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

4 **RUNNING TITLE: Polysaccharide inhibitors of malaria**

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25

26 **Abstract**

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

41

42 **INTRODUCTION**

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

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

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

75 that the dominant inhibitory activity of HLMs is mediated at the early invasion stages as
76 demonstrated with live-video microscopy of merozoite invasion that heparin blocks ‘pre-
77 invasion’ steps (7, 16). However, the capacity of HLMs to also bind proteins involved in
78 down-stream invasion steps may also contribute to effective inhibition and the observed
79 inability to select for heparin resistant parasites lines (7). Of further potential therapeutic
80 benefit, HLMs are also known to disrupt both rosetting and sequestration of infected RBCs
81 (15, 20-26), which are important mediators of pathogenesis. The ability of HLMs to inhibit
82 both merozoite invasion and sequestration/rosetting highlights the potential of these
83 molecules to reduce parasitemia and disease severity. Owing to the anticoagulant activity of
84 heparin it cannot be used as an antimalarial agent. However, it may be possible to reduce
85 anticoagulant activity of HLMs while maintaining inhibition of *P. falciparum* (7). Indeed,
86 curdlan sulfate, which has a ten-fold reduced anticoagulation activity compared to heparin,
87 has been tested in a small human trial which suggested that treatment reduced malaria disease
88 severity (27). Further, HLMs such as K5 polysaccharides, as well as other polyanions that
89 lack anticoagulant activity have been proposed as potential therapeutics for viral diseases
90 (reviewed in (28)), and can inhibit merozoite invasion (7).

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

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

104

105 **MATERIALS AND METHODS**

106 **Parasite culture**

107 *P. falciparum* 3D7 or D10-PfGFP isolates (30) were cultured as described (31, 32), in
108 culture media of RPMI-HEPES (pH 7.4) supplemented with 50 µg/ml hypoxanthine, 20
109 µg/ml gentamicin, 25 mM sodium bicarbonate (NaHCO₃) and 0.5% Albumax II (Gibco).
110 RBCs from group O+ blood donors were used to culture parasites. Cultures were gassed with
111 1% O₂, 4% CO₂, 95% N₂ and incubated at 37°C. Parasites were initially synchronized using
112 5% D-sorbitol-treatment for 5 minutes, as described (33). For invasion-inhibition assays,
113 sorbitol treatment cultures were further synchronised using heparin synchronization; heparin
114 cannot be used for the selection of heparin resistant cultures therefore it is unlikely that
115 heparin synchronization affected the testing of HLMs (7). Live video filming of merozoite
116 invasion was performed as described (7, 34).

117 **Growth inhibition assays and invasion inhibition assays**

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

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

127 Invasion inhibition assays with isolated merozoites were conducted as described (4,
128 37) (for detailed methods see Methods in Malaria Research, 2013
129 <https://www.beiresources.org/Publications/MethodsInMalariaResearch.aspx>). Highly
130 synchronized late-stage schizonts were magnet purified via Macs magnet separation column
131 (Macs; Miltenyi Biotec) and treated with E64 until mature merozoites were formed.
132 Merozoites were isolated by membrane filtration and incubated with uninfected RBCs at
133 0.5% haematocrit and test compounds at indicated concentrations, in 50µl volumes.
134 Estimated number of merozoites per test is approximately 7×10^6 merozoites. Invasion
135 occurred in agitated conditions for ten minutes, and then in static conditions for a further 20
136 minutes. Following invasion, cultures were washed twice and returned to culture media.
137 Parasites were analysed by flow cytometry at 40 hours post invasion as described for growth
138 inhibition assays.

139 **Modification of heparin-like-molecules**

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

147 3kDa low MW heparin) was purchased as Hibro manufactured by Laboratorios
148 Farmaceuticos Rovi SA.

