TITLE: Identification of heparin modifications and polysaccharide inhibitors of *Plasmodium falciparum* merozoite invasion that have potential for novel drug development

RUNNING TITLE: Polysaccharide inhibitors of malaria

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Abstract

Despite recent successful control efforts, malaria remains a leading global health burden. Alarmingly, resistance to current antimalarials is increasing, and the development of new drug families is needed to maintain malaria control. Current antimalarials target the intra-erythrocytic developmental stage of the *Plasmodium falciparum* life cycle. However, the invasive extracellular parasite form, the merozoite, is also an attractive target for drug development. We have previously demonstrated that heparin-like-molecules, including those with low molecular weights and low anti-coagulant activities are potent and specific inhibitors of merozoite invasion and blood-stage replication. Here we tested a large panel of heparin-like-molecules and sulfated polysaccharides together with various modified chemical forms for inhibitory activity against *P. falciparum* merozoite invasion. We identified chemical modifications that improve inhibitory activity and identified several additional sulfated polysaccharides with strong inhibitory activity. These studies have important implications for the further development of heparin-like-molecules as anti-malarial drugs, and for understanding merozoite invasion.

INTRODUCTION

Despite gains in malaria control, and a push to elimination in some areas, malaria remains a significant disease globally with *Plasmodium falciparum* the leading cause of malaria (1). Recent evidence of the emergence and spread of artemisinin resistance in several countries raises concerns that current therapies will lose their clinical value (2), making continued drug discovery and development a high priority. Malaria disease occurs during blood stage infection by *P. falciparum* in which the merozoite form of the parasite invades and replicates within red blood cells (RBCs). All current drugs including the artemisinin class

target the intra-RBC stage of development (3). However, targeting and blocking merozoite invasion also presents an attractive approach for therapeutics to prevent parasite invasion of RBCs, reducing parasite burden and disease (4, 5). Compounds that block invasion may be valuable in combination with current drugs providing activity at different stages of the blood-stage life cycle, and drug combinations are increasingly used for various infections to maximize efficacy and reduce the risk of developing drug resistance.

Merozoite invasion involves numerous receptor-ligand interactions, with multiple, redundant invasion pathways being identified (6). Nevertheless, sulfated carbohydrates and heparin-like-molecules (HLMs) have been identified as a group of compounds that block essential invasion events and are able to inhibit multiple invasion pathways (7). Inhibitory HLMs include heparin (7, 8), curdlan sulfate (9, 10), polyvinyl-sulfonate sodium salt (11), suramin (12), carrageenans (13), sulfated cyclodextrins (14), fucosylated chondroitin sulfate (15) and K5 polysaccharides (7). The ability of HLMs to disrupt invasion maybe due to the targeting by HLMs of multiple essential or important merozoite ligands. Merozoite invasion into RBCs proceeds through a number of steps; i) initial contact and weak deformation of the RBC involving merozoite surface antigens, ii) strong deformation of the RBC involving microneme and rhoptry proteins and the actin-myosin motor of the parasite, iii) pore opening between the parasite and the RBC, iv) tight-junction formation between the parasite and RBC and v) internalization (16). HLMs bind proteins involved 'pre-invasion' and initial attachment stages to the RBC such as merozoite surface protein 1 (MSP1) (7), along with rhoptry and microneme proteins involved in reorientation and signalling steps of invasion that trigger strong deformation of the RBCs (17-19). Although the precise mechanisms of action are not known, it is possible these merozoite proteins interact with sulfate groups on the RBC surface and HLMs may inhibit invasion by disrupting essential receptor-ligand interactions. Whilst heparin has the capacity to inhibit at multiple invasion steps, it appears

that the dominant inhibitory activity of HLMs is mediated at the early invasion stages as demonstrated with live-video microscopy of merozoite invasion that heparin blocks 'pre-invasion' steps (7, 16). However, the capacity of HLMs to also bind proteins involved in down-stream invasion steps may also contribute to effective inhibition and the observed inability to select for heparin resistant parasites lines (7). Of further potential therapeutic benefit, HLMs are also known to disrupt both rosetting and sequestration of infected RBCs (15, 20-26), which are important mediators of pathogenesis. The ability of HLMs to inhibit both merozoite invasion and sequestration/rosetting highlights the potential of these molecules to reduce parasitemia and disease severity. Owing to the anticoagulant activity of heparin it cannot be used as an antimalarial agent. However, it may be possible to reduce anticoagulant activity of HLMs while maintaining inhibition of *P. falciparum* (7). Indeed, curdlan sulfate, which has a ten-fold reduced anticoagulation activity compared to heparin, has been tested in a small human trial which suggested that treatment reduced malaria disease severity (27). Further, HLMs such as K5 polysaccharides, as well as other polyanions that lack anticoagulant activity have been proposed as potential therapeutics for viral diseases (reviewed in (28)), and can inhibit merozoite invasion (7).

In previous work, we identified a number of key structural features of HLMs for invasion-inhibitory activity by testing chemically modified K5-polysaccarides and heparins together with their oligosaccharides (7). Our findings suggest the importance of N- and O-sulfate residues, ≥2 sulfate units per disaccharide, specific spatial arrangements of sulfation requiring sulfate groups positioned together on a single saccharide unit, and a minimum chain length of 6 monosaccharide residues for optimal inhibitory activity (7). Structure/function studies have also successfully been used to develop small drug HLMs for other clinical applications, such as the pentasaccharide anticoagulant fondaparinux (29). Here we build on this knowledge by testing HLMs with specific modifications to further investigate structural

features that mediate high inhibitory activity and identify chemical modifications that increase activity. Further, we tested a large panel of sulfated polysaccharides prepared from a wide range of sources to identify inhibitory compounds. We aimed to identify compounds with strong invasion inhibitory activity that may have potential for therapeutic development.

MATERIALS AND METHODS

Parasite culture

P. falciparum 3D7 or D10-PfGFP isolates (30) were cultured as described (31, 32), in culture media of RPMI-HEPES (pH 7.4) supplemented with 50 μg/ml hypoxanthine, 20 μg/ml gentamicin, 25 mM sodium bicarbonate ($NaHCO_3$) and 0.5% Albumax II (Gibco). RBCs from group O+ blood donors were used to culture parasites. Cultures were gassed with 1% O_2, 4% CO_2, 95% N_2 and incubated at 37°C. Parasites were initially synchronized using 5% D-sorbitol-treatment for 5 minutes, as described (33). For invasion-inhibition assays, sorbitol treatment cultures were further synchronised using heparin synchronization; heparin cannot be used for the selection of heparin resistant cultures therefore it is unlikely that heparin synchronization affected the testing of HLMs (7). Live video filming of merozoite invasion was performed as described (7, 34).

Growth inhibition assays and invasion inhibition assays

High throughput growth inhibition assays were performed as described (30, 32, 35, 36). Duplicate suspensions of synchronised parasites at 2% parasitemia and 1% hematocrit were incubated with compounds in 96 well sterile U-bottom plates (Falcon) for 44 hours for one-cycle assays, or 72 hours for two-cycle assays and analysed by flow cytometry with staining of parasites with 10 μg/ml ethidium bromide (BioRad) for one hour in darkness.

Parasitemia was measured using BD FACSCalibur or BD FACSCantoII flow cytometer. Samples were analysed using FlowJo (Tree Star) gating on intact RBCs and then determining parasitemia by ethidium bromide positive RBCs. Inhibitory effects of compounds were normalised as % growth of controls for each assay.

Invasion inhibition assays with isolated merozoites were conducted as described (4, 37) (for detailed methods see Methods in Malaria Research, 2013 https://www.beiresources.org/Publications/MethodsinMalariaResearch.aspx). Highly synchronized late-stage schizonts were magnet purified via Macs magnet separation column (Macs; Miltenyi Biotec) and treated with E64 until mature merozoites were formed. Merozoites were isolated by membrane filtration and incubated with uninfected RBCs at 0.5% haematocrit and test compounds at indicated concentrations, in 50µl volumes. Estimated number of merozoites per test is approximately 7 X 10^6 merozoites. Invasion occurred in agitated conditions for ten minutes, and then in static conditions for a further 20 minutes. Following invasion, cultures were washed twice and returned to culture media. Parasites were analysed by flow cytometry at 40 hours post invasion as described for growth inhibition assays.

