (2-16 monosaccharide units), tested at 100 $\mu$g/ml. Data are mean growth ± s.e.m. (three assays in duplicate).

Figure 7: Structural requirements of heparin-like molecules for binding of MSP1-42. Binding of MSP1-42 to heparin-agarose beads was inhibited by heparin but not by de-N-sulfated (de-NS-) and de-6O-sulfated (de-6OS-) heparin. Binding of MSP1-42 was also inhibited by K5 polysaccharides with high
level N and O sulfation (K5-NSOS-H), but not by other K5 polysaccharides having different sulfation levels or patterns, or by EK5 polysaccharides. Heparin-agarose bound proteins were detected by Western blot using antibodies to MSP1-19. K5-NSOS-H was the most inhibitory molecule in parasite invasion inhibition assays, whereas the other molecules had little or no invasion-inhibitory activity.
Figure 1

A

% parasitemia (schizonts)

B

% parasitemia (rings)

Time point (hrs)

0 3 6 9 12 15

Time point (hrs)

0 3 6 9 12 15

Control

Heparin

C

D

From www.bloodjournal.org
Figure 2

(A) Growth (% of control) vs. Concentration (µg/mL) for 3D7: CSC, W2mef: CSC, 3D7: Hep, and W2mef: Hep.

(B) Growth (% of control) vs. Heparin concentration (µg/mL) for untreated, trypsin, chymotrypsin, and neuraminidase.

(C) Growth (% of control) vs. Heparin concentration (µg/mL) for Untreated, Trypsin, and ChymoT.

(D) Growth (% of control) vs. Heparin concentration (µg/mL) for parent line, heparin selected - 1, and heparin selected - 2.
Figure 5

A

CH$_2$O$-\text{SO}_3^-$

OH

O$-\text{SO}_3^-$

OH

B

COO$^-$

OH

OH

CH$_2$OH

CH$_3$COOH

OH

OH

NH$\text{-SO}_3^-$

Ac
Figure 7

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>PBS</th>
<th>Hep</th>
<th>CSC</th>
<th>de-NS-hep</th>
<th>de-6OS-hep</th>
</tr>
</thead>
</table>

- MSP1-42

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>K5-OS-H</th>
<th>K5-NSOS-L</th>
<th>K5-NSOS-H</th>
<th>EK5-NOS-L</th>
<th>EK5-NSOS-H</th>
</tr>
</thead>
</table>

- MSP1-42
Interactions with heparin-like molecules during erythrocyte invasion by *P. falciparum* merozoites

Michelle J. Boyle, Jack S. Richards, Paul R. Gilson, Wengang Chai and James G. Beeson