149 Heparin compounds were modified with published methods as per following: Glycol-split
150 heparins and partially (50%) glycol split heparin were prepared by periodate oxidation
151 followed by NaBH₄ reduction as described previously (38-40). Fully de-sulfated heparin was
152 prepared by the treatment of their pyridinium salts with dimethyl sulfoxide containing 10% of
153 water at 100⁰C by the method of Nagasawa et al 1977 (41). De-N-sulfated and partially de-N-
154 sulfated heparins were prepared by treatment of their pyridinium salts with dimethyl
155 sulfoxide containing 5% of methanol for upto 1.5 h at 50⁰C (42). De-N-sulfated heparins
156 were N-acetylated by treatment of the heparin with acetic anhydride in 0.5M NaHCO₃ at 4⁰C
157 (43). 2-*O*-Desulfated heparins and glycol-split heparins were prepared as described by
158 dissolving the heparin in 0.2M NaOH followed by lyophilization (44), using an adaption
159 (45). Mucosal heparin (porcine) lacking 6-*O* sulfate (MH de 6S) was prepared by the
160 treatment of the pyridinium salt of heparin with N,*O*-bis(trimethylsilyl)acetamide in pyridine
161 for 2 h at 60⁰C (46). Heparin were decarboxylated by treatment with 1-ethyl-3-(3-dimethyl-
162 aminopropyl) carbodiimide and subsequent sodium borohydride reduction as described
163 previously ((47), adapted from (48)).

164 Mucosal heparin (porcine) (5-kDa MH, Smith degradation) was prepared by treatment
165 of periodate-oxidized mucosal heparin (porcine) with sodium hydroxide, followed by
166 reduction with sodium borohydride and acid hydrolysis (49). *O*-acylated derivatives (butyl
167 and hexyl) of heparin fragments were prepared from their tributylammonium salts in N,*N*-
168 dimethylformamide using carboxylic acid anhydrides and 4-(dimethylamino)pyridine as
169 catalyst (50, 51). MH peroxide 3-4kDa MH (H₂O₂) was prepared by hydrogen peroxide
170 induced free radical degradation (52). 3kDa MHgc (glycol split mucosal heparin (porcine))
171 was prepared by limited (10mins) nitrous oxide degradation of glycol split heparin at pH 4

172 (Reaction A, which cleaves both at glucosamine (Glc)NS and GlcNH) using the method of
173 Lindahl, U et al, 1973 (53) followed by sodium borohydride reduction. 3kDa MH gc-CHO
174 RT was prepared by the above method without subsequent borohydride reduction to leave a
175 terminal reactive aldehyde moiety. Hydrazone derivatives of 3kDa MH gc-CHO with 4-
176 phenylsemicarbazide or Benzhydrazide were prepared with a 5 fold molar excess of 4-
177 phenylsemicarbazide or Benzhydrazide in 100mM sodium acetate, pH 6 overnight at room
178 temperature (20⁰C). Reductive amination of 3kDa MH gc-CHO with Anthranilic acid or
179 ANTS (1,3,6 triSO₃-aminonaphthaline) were prepared with a 5 fold molar excess of
180 Anthranilic acid or ANTS (1,3,6 triSO₃-aminonaphthaline) and a 25 molar excess of sodium
181 cyanoborohydride (NaBH₃CN) in 100mM sodium acetate, pH 6 overnight at room
182 temperature (20⁰C).

183 **Confirmation of chemical modifications**

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

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

200 **Preparation of oligosaccharide fractions from heparin, heparan sulfate and chondroitin** 201 **sulfate E**

202 Heparin and HS oligosaccharide fragments were prepared as described (58). Briefly,
203 heparin (200 mg, from porcine intestinal mucosa, Sigma) was incubated with heparin lyase I
204 (100U, Sigma) and HS (200 mg, from porcine intestinal mucosa, Celsus) with heparinase III
205 (650 mU, IBEX Technologies, Montreal, Canada) (59) in 5 mM sodium phosphate buffer
206 (pH 7.1) containing 0.2 M NaCl. The digestion was carried out at 30 °C and stopped when
207 the reaction was 40% complete. After desalting on a short Sephadex G10 column, the
208 oligosaccharides were fractionated on a Bio-Gel P-4 column (1.6 × 90 cm) with elution by
209 0.1 M NH₄Cl (pH 3.5).

210 CSE (2 mg, from squid cartilage, AMS Biotechnology, Abingdon, England) was
211 digested with 20 mU of chondroitinase ABC (Sigma) in the same phosphate buffer (400 µl)
212 under identical conditions described above. Oligosaccharide fractionation was carried out on
213 a Superdex Peptide column (Amersham Biosciences, Little Chalfont, England) eluted by
214 0.05M ammonium acetate.

215 The tetra- and hexasaccharide fractions were subfractionated by strong-anion exchange
216 on a short cartridge column (HiTrap Q-Sepharose HP, 1 ml, Amersham Biosciences) with
217 detection at UV 232 nm. Elution was carried out with a linear gradient of NaCl (solvent A,
218 0.1 M NaCl; solvent B, 1.5 M NaCl; pH 3.5) as described (60). Oligosaccharide subfractions
219 were collected desalted and freeze-dried before quantitation by carbazole assay for hexuronic
220 acid content (61).