Modification of heparin-like-molecules

Porcine mucosal heparan sulfates (HS) (HO-10595, a 12-15kDa Highly Sulfated HS, and HS1098 a15kDa lowly sulfated HS) and 12.5-kDa heparin (MH) were purchased from Celsus Laboratories, Inc. (Cincinnati, OH, USA). Bovine lung heparin (LH) was from Calbiochem (Melbourne, Australia), Sulodexide (a low sulfated heparin/low MW dermatan sulfate, 80:20 ratio) was purchased as Vessel™, manufactured by Alfa Wasserman, Bologna, Italy, Arixtra (a synthetic heparin pentasaccharide) was from GlaxoSmithKline, Enoxaparin (a 3kDa low MW heparin) was purchased as Clexane from Sanofi-Adventis and Bemiparin (a

3kDa low MW heparin) was purchased as Hibro manufactured by Laboratorios Farmaceuticos Rovi SA.

Heparin compounds were modified with published methods as per following: Glycol-split heparins and partially (50%) glycol split heparin were prepared by periodate oxidation followed by NaBH4 reduction as described previously (38-40). Fully de-sulfated heparin was prepared by the treatment of their pyridinium salts with dimethyl sulfoxide containing 10% of water at 100^0C by the method of Nagasawa et al 1977 (41). De-N-sulfated and partially de-N-sulfated heparins were prepared by treatment of their pyridinium salts with dimethyl sulfoxide containing 5% of methanol for upto 1.5 h at 50^0C (42). De-N-sulfated heparins were N-acetylated by treatment of the heparin with acetic anhydride in 0.5M $NaHCO_3$ at 4^0C (43). 2-*O*-Desulfated heparins and glycol-split heparins were prepared as described by dissolving the heparin in 0.2M NaOH followed by lyophilization (44), using an adaption (45). Mucosal heparin (porcine) lacking 6-O sulfate (MH de 6S) was prepared by the treatment of the pyridinium salt of heparin with N,O-bis(trimethylsilyl)acetamide in pyridine for 2 h at 60°C (46). Heparin were decarboxylated by treatment with 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide and subsequent sodium borohydride reduction as described previously ((47), adapted from (48)).

Mucosal heparin (porcine) (5-kDa MH, Smith degradation) was prepared by treatment of periodate-oxidized mucosal heparin (porcine) with sodium hydroxide, followed by reduction with sodium borohydride and acid hydrolysis (49). O-acylated derivatives (butyl and hexyl) of heparin fragments were prepared from their tributylammonium salts in N,N-dimethylformamide using carboxylic acid anhydrides and 4-(dimethylamino)pyridine as catalyst (50, 51). MH peroxide 3-4kDa MH (H_2O_2) was prepared by hydrogen peroxide induced free radical degradation (52). 3kDa MHgc (glycol split mucosal heparin (porcine)) was prepared by limited (10mins) nitrous oxide degradation of glycol split heparin at pH 4

(Reaction A, which cleaves both at glucosamine (Glc)NS and GlcNH) using the method of Lindahl, U et al, 1973 (53) followed by sodium borohydride reduction. 3kDa MH gc-CHO RT was prepared by the above method without subsequent borohydride reduction to leave a terminal reactive aldehyde moiety. Hydrazone derivatives of 3kDa MH gc-CHO with 4-phenylsemicarbazide or Benzhydrazide were prepared with a 5 fold molar excess of 4-phenylsemicarbazide or Benzhydrazide in 100mM sodium acetate, pH 6 overnight at room temperature (20^0C). Reductive amination of 3kDa MH gc-CHO with Anthranilic acid or ANTS (1,3,6 triSO3-aminonaphthaline) were prepared with a 5 fold molar excess of Anthranilic acid or ANTS (1,3,6 triSO3-aminonaphthaline) and a 25 molar excess of sodium cyanoborahydride (NaBH3CN) in 100mM sodium acetate, pH 6 overnight at room temperature (20^0C).

Confirmation of chemical modifications

Following de-N-sulfation of glucosamine residues in the heparin derivatives and their subsequent re-N-acetylation, the presence or absence of un-substituted glucosamines GlcNH was determined by degradation of the derivative by nitrous acid at pH 4 using Reaction B (53) which only cleaves adjacent to unsubstituted glucosamine residues and analysis on PAGE to determine reduction of size. The reaction was also quantified by colorimetric analysis of the resultant anhydromannose residues by reaction with 3-methyl-2-benzothiazolinone hydrazone (54). Size analysis by PAGE was used to demonstrate no degradation of the modified heparins had occurred following glycol splitting. The apparent size of heparin fragments cleaved by peroxide, periodate of nitrous acid cleavage were determined by PAGE analysis using a mini-gel apparatus (Bio-Rad, Hercules, CA) and fractionated on 15% resolving gels or 30% Tris-glycine gels (55, 56) using known heparin-derived molecular mass standards of 16.7, 10.6, 6.7 and 3.1kDa which were a generous gift from Nova Nordisk (Gentofte, Denmark) (57). In addition the structures of carboxyl-reduced

heparins and de-2-O-sulfate heparins and glycol-split derivatives were analysed by poly-acrylamide gel electrophoresis, and chemical structures determined by 1H NMR spectroscopy, as previously published (44, 47).

Preparation of oligosaccharide fractions from heparin, heparan sulfate and chondroitin sulfate E

Heparin and HS oligosaccharide fragments were prepared as described (58). Briefly, heparin (200 mg, from porcine intestinal mucosa, Sigma) was incubated with heparin lyase I (100U , Sigma) and HS (200 mg, from porcine intestinal mucosa, Celsus) with heparinise III (650 mU, IBEX Technologies, Montreal, Canada) (59) in 5 mM sodium phosphate buffer (pH 7.1) containing 0.2 M NaCl. The digestion was carried out at 30 ^{o}C and stopped when the reaction was 40% complete. After desalting on a short Sephadex G10 column, the oligosaccharides were fractionated on a Bio-Gel P-4 column (1.6 × 90 cm) with elution by 0.1 M NH_4Cl (pH 3.5).

CSE (2 mg, from squid cartilage, AMS Biotechnology, Abingdon, England) was digested with 20 mU of chondroitinase ABC (Sigma) in the same phosphate buffer (400 µl) under identical conditions described above. Oligosaccharide fractionation was carried out on a Superdex Peptide column (Amersham Biosciences, Little Chalfont, England) eluted by 0.05M ammonium acetate.

The tetra- and hexsaccharide fractions were subfractionated by strong-anion exchange on a short cartridge column (HiTrap Q-Sepharose HP, 1 ml, Amersham Biosciences) with detection at UV 232 nm. Elution was carried out with a linear gradient of NaCl (solvent A, 0.1 M NaCl; solvent B, 1.5 M NaCl; pH 3.5) as described (60). Oligosaccharide subfractions were collected desalted and freeze-dried before quantitation by carbazole assay for hexuronic acid content (61).

Heparin binding assays

For heparin-agarose bead binding assays proteins were extracted from *P. falciparum* schizonts into 1% Triton X-100 in PBS as described (62). Proteins from culture supernatants were collected by allowing highly synchronous schizonts to rupture into protein-free culture medium and cells removed by centrifugation. Binding of solubilised proteins to heparin-agarose beads was performed as described (7); heparin-agarose beads were washed twice in PBS, then blocked with 1% casein PBS overnight at 4°C. Schizont protein extracts were incubated overnight at 4°C with beads containing 0.1% casein and 200 μg/ml of test inhibitor (heparin from Sigma-Aldrich, de-6-OS-heparin from Iduron, Alderley Edgy, U.K.), CSE from Sikagaku, Tokyo, Japan, and CSC from Sigma-Aldrich), 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 5 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 sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Bound and unbound proteins were separated by SDS-PAGE under reducing conditions and Western blotted onto polyvinylidene difluoride membranes for probing with antibodies to detect MSP1-19. MSP1-19 antibodies were raised in rabbits and purified as described (63).

Heparin binding to recombinant MSP1-42 in ELISA was performed as previsouly described (7). Recombinant MSP1-42 (expressed as His-tagged proteins in *E. coli* (64), from Carol Long – National Institute for Health) was coated (1ug/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, along with increasing concentrations of soluble inhibitors of heparin, CSC and CSE. Plates were washed, and bound heparin-BSA/BSA was detected with anti-BSA antibodies (rabbit, Sigma-Aldrich), followed by antirabbit-HRP, and 2,2’- azino-bis(3-ethylbenzthiazoline-6-sulphonic acid; Sigma-Aldrich). All incubations were

247 performed in PBS with 0.1% casein and 0.05% Tween 20, 1 hour at room temperature.