221 **Heparin binding assays**

222 For heparin-agarose bead binding assays proteins were extracted from *P. falciparum*
223 schizonts into 1% Triton X-100 in PBS as described (62). Proteins from culture supernatants
224 were collected by allowing highly synchronous schizonts to rupture into protein-free culture
225 medium and cells removed by centrifugation. Binding of solubilised proteins to heparin-
226 agarose beads was performed as described (7); heparin-agarose beads were washed twice in
227 PBS, then blocked with 1% casein PBS overnight at 4°C. Schizont protein extracts were
228 incubated overnight at 4°C with beads containing 0.1% casein and 200 µg/ml of test inhibitor
229 (heparin from Sigma-Aldrich, de-6-OS-heparin from Iduron, Alderley Edgy, U.K.), CSE
230 from Sikagaku, Tokyo, Japan, and CSC from Sigma-Aldrich), or PBS control (50 µl of
231 packed beads plus 100 µl of protein supernatant). Unbound proteins in the supernatant were
232 collected through Micro Bio-Spin Chromatography Columns (Bio-Rad) and beads washed 5
233 times with PBS containing 0.1% casein, 1% Triton X-100, and protease inhibitors. Bound
234 proteins were eluted from beads with 50 µl of warmed reducing sodium dodecyl sulfate–
235 polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Bound and unbound proteins
236 were separated by SDS-PAGE under reducing conditions and Western blotted onto
237 polyvinylidene difluoride membranes for probing with antibodies to detect MSP1-19. MSP1-
238 19 antibodies were raised in rabbits and purified as described (63).

239 Heparin binding to recombinant MSP1-42 in ELISA was performed as previously
240 described (7). Recombinant MSP1-42 (expressed as His-tagged proteins in *E. coli* (64), from
241 Carol Long – National Institute for Health) was coated (1µg/mL) onto 96-well plates (Nunc
242 Maxisorb) in PBS overnight at 4°C. Plates were washed and blocked with 1% casein, then
243 incubated with heparin-BSA or BSA, along with increasing concentrations of soluble
244 inhibitors of heparin, CSC and CSE. Plates were washed, and bound heparin-BSA/BSA was
245 detected with anti-BSA antibodies (rabbit, Sigma-Aldrich), followed by antirabbit-HRP, and
246 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}H₂O. The dialysed solution was frozen and
263 lyophilised before size exclusion chromatography was performed using HPLC grade H₂O
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 Omnic

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

277 **Assessing anticoagulation activity by activated partial thromboplastin time**

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

286 **Statistical analysis**

287 Statistical analysis was performed in Graph Pad Prism 6. Comparison of activity
288 between individual parent and their modified HLMs in growth inhibition assays was
289 performed using paired t-tests for each combination of compounds. P values were adjusted
290 using the Holm-Sidak method within each individual modification, to decrease the risk of
291 false discovery rates due to performing multiple comparisons. Overall impact of a specific
292 modification (for example, de-2-sulfation) was assessed by Wilcoxon matched-pair sign rank
293 test of all compounds with parent and modified compounds paired. For all comparisons
294 $p < 0.05$ is considered statistically significant.

295

296 **RESULTS**

297 **Heparin can be modified to increase inhibitory activity and remove anticoagulant**
298 **activity**

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

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

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

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

338 **Highly sulfated chondroitin sulfates inhibit *P. falciparum* growth**

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

344 2S at uronate and either 4S or 6S at galactosamine. CSE had substantial inhibitory activity
345 (IC_{50} approximately 25 $\mu\text{g/ml}$) (Figure 2a). Highly sulfated CSB-2,6-OS also had modest
346 inhibitory activity at high concentrations, whereas CSD was not inhibitory. This suggests
347 that, as for K5-heparin like molecules tested previously (7), location of at least 2 sulfates
348 together on a single oligosaccharide enhances inhibitory activity. To further investigate the
349 inhibitory activity of CSE a number of CSE hexasaccharides with different sulfation levels
350 were tested in invasion inhibition assays with purified merozoites. This assay differs from
351 standard growth assays as compounds are only incubated with merozoites and RBCs for 30
352 minutes while invasion is occurring, compared to growth assays which incubated drugs with
353 cultures over the course of the entire 48 hour blood-stage development cycle and invasion
354 inhibition assays are more sensitive than standard growth assays (37). Inhibition appeared to
355 increase with sulfation level; 50% inhibition was observed with CSE hexasaccharides having
356 7 sulfate groups, when tested at 100 $\mu\text{g/ml}$ (Figure 2b). However, CSE hexasaccharides had
357 no activity in standard growth inhibitory assays at these concentrations, possibly due to short
358 chain CS oligosaccharides having too few sulfate groups to mediate complete inhibition. The
359 discrepancy between standard growth and invasion inhibition assays is likely due to the
360 increased sensitivity of invasion assays that use purified merozoites (37), suggesting that the
361 inhibitory activity of compounds is at the threshold of that detected by standard growth
362 assays.

363 To characterize the functional mechanism of CSE inhibition, schizont rupture and
364 merozoite invasion in the presence of CSE was analysed via flow cytometry with
365 differentiation of parasite stages with ethidium bromide staining (7) and live video imaging
366 (16, 34). As with the inhibitory mechanism of heparin (7), cultures incubated with CSE
367 showed evidence of a slight delay of schizont rupture compared to uninhibited cultures
368 (Figure 3a), but the predominant mechanism of inhibition appeared to be merozoite invasion

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

382 **Identification of inhibitory sulfated carbohydrates**

383 Having shown that inhibitory activity appears to be reliant on sulfation level and that
384 longer chain lengths are needed for substantial activity, we tested a large panel of
385 polysaccharides to test the impact of sulfation and re-sulfation on inhibitory activity, and to
386 attempt to identify polysaccharides that have potential to be used as the base compounds for
387 novel drug development (for the full list of compounds and sources see Table S2).
388 Polysaccharides were tested in standard growth inhibitory assays at 2, 10, 20 and 100 $\mu\text{g/ml}$.
389 We tested 87 compounds prepared from a variety of sources with different levels and patterns
390 of sulfation. Initial testing identified 50 compounds with inhibitory activity of greater than
391 20% when tested at 20 $\mu\text{g/ml}$ or lower, with 14 compounds being highly inhibitory at
392 concentrations of 2 $\mu\text{g/ml}$ (Table 4). The other 37 compounds showed weak or no inhibitory
393 activity and were not further studied (Table 5). The stage-specificity of inhibition of

394 merozoite invasion of inhibitory compounds with an $IC_{50} < 10 \mu\text{g/ml}$ in growth inhibition
395 assays was confirmed in direct invasion inhibition assays using purified merozoites (Figure
396 4) (4, 37). The most highly inhibitory compounds with an IC_{50} of $< 2 \mu\text{g/ml}$ determined in
397 standard growth inhibition assays and that had confirmed invasion inhibitory activity were
398 chemically over-sulfated *i*-carrageenan, inulin sulfate, propyleneglycol alginic sulfate,
399 psyllium sulfate, scleroglucan sulfate, tragacanth sulfate, xylan sulfate (also known as
400 pentosan polysulfate), chemically over-sulfated λ -carrageenan, pullulan sulfate and
401 chemically over-sulfated dextran. De-N-acetylated dermatan sulfate and de-N-acetylated
402 heparin (bovine) also had inhibitory activity in standard 48 hour growth assays; however, this
403 was not confirmed in direct invasion inhibitory assays, suggesting that inhibition seen in
404 growth assays was not specific to merozoite invasion and may be due to non-specific activity
405 of the sample or other mechanisms of inhibition. The anti-coagulation activity of highly
406 inhibitory compounds was tested by assessing activated partial thromboplastin time. All
407 compounds had reduced anti-coagulation activity compared to heparin (Supplementary Table
408 3), indicating that these compounds may be more suitable base compounds for future drug
409 development.

410

411 **Discussion**

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

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

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

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

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

456 A major priority for future development of drugs based on this approach is the
457 generation of compounds with much greater potency. This might be achieved through
458 chemical modification of compounds, or through the synthesis of mimetics with a similar
459 mechanism of action. Further, inhibitory HLMs and the identified CS oligosaccharide
460 compounds with inhibitory activity may in the future be the basis for the development of
461 modified HLMs with increased bioavailability and improved inhibition activity.
462 Modifications may include those identified above, including periodate treatment and
463 esterification. The activity of 4-mer and 6-mer oligosaccharides suggests it may be possible
464 to identify and optimise short saccharides with high invasion inhibition activity; while our
465 data suggests that a 6-mer oligosaccharide is needed for inhibitory activity of heparin,
466 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-cysteine
472 (70) and deoxycholic 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 $IC_{50} < 10\mu\text{g/ml}$ 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 sulfate, glycogen sulfate, guar sulfate, inulin
482 sulfate, konjac glucomannan sulfate, levan sulfate, paramylon sulfate, penoxycetyl cellulose
483 sulfate, 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 $10\mu\text{g/ml}$. 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