248

249 **Chemical sulfation of polysaccharides**

250 The sulfation of all non-glycosaminoglycan based carbohydrates was achieved
251 utilising chlorosulfonic acid, except for those indicted † and $, where sulfation was carried out
252 essentially as described by Yoshida et al. using pyridine sulfur trioxide complex and
253 piperidine-*N*-sulfonic acid respectively (65). Carbohydrates were purchased from Sigma-
254 Aldrich, Dextra Laboratories, Celsus Glycoscience, Novartis, WAKO Chemicals, and EDQM
255 (Conseil de l'Europe) as indicated in Supplementary Table S2. Precursor carbohydrates
256 requiring sulfation (500 mg) were added to pre-chilled dry pyridine (VWR) in advance of the
257 addition of chlorosulfonic acid (1:16 v/v; VWR). The mixture was incubated at 95°C for 2
258 hours prior to cooling with the assistance of an ice-bath. Sodium hydroxide (10 M; Fisher)
259 was added to the mixture with stirring until precipitation occurred. The contents were
260 subsequently transferred to ice cold ethanol (VWR) pre-saturated with sodium acetate
261 (VWR). The precipitate was washed extensively before dissolution in and dialysis (3.5 kDa
262 cut-off; Medicell Membranes) against $_{dd}H_2O$. The dialysed solution was frozen and
263 lyophilised before size exclusion chromatography was performed using HPLC grade H_2O
264 (Fisher) and a pre-packed PD-10 column (GE Healthcare), as per the manufacturer's
265 instructions. Sulfation of highly inhibitory compounds was confirmed by recording
266 attenuated total reflectance FTIR spectra using a Nicolet iS5 IR-TF (Thermo Fisher)
267 spectrometer at the Institute of Science and Technology for Medicine facility of Keele
268 University, scanning in the 4000–400 cm^{-1} region with a spectral resolution of 2 cm^{-1} over 32
269 scans (Supplementary Figure 1). A background air spectrum was obtained and subtracted
270 from all spectra. All carbohydrate spectra were recorded using ThermoFisher Omnics

software. In order to further improve the comparison between samples, the mean of 5 FTIR spectra per sample was normalized to relative absorbance (i.e. dividing the absorbance value of each point of the spectrum by the ratio of a mutually common and identical spectral region for each precursor and modified polysaccharide pair). First derivatives of all spectral data for precursor/modified polysaccharide pairs were plotted and overlaid using Prism software (GraphPad Software, Inc.).

Assessing anticoagulation activity by activated partial thromboplastin time

Anticoagulation activity of a subset of sulfated polysaccharides, which had high merozoite inhibitory activity, was assessed by measuring activated partial thromboplastin time. Cuvettes, ball bearings, 50 mM calcium chloride and test compounds (or controls) were all pre-warmed to 37°C using a Thrombotrack Solo coagulation analyzer (Axis-Shield). Into a cuvette, 50 µl of normal human citrated plasma, 25 µl of aqueous test sample or an HPLC grade water control and 50 µl Pathromtin SL reagent (Siemens) were incubated for 2 minutes at 37°C. The time for clot formation to occur was ascertained immediately following the addition of 25 µl of a 50 mM calcium chloride solution to the cuvette.

Statistical analysis

Statistical analysis was performed in Graph Pad Prism 6. Comparison of activity between individual parent and their modified HLMs in growth inhibition assays was performed using paired t-tests for each combination of compounds. P values were adjusted using the Holm-Sidak method within each individual modification, to decrease the risk of false discovery rates due to performing multiple comparisons. Overall impact of a specific modification (for example, de-2-sulfation) was assessed by Wilcoxon matched-pair sign rank test of all compounds with parent and modified compounds paired. For all comparisons $p<0.05$ is considered statistically significant.

RESULTS

Heparin can be modified to increase inhibitory activity and remove anticoagulant activity

Due to the high anti-coagulant activity of heparin, it cannot be used directly as an anti-malarial agent. Different modifications of heparin compounds can reduce the off-target effects of compounds such as anticoagulation activity, and increase bioavailability and half-life. We investigate a panel of compounds comprising modified heparin and HLMs for inhibitory activity. These included HLMs with non-sulfated uronic acid ring opened and cleaved at the diol site after periodate oxidation treatment; HLMs with carboxyl groups of hexuronic acid residues reduced and HLMs with hydroxyl groups acylated (for the full list of compounds see Table S1). Periodate oxidation of non-sulfated uronic acid residues, which has been reported to abolish anticoagulation activity (40), increased the activity of some but not all compounds (compounds with improved inhibition following treatment – mucosal heparin de2S, mucosal heparin de6S, mucosal heparin 3kDa length; compounds with decreased inhibition following treatment – bemiparin and fondaparinux; overall impact of periodate treatment p=0.195, Table 1). The molecular basis for the increased activity is currently unknown, but one possibility is that increased conformational flexibility of these modified compounds may allow for higher capacity to bind merozoite target antigens. We also assessed the impact of esterification of hydroxyl groups by testing inhibitory activity of mucosal heparin (porcine) that was both periodate treated and esterified (MH gc butyrate). Compared to non-esterified parent compound (MH gc), esterification of hydroxyl groups resulted in an 32% increase in inhibitory activity compared to parent compound (% inhibition at 20 μg/ml – MH gc; 65% s.e.m 4.7%. MH gc butyrate; 97% s.e.m. 0.1%, $p<0.001$). Indeed, periodate treated and esterified heparin was one of the most highly inhibitory compounds

tested. These results demonstrated the potential for developing compounds with increased inhibitory activity based on heparin and modified molecules.

Inhibitory activity of HLMs requires sulfation and activity occurs across a range of sizes

We next compared parent and modified compounds to identify features important in inhibitory activity. Consistent with our prior reports (7), sulfation was a key feature of inhibitory compounds, with reduced activity in de-O- and -N-sulfated compounds compared to the parent compounds (overall impact of de-sulfation $p<0.0001$, Table 2). Further, overall longer chain heparin molecules (>3-25kDa) showed a trend towards having higher inhibitory activity than shorter chain compounds (3kDa) (Mann-Whitney test $p=0.06$, Table 3). However, inhibition was not strictly size dependent; low molecular weight heparin such as enoxaparin (~3kDa) had comparable activity to full-length heparin (3-25 kDa) (Table 3). Further, a number of size fractionated highly sulfated small HLMs (di-, tetra- and hexasaccharides) had substantial growth inhibitory activity, including HS derived hexasaccharides (Figure 1). However, it should be noted that heparin oligosaccharides <6-mers in general have little inhibitory activity (7), suggesting that inhibition by the hexasaccharides tested here may be due to specific sulfation conformation or patterns of these compounds that convey higher than usual inhibitory activity.

Highly sulfated chondroitin sulfates inhibit *P. falciparum* growth

Having shown that a key feature of inhibitory HLMs is a high level of sulfation, we investigated whether highly sulfated CS compounds inhibited merozoite invasion. We have previously shown that CSC and CSA with low degree of sulfation are non-inhibitory (7). Here we tested CSD (low level sulfation) and highly sulfated CSE and CSB. CSD has sulfation at uronate 2S, and galactosamine 6-S, CSE at 4S and 6S of galactosamine and CSB

2S at uronate and either 4S or 6S at galactosamine. CSE had substantial inhibitory activity (IC_{50} approximately 25 μg/ml) (Figure 2a). Highly sulfated CSB-2,6-OS also had modest inhibitory activity at high concentrations, whereas CSD was not inhibitory. This suggests that, as for K5-heparin like molecules tested previously (7), location of at least 2 sulfates together on a single oligosaccharide enhances inhibitory activity. To further investigate the inhibitory activity of CSE a number of CSE hexasaccharides with different sulfation levels were tested in invasion inhibition assays with purified merozoites. This assay differs from standard growth assays as compounds are only incubated with merozoites and RBCs for 30 minutes while invasion is occurring, compared to growth assays which incubated drugs with cultures over the course of the entire 48 hour blood-stage development cycle and invasion inhibition assays are more sensitive that standard growth assays (37). Inhibition appeared to increase with sulfation level; 50% inhibition was observed with CSE hexasaccharides having 7 sulfate groups, when tested at 100μg/ml (Figure 2b). However, CSE hexasaccharides had no activity in standard growth inhibitory assays at these concentrations, possibly due to short chain CS oligosaccharides having too few sulfate groups to mediate complete inhibition. The discrepancy between standard growth and invasion inhibition assays is likely due to the increased sensitivity of invasion assays that use purified merozoites (37), suggesting that the inhibitory activity of compounds is at the threshold of that detected by standard growth assays.