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

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

515

516 **LIST OF ABBREVIATIONS:**

517 CS – chondroitin sulfate
518 gc – glycol splitting
519 Glc – glucosamine
520 HLMs – heparin-like-molecules
521 HS – heparan sulfate
522 LH – lung heparin
523 MH – mucosal heparin
524 MSP1 – merozoite surface protein 1
525 RBC – red blood cell

526

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541

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811

812

813

814 **Figure legends:**

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

817 Fractionated heparin and heparin sulfate tetra- and hexa-saccharides were tested for growth
818 inhibitory activity in two cycle assays. Data are mean \pm s.e.m. from two assays in duplicate.

819 Abbreviations: HS, heparan-sulfate, hep heparin, 4mer tetrasaccharide, 6mer

820 hexasaccharides. N-sulfation is indicated as Ac1-2, while lack of N-sulfation is Ac0. O-

821 sulfation is indicated as S0-9. Compounds listed with * are different preparations of the same

822 fraction.

823

824 **Figure 2: Identification of chondroitin sulfates with inhibitory activity against *P.***

825 ***falciparum* merozoite invasion.**

826 **A.** CSD, CSE and highly sulfated CSB polysaccharides were tested in growth inhibition

827 assays at concentrations 0-100 μ g/ml. Data are means \pm s.e.m. of three assays in duplicate. **B.**

828 Fractionated CSE hexasaccharides were tested in invasion inhibition assays. Degree of

829 sulfation is 5, 6, or 7 sulfate groups per molecule. Data are mean \pm range of one assay in

830 duplicate.

831

832 **Figure 3: CSE disrupts initial contact of the merozoite to the RBC, but not heparin**

833 **binding to MSP1-42.**

834 Flow cytometry of late stage parasite cultures with parasite stages differentiated based on

835 ethidium bromide staining was used to track parasite rupture as % schizonts (**A**), and

836 merozoites invasion as % ring forms (**B**) in CSE (100 μ g/ml and PBS/uninhibited cultures.

837 After 3 hours of incubation there were increased frequencies of schizonts and decreased ring
838 forms in CSE incubated cultures. Data are mean \pm s.e.m of two assays performed in
839 duplicated, * $p < 0.05$. (C) Live video microscopy of merozoite invasion in the presence of
840 CSE. Merozoites were able to make initial contact with the RBC, but contact was not
841 sustained, and merozoites disassociated from the RBC surface. Seconds is indicated in lower
842 right corner, and the white arrow highlights a single merozoite that attached and then
843 disassociated from the RBC. (D) Heparin-bead binding assays with *P. falciparum* protein
844 extract. Protein extract was incubated with heparin-beads along with soluble inhibitors as
845 indicated. Unbound and bead-bound fractions were probed for MSP1-42 binding via western
846 blots. MSP1-42 was found in the unbound fraction when incubated with heparin as a soluble
847 inhibitor, indicating that soluble heparin was able to out-compete binding. However, MSP1-
848 42 was found in the bound fraction when incubated with soluble de-6-OS heparin, CSE or
849 CSC indicating that this compounds were not able to compete with heparin-binding. (E)
850 MSP1-42 coated on ELISA plates was incubated with heparin-BSA along with soluble
851 heparin, CSC and CSE at increasing concentrations. Binding of heparin BSA was detected
852 with anti-BSA antibodies. Soluble heparin, but not CSE or CSC inhibited binding of heparin-
853 BSA to MSP1-42.

854

855 **Figure 4: Invasion inhibition activity of sulfated polysaccharides.**

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

862

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

865

Modification	Parent compounds	Inhibition % (s.e.m.)		Gain of inhibition
		Parent	Modified	
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 ₂ O ₂	57 (4)	62 (4)	5
	Enoxaparin	64 (5)	80 (3)	14
	Bemiparin	45 (2)	31 (5)	- 14
	Fondaparinux	34 (0)	11 (4)	- 23 *

866

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

876

877

878

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**

Modification	Parent compounds	Inhibition % (s.e.m.)		Loss in inhibition
		Parent	Modified	
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 ₂ O 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 ₂ O ₂	57 (4)	15 (3)	42 *
	MH H ₂ O 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 *
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881

882 Modified heparin compounds were tested for inhibition of *P. falciparum* in growth inhibition

883 assays at 100 µg/ml. Comparisons were made between parent and de-sulfated modified

884 compounds. In the majority of cases de-sulfation result in reduction in inhibition activity.

885 Loss of inhibitory activity is calculated as the (inhibition of the parent – inhibition of

886 modified). Positive values indicate reduced inhibitory activity in the modified compound

887 compared to the parent. Negative values indicate increased inhibitory activity in the modified

888 compound compared to the parent. Significant differences in inhibitory activity between

889 parent and modified compounds are indicated with * ($p > 0.05$, corrected for multiple

890 comparisons by Holm-Sidak method). Significant difference of modification across groups of

891 modified compounds is indicated with %. Data is mean inhibition \pm s.e.m. of two assays in

892 duplicate.. Abbreviations: MH, mucosal heparin (porcine); LH, lung heparin (bovine), gc,

893 glycol splitting.

894

895 **Table 3: Invasion inhibition activity of heparin compounds of different sizes against**
 896 **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)

897
 898 Heparin compounds of different oligosaccharide chain length were tested for inhibition of *P.*
 899 *falciparum* in growth inhibition assays at 100 µg/ml. Data is mean inhibition ± s.e.m. of two
 900 assays in duplicate. Mucosal heparin is from porcine and lung heparin is from bovine
 901 sources.

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

Very strong inhibitors	Inhibition	Strong inhibitors	Inhibition	Moderate inhibitors	Inhibition
Estimated IC₅₀	% (s.e.m)	Estimated IC₅₀	% (s.e.m.)	Estimated IC₅₀	% (s.e.m)
<2µg/ml	(2 µg/ml)	2-10 µg/ml	(10 µg/ml)	10-20 µg/ml	(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 ^s	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 ^s (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)

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

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

913

Weakly inhibitory		Non-inhibitory	
Estimated IC ₅₀ 20-100 µg/ml	Inhibition % (SD) (100 µg/ml)	<20% inhibition at 100 µg/ml	Inhibition % (SD) (100 µg/ml)
Alginate 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)

914

915 Compounds were tested in growth inhibition assays at 2, 10, 20 and 100 $\mu\text{g/ml}$. Weakly
916 inhibitory and non-inhibitory compounds are listed according to estimated IC_{50} of between 20
917 and 100 $\mu\text{g/ml}$ or non-inhibitory if $<20\%$ growth inhibition at 100 $\mu\text{g/ml}$. Inhibition activity at
918 100 $\mu\text{g/ml}$ is indicated. Data are means of two assays performed in duplicate \pm 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