To characterize the functional mechanism of CSE inhibition, schizont rupture and merozoite invasion in the presence of CSE was analysed via flow cytometry with differentiation of parasite stages with ethidium bromide staining (7) and live video imaging (16, 34). As with the inhibitory mechanism of heparin (7), cultures incubated with CSE showed evidence of a slight delay of schizont rupture compared to uninhibited cultures (Figure 3a), but the predominant mechanism of inhibition appeared to be merozoite invasion

inhibition, resulting in very low rates of ring formation (Figure 3b). Live-video imaging demonstrated that CSE prevented the invasion of merozoites into RBCs at early invasion steps; we observed schizont rupture, merozoite dispersal and initial contact of merozoite with RBCs. However, no oscillatory deformation was observed and merozoites dissociated from the RBC surface without clear re-orientation of merozoites and echinocytosis of the RBC (Figure 3c). A single invasion event was observed in 6000 seconds of observation, over 9 schizont rupture events; whereas 21 invasion events in 13 schizont ruptures and 5442 seconds of filming was recorded in uninhibited cultures. Having observed that the CSE inhibitory mechanism appeared similar to heparin, we tested the ability of CSE to disrupt MSP1 binding to heparin, a target of heparin inhibition (7). CSE was unable to disrupt the binding of native or recombinant MSP1-42 to heparin (Figure 3d/e). This suggests that CSE may be inhibiting initial steps of merozoite invasion via targeting alternative merozoite surface proteins or invasion ligands.

Identification of inhibitory sulfated carbohydrates

Having shown that inhibitory activity appears to be reliant on sulfation level and that longer chain lengths are needed for substantial activity, we tested a large panel of polysaccharides to test the impact of sulfation and re-sulfation on inhibitory activity, and to attempt to identify polysaccharides that have potential to be used as the base compounds for novel drug development (for the full list of compounds and sources see Table S2). Polysaccharides were tested in standard growth inhibitory assays at 2, 10, 20 and 100 μg/ml. We tested 87 compounds prepared from a variety of sources with different levels and patterns of sulfation. Initial testing identified 50 compounds with inhibitory activity of greater than 20% when tested at 20μg/ml or lower, with 14 compounds being highly inhibitory at concentrations of 2μg/ml (Table 4). The other 37 compounds showed weak or no inhibitory activity and were not further studied (Table 5). The stage-specificity of inhibition of

merozoite invasion of inhibitory compounds with an IC_{50}<10μg/ml in growth inhibition assays was confirmed in direct invasion inhibition assays using purified merozoites (Figure 4) (4, 37). The most highly inhibitory compounds with an IC_{50} of <2 μg/ml determined in standard growth inhibition assays and that had confirmed invasion inhibitory activity were chemically over-sulfated *i*-carrageenan, inulin sulfate, propyleneglycol alginic sulfate, psyllium sulfate, scleroglucan sulfate, tragacanth sulfate, xylan sulfate (also known as pentosan polysulfate), chemically over-sulfated λ-carrageenan, pullulan sulfate and chemically over-sulfated dextran. De-N-acetylated dermatan sulfate and de-N-acetylated heparin (bovine) also had inhibitory activity in standard 48 hour growth assays; however, this was not confirmed in direct invasion inhibitory assays, suggesting that inhibition seen in growth assays was not specific to merozoite invasion and may be due to non-specific activity of the sample or other mechanisms of inhibition. The anti-coagulation activity of highly inhibitory compounds was tested by assessing activated partial thromboplastin time. All compounds had reduced anti-coagulation activity compared to heparin (Supplementary Table 3), indicating that these compounds may be more suitable base compounds for future drug development.

Discussion

Merozoite invasion of the RBC is a critical step during parasite infection, and an attractive target for therapeutics that may have potential for use in combination with current antimalarials (4, 5, 7). While heparin has been used as adjunctive treatment for malaria complications, its use as a therapeutic in malaria is no longer recommended due to the risk of serious bleeding-related side effects from high anti-coagulant activity (66). Here we identify chemical modifications of heparin that increase inhibitory activity of merozoite invasion,

such as periodate oxidation of non-sulfated uronic acid residues and treatment to esterify hydroxyl groups. Importantly, periodate treatment has been reported to greatly reduce anticoagulation activity of heparin (40), suggesting that the development of HLMs that have high anti-malarial activity and reduced anticoagulation maybe possible. Similarly treated HLMs have been recently tested for inhibition of lung cancer growth in mice and have no anticoagulation activity nor toxicity in heart, liver, kidney or lung tissue (67). On the other hand, de-sulfation and de-N-acetylation significantly decreased inhibitory activity, consistent with sulfation mediating inhibition and our prior published studies (7). We demonstrated that highly sulfated heparin and chondroitin sulfate compounds have substantial inhibitory activity against *P. falciparum* merozoite invasion. Short chain heparins and heparin and HS tetra- and hexasaccharides, along with chemically over sulfated CSB polysaccharides and naturally sulfated CSE polysaccharides and oligosaccharides were identified with inhibitory activity.

As with heparin-like compounds, chondrotin sulfate compounds have been proposed as the basis for drug development with a number of applications, suggesting that these compounds may have use as base molecules for anti-malarial drug development (68). CSE demonstrated significant inhibitory activity that appeared to be targeting initial contact events of merozite invasion, with a small inhibitory activity against schizont rupture, similar to the previously reported mechanisms of inhibition of heparin (7). However, CSE was unable to inhibit the binding of MSP1-42, which was previously identified as a target of heparin inhibition (7), suggesting that CSE may target another merozoite surface protein. Multiple merozoite microneme and rhoptry proteins have been reported to bind heparin (17-19). However, the timing of CSE inhibitory activity is at the initial contact/pre-invasion steps prior to substantial deformation of the RBC that is triggered by these proteins (16), which

suggests that the CSE inhibitory function is targeting merozoite surface proteins that are thought to be involved in these initial stages of invasion.

The targeting of inhibitory HLMs in preventing the early stages of invasion suggests that HLMs inhibit the binding of the merozoite to the RBC by disruption of receptor-ligand interaction to sulfated receptors. These initial contact events are thought to be mediated by multiple merozoite surface proteins, via low affinity interactions with the RBC surface. As many of these interactions are likely to be to sulfated surface receptors, the ability of HLMs to disrupt multiple interactions, across multiple invasion steps is likely to ensure the efficacy of HLMs across all parasite strains, and limit the emergence of drug resistance. Indeed, previous attempts to induce heparin-resistance *in vitro* have failed (7). It is possible that HLMs are active at different stages of the parasite life cycle, as suggested by the small inhibitory effect on schizont rupture. It is possible that HLMs may also function by coating the RBC surface, rather than the merozoite, further contributing to the inability to induce resistant parasites.

A major priority for future development of drugs based on this approach is the generation of compounds with much greater potency. This might be achieved through chemical modification of compounds, or through the synthesis of mimetics with a similar mechanism of action. Further, inhibitory HLMs and the identified CS oligosaccharide compounds with inhibitory activity may in the future be the basis for the development of modified HLMs with increased bioavailability and improved inhibition activity. Modifications may include those identified above, including periodate treatment and esterification. The activity of 4-mer and 6-mer oligosaccharides suggests it may be possible to identify and optimise short saccharides with high invasion inhibition activity; while our data suggests that a 6-mer oligosaccharide is needed for inhibitory activity of heparin, stachyose sulfate (a tetramer) was also identified as a strong inhibitor of invasion. At the

467 present time, there is little information available concerning the oral availability of the active
468 compounds reported here. However, there have been efforts to improve the oral availability
469 of heparin derivatives (reviewed in (69)), and the expectation is that such approaches would
470 also prove effective for these compounds if required. Among the successful methods that
471 have been reported are the use of conjugates with polycarbophil-cycteine
472 (70) anddeoxycholic acid (71). Further, the use of nanoparticles has been reported to improve
473 both oral availability, as well as prolong HLM drug activity (72) which may allow heparin
474 based compounds to remain active for multiple parasite life cycles.