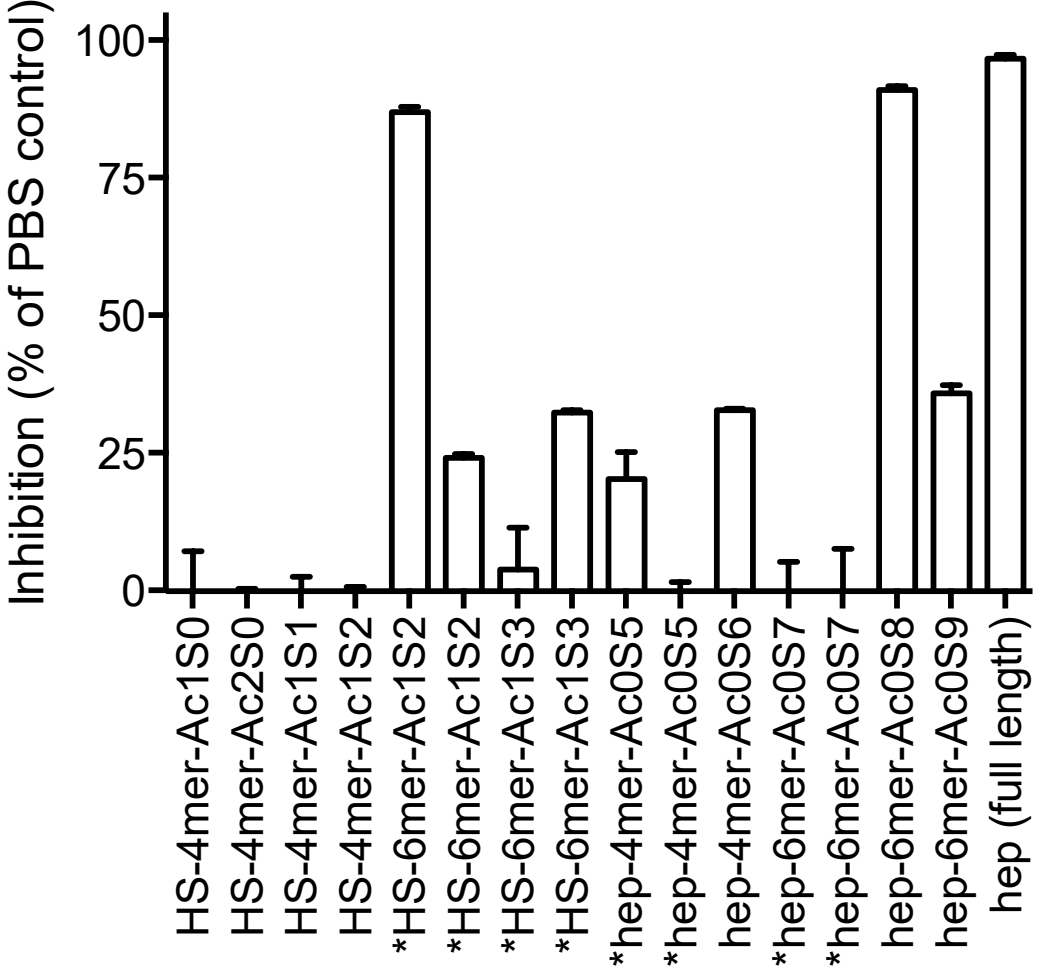


Figure 2

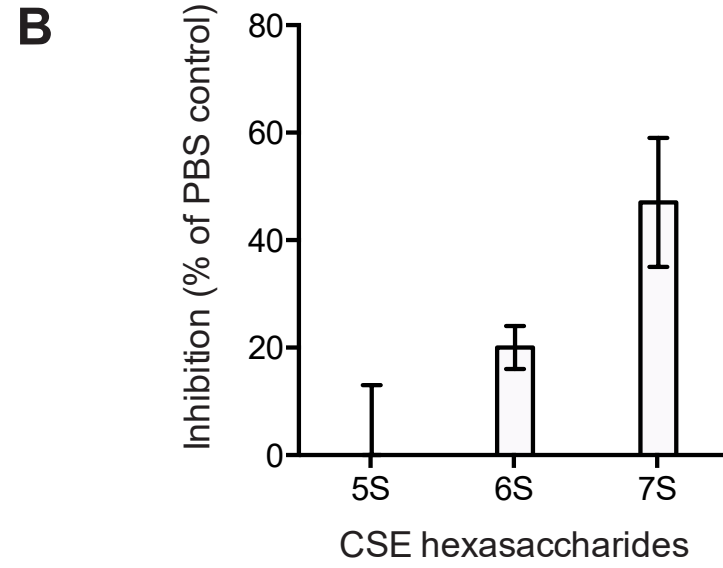
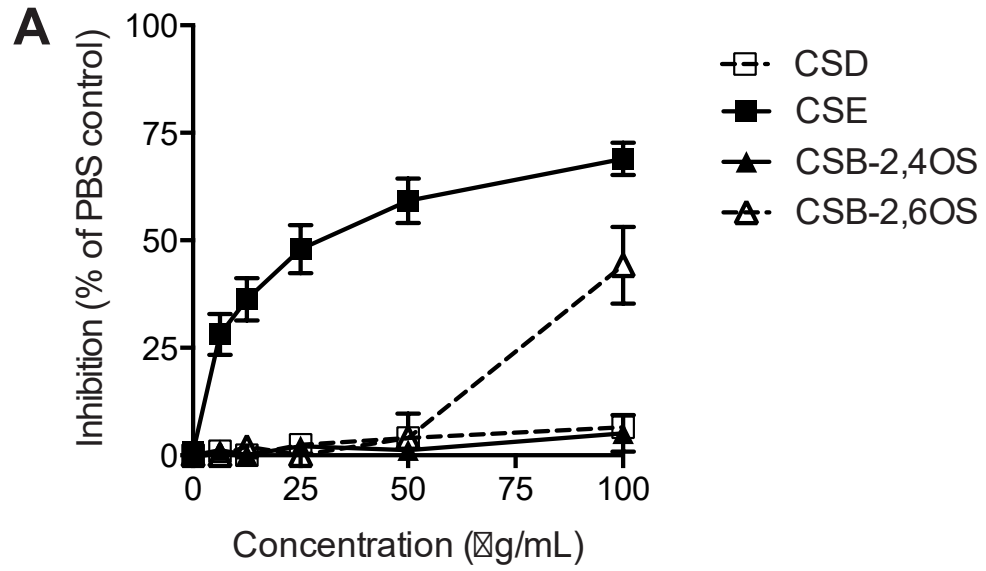