475 Alternatively, inhibitory sulfated polysaccharides identified here from testing a large
476 panel of sulfated polysaccharides prepared from a wide range of sources may be used as base
477 molecules for future drug development. We identify a number of highly inhibitory
478 compounds with estimated IC_{50} <10μg/ml targeting merozoite invasion; agarose sulfate,
479 alginic sulfate, amylopectin sulfate, arabic sulfate, cyclodextrin sulfate, chemically over-
480 sulfated *i*-carrageenan, λ-carrageenan, chemically oversulfated λ-carrageenan, dextran
481 sulfate, dextrin sulfate, gellan sulfate, ghatti sulfatie, glycogen sulfate, guar sulfate, inulin
482 sulfate, konjac glucomannan sulfate, levan sulfate, paramylon sulfate, penoxyacetyl cellulos
483 suflate, pullulan sulfate, propyleneglycol alginic sulfate, psyllium sulfate, scleroglucan
484 sulfate, tragacanth sulfate, taramind sulfate, welan sulfate and xylan sulfate. While dextran
485 sulfates, carrageenans, gellan sulfates and xylan sulfate have been previously reported to
486 inhibit *P. falciparum in vitro* growth (13, 73-75), the remaining compounds have not been
487 previously identified as *P. falciparum* inhibitors. All of these compounds had greater
488 inhibitory activity than heparin, with IC_{50} values less than 10 μg/ml. Importantly, these
489 compounds have reduced anticoagulation potential than heparin, suggesting that these
490 compounds are more suitable for future drug development as they would avoid bleeding-
491 related complications. Base compounds may be extracted from natural sources for future drug

development. Indeed, the extraction of sulfated seaweed polysaccharides such as carrageenans from algae has become routine due to their broad application (reviewed in (76)). Compounds identified here may also be used as the basis for future structure/functional studies and the development of small molecule inhibitors that can be synthetically developed. The synthetic and chemically modified non-glycosaminoglycan based compounds investigated in this study have the additional advantages of being isolated from non-mammalian sources, circumventing possible concerns with prion diseases or the provenance of supplies of mammalian origin. This is of particular relevance following recent reports regarding the contamination of pharmaceutical grade heparin (77, 78). Further, while this work has focused on the inhibition of merozoite invasion, compounds identified here may also have further therapeutic benefit by disrupting parasite sequestration and rosette formation (15, 20-26). The combined ability to disrupt two separate stages of the parasite life-cycle increases the time window of activity of any dual acting compounds. Further studies are needed to assess whether the structural features required for merozoite invasion inhibition are also important in sequestration and rosette inhibition. However, the pursuit of sulfate HLMs as base compounds for novel drug development is supported by several reports of the inhibitory capacity of similar compounds to both disrupt parasite invasion as well as sequestration in *in vivo* models (11, 14, 25, 73, 79, 80).

In conclusion, this work has identified a number of carbohydrate compounds with high inhibitory activity against merozoite invasion of RBCs and a number of modifications were identified that enhance inhibitory activity. Optimisation of highly inhibitory compounds based on these observations may provide opportunities for the development of novel therapeutics useful in combating malarial disease.

LIST OF ABBREVIATIONS:

CS – chondroitin sulfate

gc – glycol splitting

Glc – glucosamine

HLMs – heparin-like-molecules

HS – heparan sulfate

LH – lung heparin

MH – mucosal heparin

MSP1 – merozoite surface protein 1

RBC – red blood cell

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Figure legends:

Figure 1: Growth inhibitory activity of fractionated heparin and heparan-sulfate compounds.

Fractionated heparin and heparin sulfate tetra- and hexa-saccharides were tested for growth inhibitory activity in two cycle assays. Data are mean ± s.e.m. from two assays in duplicate. Abbreviations: HS, heparan-sulfate, hep heparin, 4mer tetrasaccharide, 6mer hexasaccharides. N-sulfation is indicated as Ac1-2, while lack of N-sulfation is Ac0. O-sulfation is indicated as S0-9. Compounds listed with * are different preparations of the same fraction.

Figure 2: Identification of chondroitin sulfates with inhibitory activity against *P. falciparum* merozoite invasion.

A. CSD, CSE and highly sulfated CSB polysaccharides were tested in growth inhibition assays at concentrations 0-100 μg/ml. Data are means ± s.e.m. of three assays in duplicate. **B.** Fractionated CSE hexasaccharides were tested in invasion inhibition assays. Degree of sulfation is 5, 6, or 7 sulfate groups per molecule. Data are mean ± range of one assay in duplicate.

Figure 3: CSE disrupts initial contact of the merozoite to the RBC, but not heparin binding to MSP1-42.

Flow cytometry of late stage parasite cultures with parasite stages differentiated based on ethidium bromide staining was used to track parasite rupture as % schizonts **(A),** and merozoites invasion as % ring forms **(B)** in CSE (100μg/ml and PBS/uninhibited cultures.

After 3 hours of incubation there were increased frequencies of schizonts and decreased ring forms in CSE incubated cultures. Data are mean ± s.e.m of two assays performed in duplicated, * $p<0.05$. **(C)** Live video microscopy of merozoite invasion in the presence of CSE. Merozoites were able to make initial contact with the RBC, but contact was not sustained, and merozoites disassociated from the RBC surface. Seconds is indicated in lower right corner, and the white arrow highlights a single merozoite that attached and then disassociated from the RBC. **(D)** Heparin-bead binding assays with *P. falciparum* protein extract. Protein extract was incubated with heparin-beads along with soluble inhibitors as indicated. Unbound and bead-bound fractions were probed for MSP1-42 binding via western blots. MSP1-42 was found in the unbound fraction when incubated with heparin as a soluble inhibitor, indicating that soluble heparin was able to out-compete binding. However, MSP1-42 was found in the bound fraction when incubated with soluble de-6-OS heparin, CSE or CSC indicating that this compounds were not able to compete with heparin-binding. **(E)** MSP1-42 coated on ELISA plates was incubated with heparin-BSA along with soluble heparin, CSC and CSE at increasing concentrations. Binding of heparin BSA was detected with anti-BSA antibodies. Soluble heparin, but not CSE or CSC inhibited binding of heparin-BSA to MSP1-42.

Figure 4: Invasion inhibition activity of sulfated polysaccharides.

Highly active HLMs and sulfated carbohydrates were tested in invasion inhibition assays to confirm activity against merozoite invasion. All compounds were tested at 10 μg/ml. Data are expressed as inhibition from one assay in duplicate, relative to PBS as a reference control. Three CSC negative controls were included in assay and all were non inhibitory (data not shown). [†] Prepared using pyridine sulfur trioxide complex. [$] Prepared using piperidine-*N*-sulfonic acid.

Table 1: Effect of glycol splitting by periodate treatment on inhibitory activity of heparin against merozoite invasion in growth inhibition assays.

		Inhibition % (s.e.m.)		
Modification	**Parent compounds**	**Parent**	**Modified**	**Gain of inhibition**
Glycol split	MH	68 (7)	77 (9)	9
	MH de2S	27 (2)	54 (6)	27 *
	MH de6S	5 (1)	61 (5.8)	56 *
	MH 3kDa	37 (2)	70 (8)	33 *
	MH H_2O_2	57 (4)	62 (4)	5
	Enoxaparin	64 (5)	80 (3)	14
	Bemiparin	45 (2)	31 (5)	- 14
	Fondaparinux	34 (0)	11 (4)	- 23 *

A panel of modified heparin compounds was tested for inhibition of *P. falciparum* in standard growth inhibition assays at 100 μg/ml. Comparisons were made between parent and periodate treated compounds. Gain of inhibition is calculated as the (inhibition of the modifed – inhibition of parent). Positive values indicate increased inhibitory activity in the modified compound compared to the parent. Negative values indicate reduced inhibitory activity in the modified compound compared to the parent. Significant differences in inhibitory activity between parent and modified compounds are indicated with * ($p>0.05$, corrected for multiple comparisons by Holm-Sidak method). Data is mean inhibition ± s.e.m. of two assays in duplicate. Abbreviations: MH, mucosal heparin (porcine);

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879 **Table 2: Effect of de-2, -6, or –N sulfation (with and without re-NAc) on inhibitory**

880 **activity of heparin against merozoite invasion in growth inhibition assays**

		Inhibition % (s.e.m.)		
Modification	**Parent compounds**	**Parent**	**Modified**	**Loss in inhibition**
De-2-S [%]	MH	68 (7)	26 (2)	42 *
	MH gc	77 (9)	54 (6)	23
	LH	74 (7)	49 (0)	25
	MH 5kDa	75 (6)	45 (4)	30
	MH 5kD gc	73 (8)	41 (1)	32
	MH H_20_2 gc	62 (4)	7 (1)	55 *
	MH 3kDa	37 (0)	15 (4)	22 *
	MH 3kDa gc	70 (8)	11 (5)	59 *
De-6S	MH	68 (7)	5 (1)	63 *
	MH gc	77 (9)	61 (6)	16
	MH gc de2s	54 (6)	21 (1)	33 *
	MH 5kDa	75 (6)	41 (4)	34 *
De-NS (NH) [%]	MH gc	77 (9)	17 (4)	60 *
Partial NAc [%]	MH gc	77 (9)	62 (6)	15
	MH 5kD gc	73 (8)	64 (2)	9
	MH H_20_2	57 (4)	15 (3)	42 *
	MH H_20_2 gc de2S	7 (1)	3 (1)	4
	MH gc de2s	54 (6)	24 (8)	30
	MH de2S	26 (2)	28 (3)	-2
	LH de2S	49 (0)	8 (6)	41 *
Total NAc	MH	68 (7)	47 (4)	21
	MH de2S	26 (2)	23 (2)	3
	MH gc	77 (9)	8 (3)	69 *
	MH gc de2S	54 (6)	0 (3)	54 *

LH de2S	49 (0)	-8 (6)	57 *

Modified heparin compounds were tested for inhibition of *P. falciparum* in growth inhibition assays at 100 μg/ml. Comparisons were made between parent and de-sulfated modified compounds. In the majority of cases de-sulfation result in reduction in inhibition activity. Loss of inhibitory activity is calculated as the (inhibition of the parent – inhibition of modified). Positive values indicate reduced inhibitory activity in the modified compound compared to the parent. Negative values indicate increased inhibitory activity in the modified compound compared to the parent. Significant differences in inhibitory activity between parent and modified compounds are indicated with * ($p>0.05$, corrected for multiple comparisons by Holm-Sidak method). Significant difference of modification across groups of modified compounds is indicated with %. Data is mean inhibition ± s.e.m. of two assays in duplicate.. Abbreviations: MH, mucosal heparin (porcine); LH, lung heparin (bovine), gc, glycol splitting.

Table 3: Invasion inhibition activity of heparin compounds of different sizes against merozoite invasion in growth inhibition assays.

Size group	Compounds (size estimate)	Inhibition % (s.e.m.)
Long chain	Mucosal heparin (>3-25kDa)	68 (7)
	Lung heparin (>3-25kDa)	74 (5)
	Mucosal heparin 5kDa (>3-8kDa)	75 (4)
Short chain	Mucosal heparin (3kDa)	37 (0)
	Enoxaparin (3kDa)	64 (6)
	Bemiparin (3kDa)	45 (3)
	Fondaparinux (3kDa) (pentasaccharide)	34 (0)

Heparin compounds of different oligosaccharide chain length were tested for inhibition of *P. falciparum* in growth inhibition assays at 100 μg/ml. Data is mean inhibition ± s.e.m. of two assays in duplicate. Mucosal heparin is from porcine and lung heparin is from bovine sources.

902 **Table 4: Inhibitory activity of heparin like molecules and sulfated polysaccharides of merozoite invasion in growth inhibition assays**

Very strong inhibitors **Estimated IC_{50}** **<2μg/ml**	**Inhibition % (s.e.m) (2 μg/ml)**	**Strong inhibitors** **Estimated IC_{50}** **2-10 μg/ml**	**Inhibition % (s.e.m.) (10 μg/ml)**	**Moderate inhibitors** **Estimated IC_{50}** **10-20 μg/ml**	**Inhibition % (s.e.m) (20 μg/ml)**
Inulin sulfate	93 (4.5)	Cyclodextrin sulfate	96 (2)	Karaya sulfate	97 (0.1)
* De-N-Ac Hep (bovine)	92 (2.5)	Welan sulfate	96 (1.3)	Dextrin sulfate	96 (0)
Dextran sulfate[†] (73, 74)	90 (0.3)	Agarose sulfate	95 (1.4)	Fucogalactan sulfate	96 (1)
Xylan sulfate[#]	86 (6.4)	Arabic sulfate[$]	95 (1.6)	Arabic sulfate	94 (1)
Propyleneglycol alginic sulfate	77 (3.6)	Glycogen sulfate	95 (2.5)	Carboxymethyl cellulose sulfate	94 (1.1)
chemically over-sulfated N-Ac Hep	72 (1.3)	Penoxyacetyl cellulose sulfate	95 (2.3)	Amylose sulfate	86 (2.1)
* De-N-Ac Hep (porcine)	72 (1.3)	chemically over-sulfated free amino hep	95 (2.5)	Pectin sulfate[†]	84 (2)
* De-N-Ac Dermatan S	67 (13.7)	Konjac glucomannan sulfate	95 (2.8)	Locust bean gum sulfate	79 (3.7)
		Levan sulfate	94 (3.2)	Chitosan sulfate	61 (2.7)
chemically over-sulfated λ - Carrageenan [†,] (13, 75)	65 (10.4)	Pullulan sulfate[†]	94 (1.2)	Guar sulfate	59 (3.6)
Tragacanth sulfate	64 (11.7)	Taramind sulfate	94 (1.5)		
chemically over-sulfated i-carrageenan (13)	64 (6.4)	Ghatti Sulfate	92 (2.8)		
Scleroglucan sulfate	55 (8)	λ-carrageenan [$,] (13, 75)	90 (2)		
		Paramylon sulfate	90 (1.9)		

	Psyllium seed gum sulfate	89 (2)
	Stachyose sulfate	89 (4.6)
	Agarose sulfate[†]	87 (2.6)
	chemically over-sulfated κ-carrageenan [$] (13, 75)	85 (2.9)
	Gellan sulfate (81)	82 (9.6)
	Amylopectin sulfate	74 (1)
	* Tara sulfate	71 (3.7)
	Heparin (Bovine lung) (7)	70 (8.1)
	Guar sulfate[†]	68 (7.1)
	Alginic sulfate	62 (11)
	Psyllium sulfate	56 (10)
	Dextrin sulfate	56 (9.1)

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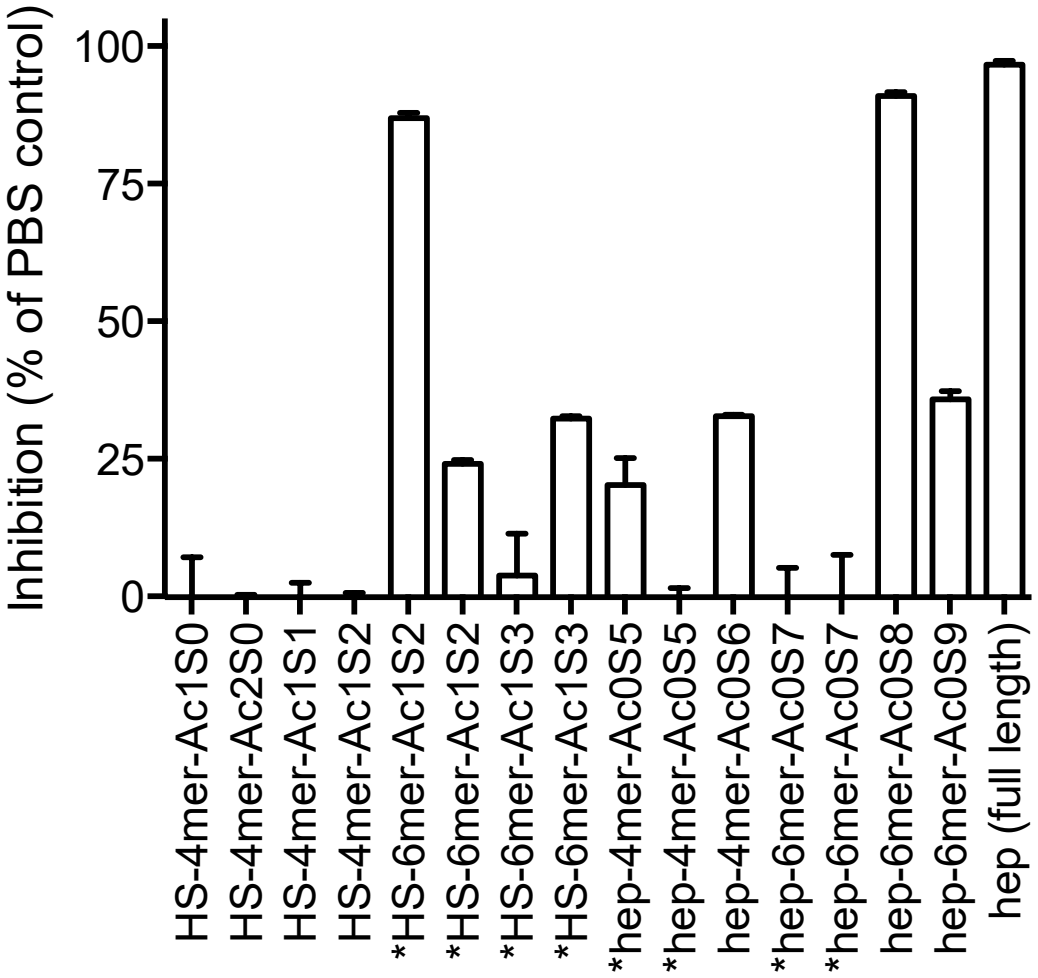
Compounds were tested in growth inhibitory assays at 2, 10 and 20 μg/ml. Inhibitory compounds are listed according to their estimated IC_{50} and ordered based on inhibitory activity. Inhibition activity at 2, 10 and 20 μg/ml is as indicated. Data is mean of two assays performed in duplicate ± s.e.m. Highly inhibitory compounds were additionally screened in invasion inhibition assays with purified merozoites to confirm targeting of merozoite invasion. Compounds marked with * were non-inhibitory in invasion inhibition assays suggesting that these samples may contain a non-specific growth inhibitory substance or act through a mechanism separate to invasion inhibition. [#] Xylan sulfate is also known as pentosan polysulfate. [†] Prepared using pyridine sulfur trioxide complex. [$] Prepared using piperidine-*N*-sulfonic acid. Abbreviations: Hep, heparin. Where published previously, references are listed beside compounds.