Figure 3

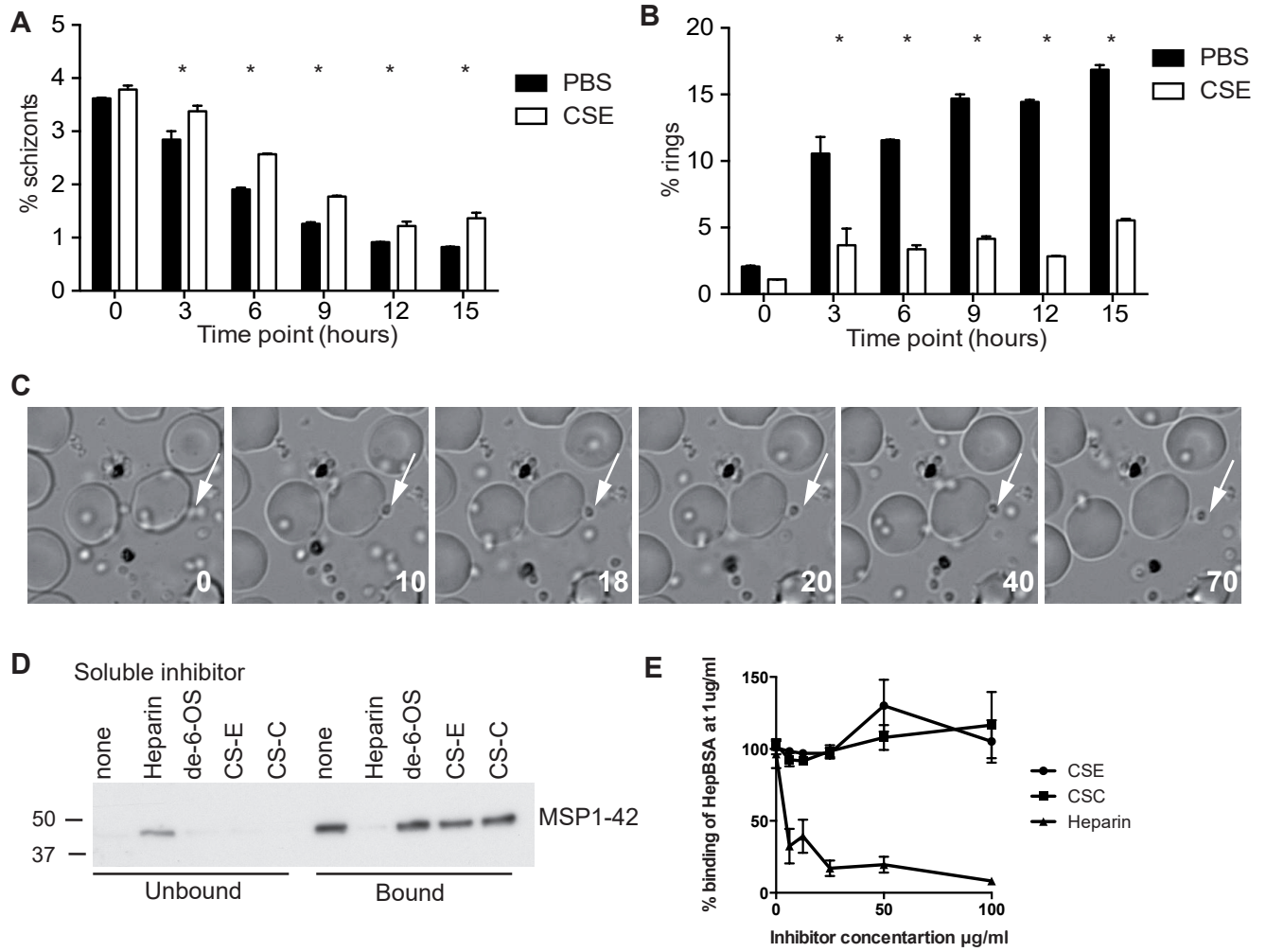


Figure 4