Table 5: Carbohydrate compounds with weak or no inhibitory activity against merozoite invasion in growth inhibition assays

Weakly inhibitory **Estimated IC_{50}** **20-100 μg/ml**	**Inhibition % (SD) (100 μg/ml)**	**Non-inhibitory** **<20% inhibition at 100 μg/ml**	**Inhibition % (SD) (100 μg/ml)**
Alginic sulfate[$]	35 (0)	Chitosan Sulfate[†]	19 (2)
Ardeparin	62 (1)	CSA	1.2 (3)
Certoparin	65 (4)	CSC (7)	0.8 (2)
Curdlan sulfate (10)	30 (0)	Curdlan sulfate[$,] (10)	11 (0)
Dalteparin (LMW heparin)	70 (5)	Danaparoid	4 (1)
De-N-sulfated enoxiparin	66 (0)	De-N-Ac Chitosan	0 (1)
Enoxiparin (LMW heparin)	63 (5)	De-N-Ac CSC	0 (1)
Ghatti Sulfate[†]	73 (3)	Ethyl cellulose sulfate	4 (8)
Hypromellose sulfate	20 (1)	Gum Rosin sulfate	3 (2)
Locust bean gum sulfate[†]	46 (3)	Hyaluronic acid	0 (3)
N-Ac enoxiparin	21 (1)	Heparan sulfate	6 (6)
Pectin sulfate[$]	28 (3)	Hydroxyethyl cellulose sulfate	0 (0)
Pullulan sulfate	41 (6)	Methylcellulose sulfate	0 (0)
Reviparin	60 (0)	N-propylated heparin (porcine)	3 (1)
Sulodexide	37 (2)	Propylmethyl sulfate	0 (3)
Tinzaparin	70 (6)	Scleroglucan Sulfate[†]	0 (0)
Tylose sulfate	23 (1)	Starch sulfate	13.3 (1)
		Storax sulfate	10 (1)
		Xanthan sulfate	6 (3)

915 Compounds were tested in growth inhibition assays at 2, 10, 20 and 100 μg/ml. Weakly
916 inhibitory and non-inhibitory compounds are listed according to estimated IC_{50} of between 20
917 and 100 μg/ml or non-inhibitory if <20% growth inhibition at 100μg/ml. Inhibition activity at
918 100 μg/ml is indicated. Data are means of two assays performed in duplicate ± s.e.m.
919 Abbreviations: CS, chondroitin sulfate, Ac, acetylated. [†] Prepared using pyridine sulfur
920 trioxide complex. [$] Prepared using piperidine-*N*-sulfonic acid.
921

Figure 1


Inhibition (% of PBS control)
100
75
50
25
0
HS-4mer-Ac1S0
HS-4mer-Ac2S0
HS-4mer-Ac1S1
HS-4mer-Ac1S2
*HS-6mer-Ac1S2
*HS-6mer-Ac1S2
*HS-6mer-Ac1S3
*HS-6mer-Ac1S3
*hep-4mer-Ac0S5
*hep-4mer-Ac0S5
hep-4mer-Ac0S6
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hep-6mer-Ac0S8
hep-6mer-Ac0S9
hep (full length)

Figure 2

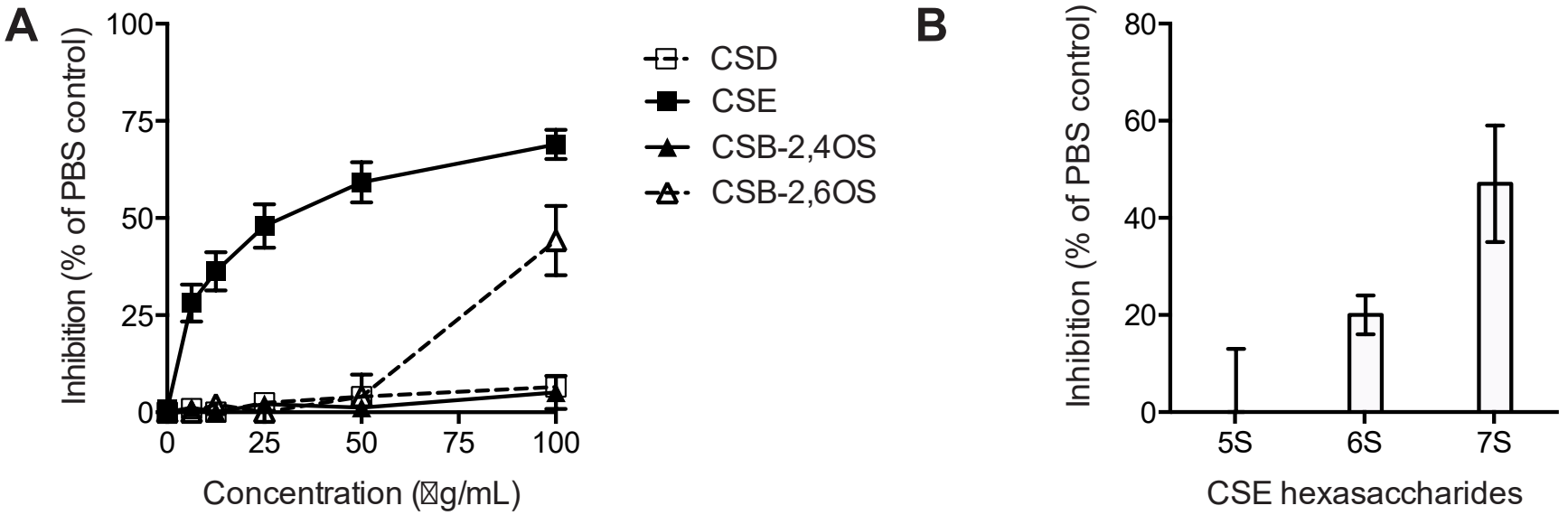

A
Inhibition (% of PBS control)
100
75
50
25
0
0
25
50
75
100
Concentration (⊠g/mL)
CSD
CSE
CSB-2,4OS
CSB-2,6OS
B
Inhibition (% of PBS control)
80
60
40
20
0
5S
6S
7S
CSE hexasaccharides

Figure 3

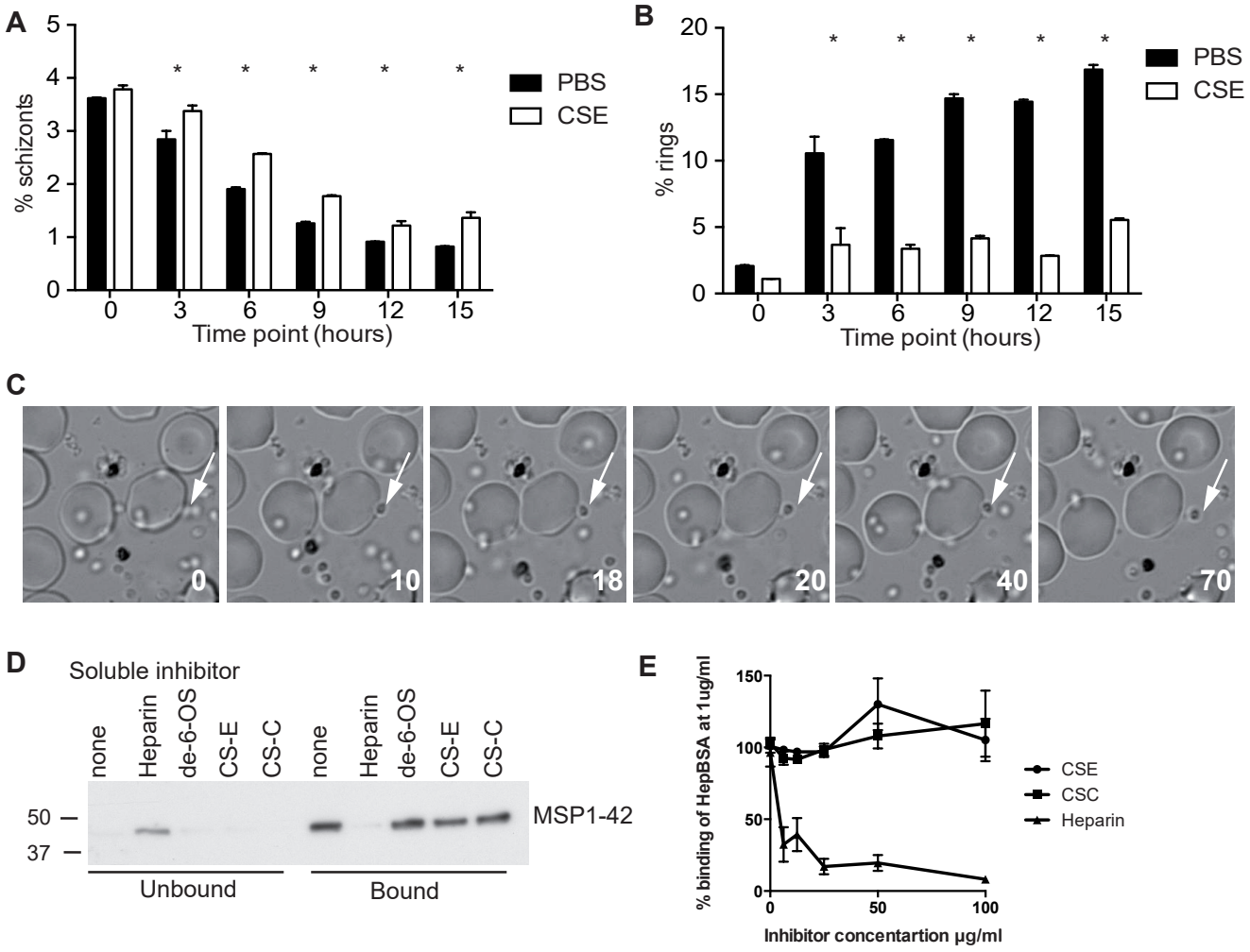
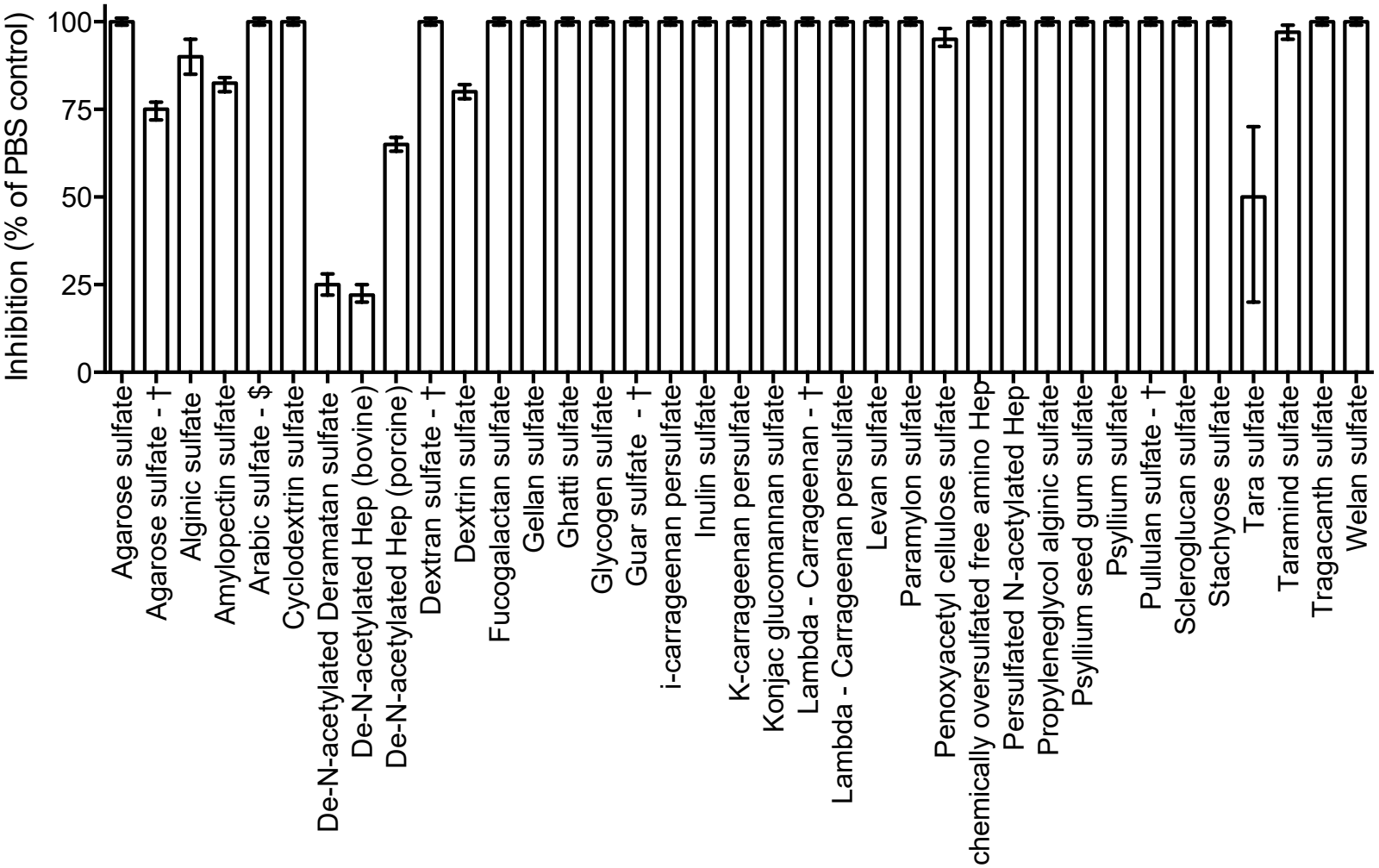

A
% schizonts
Time point (hours)
PBS
CSE
B
% rings
Time point (hours)
PBS
CSE
C
0
10
18
20
40
70
D
Soluble inhibitor
none
Heparin
de-6-OS
CS-E
CS-C
none
Heparin
de-6-OS
CS-E
CS-C
50
37
MSP1-42
Unbound
Bound
E
% binding of HepBSA at 1ug/ml
Inhibitor concentartion µg/ml
CSE
CSC
Heparin

Figure 4


Inhibition (% of PBS control)
100
75
50
25
0
Agarose sulfate
Agarose sulfate - †
Alginic sulfate
Amylopectin sulfate
Arabic sulfate - $
Cyclodextrin sulfate
De-N-acetylated Deramatan sulfate
De-N-acetylated Hep (bovine)
De-N-acetylated Hep (porcine)
Dextran sulfate - †
Dextrin sulfate
Fucogalactan sulfate
Gellan sulfate
Ghatti sulfate
Glycogen sulfate
Guar sulfate - †
i-carrageenan persulfate
Inulin sulfate
K-carrageenan persulfate
Konjac glucomannan sulfate
Lambda - Carrageenan - †
Lambda - Carrageenan persulfate
Levan sulfate
Paramylon sulfate
Penoxyacetyl cellulose sulfate
chemically oversulfated free amino Hep
Persulfated N-acetylated Hep
Propyleneglycol alginic sulfate
Psyllium seed gum sulfate
Psyllium sulfate
Pullulan sulfate - †
Scleroglucan sulfate
Stachyose sulfate
Tara sulfate
Taramind sulfate
Tragacanth sulfate
Welan sulfate