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Sulfated cyclodextrins inhibit the entry of *Plasmodium* into red blood cells
Implications for malarial therapy

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ABSTRACT

The effect of sulfated cyclodextrins on *Plasmodium falciparum* cultures was determined. α-, β-, and γ-Cyclodextrins having equal degrees of sulfation inhibited parasite viability to a similar degree, a result suggesting that the ring size of the cyclodextrin is not a critical factor for inhibitory activity. β-Cyclodextrins containing fewer than two sulfate groups had no inhibitory activity, however, compounds containing 7–17 sulfates were found to be active in the μM range. Examination of treated cultures indicated that intracellular forms of the parasite were unaffected; however, increased numbers of extracellular merozoites were present. Active compounds produced enhanced erythrocyte staining with cationic dyes that could be reduced by stilbene disulfonates, a result suggesting that sulfated cyclodextrins inhibit parasite growth by interacting with the anion transport protein, AE1. Compounds that were found to be active in *P. falciparum* cultures were also found to inhibit *P. berghei* merozoite entry and could reduce the parasitemia of *P. berghei* infection in a mouse model, results suggesting that these compounds inhibit a common step in the merozoite invasion process of at least two *Plasmodium* species.

1. Introduction

Despite optimism in the 1960s and 1970s that malaria might be eradicated, this parasitic infection continues to cause widespread morbidity and mortality [1,2]. Chloroquine has formed the cornerstone of malaria treatment for over five decades [3]; however, resistance to chloroquine has emerged and is now widespread [2]. The spread of chloroquine-resistant malaria has led to a global health disaster and has created an urgent need for novel and inexpensive antimalarials [4]. The intra-erythrocytic development of *Plasmodium falciparum*, the causative agent of the most lethal of human malarias, takes 48 h and leads to the production of merozoites, which then emerge and rapidly invade new host cells [5]. The ligands and receptors responsible for the invasion process have not been completely characterized [6]; however, individual *Plasmodium* species are frequently limited to a particular host, or even a particular stage of development of

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an erythrocyte, by the specificity of the ligand/receptor interactions involved in erythrocyte invasion [7]. Previous reports have indicated that saccharide anions can inhibit the invasion of erythrocytes by merozoites [8,9], the invasion of hepatic cells by sporozoites [10,11], the formation of rosettes with unparasitized erythrocytes [12], and the adherence of parasitized erythrocytes to chondroitin sulfate A [9]. Heparin, dextran sulfate, fucoidin, and chondroitin sulfates all inhibit one or several parasite/host interactions [8]; however, their large polydisperse nature and other *in vivo* activities have hampered their development as therapeutic agents. We have previously reported [13] that short-chain aliphatic polysulfonates inhibit merozoite invasion in *P. falciparum* cultures, and slow the increase of parasitemia in mice infected with *P. berghei*. In the present study we have examined the effect of sulfated cyclodextrins on merozoite invasion in an attempt to better define the structural features that inhibit the merozoite/red-cell interaction. While we cannot rule out an interaction between our active compounds and a merozoite, at a minimum our results suggest that sulfated cyclodextrins interact with a component of the erythrocyte membrane and may thereby disrupt the invasion process. Compounds that inhibited *P. falciparum* cultures were also found to be active in a mouse model of malaria suggesting that polysulfated materials inhibit an aspect of merozoite invasion that is common to at least two *Plasmodium* species.

2. Materials and methods

2.1. *Chemical synthesis and structural characterization of cyclodextrin derivatives*

2.1.1. *General*

β-Cyclodextrin (**3**) and all other reagents were obtained from Aldrich. ^{1}H and ^{13}C NMR spectra were recorded at room temperature on a Bruker AVANCE 300 spectrometer at 300 and 75 MHz, respectively. The signals arising from residual protons in the deuterated solvents were used as internal standards. Chemical shifts (δ) are reported in ppm from tetramethylsilane. Mass spectra were recorded using a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a standard nitrogen laser (337 nm). Positive-ion spectra were acquired with delayed extraction and reflectron mode. The sample spot was scanned with a laser beam under video observation, and spectra were acquired by averaging 200–500 individual laser shots and the data were processed using Data Explorer software (Applied Biosystems). Matrix A: a 10 mg/mL solution of 2,5-dihydroxybenzoic acid in CH_3CN–H_2O (33:67, v/v) containing 0.1% trifluoroacetic acid; matrix B: a 10 mg/mL solution of 3-hydroxypicolinic acid in CH_3CN–H_2O (33:67, v/v) containing 0.1% trifluoroacetic acid; matrix C: a 5 mg/mL solution of the sample (**1**–**6**, or **8**) in CH_3CN–H_2O (33:67, v/v) containing 0.1% trifluoroacetic acid. A 1:1:2 mixture of A, B, and C was vortexed for 1 min and then 1 μL of this mixture was applied on the sample plate and the plate was air-dried before being placed in the mass spectrometer for MALDI-MS analysis. For sample **7**, MALDI-MS analysis was performed in a different matrix mixture; matrix D: a 10 mg/mL solution of 2-cyano-4-hydroxycinnamic acid in CH_3CN–H_2O (30:70, v/v) containing 0.1% trifluoroacetic acid; matrix E: a 10 mg/mL solution of 3-hydroxypicolinic acid in CH_3CN–H_2O (30:70, v/v) containing 0.1% trifluoroacetic acid; matrix F: a 10 mg/mL solution of the sample (**7**) in CH_3CN–H_2O (30:70, v/v). A 1:1:1 mixture of D, E, and F was vortexed for 1 min and then 1 μL of this mixture was applied on the sample plate and the plate was air-dried before being placed in the mass spectrometer for MALDI-MS analysis. Mass peaks corresponding to $[M + Na]^+$ were obtained for the mixtures of sulfated cyclodextrins. Elemental analyses were performed by Canadian Microanalytical Service Ltd. (Delta, BC). Because of the possibility of hydrated forms in each sample mixture, the determination of the degree of sulfation was based on the ratio of %S to %C.

2.1.2. *Sodium α-cyclodextrin sulfate (0.8 average degree of sulfation) (**1**)*

To a solution of α-cyclodextrin (2.0 g, 2.1 mmol) in pyridine (40 mL) was added sulfur trioxide–pyridine complex (2.2 g, 13.8 mmol, 6.6 equiv.) at room temperature under argon. The reaction mixture was heated at 50–55 °C and stirred at this temperature for 2 h. After the removal of pyridine, the residue was washed sequentially with diethyl ether (2× 30 mL) and Et_2O–$CHCl_3$ (1:1, v/v, 30 mL), and then dissolved in H_2O (30 mL). The solution was basified to pH 9 by the addition of 5N NaOH and poured into EtOH (500 mL). The precipitate was collected by filtration and dissolved in H_2O (30 mL). Sulfate ions were removed by addition of a solution of $Ba(OH)_2{\cdot}H_2O$ (4.3 g) in H_2O (10 mL). The precipitate of $BaSO_4$ was removed by filtration, and barium ions were removed by addition of Na_2CO_3 (2.4 g). The precipitate of $BaCO_3$ was removed by filtration and the filtrate was acidified to pH 3 using Amberlite ion-exchange resin (H^+ form). The resulting solution was concentrated to 20 mL. The solution was basified to pH 10 using 2N NaOH, and poured into EtOH (500 mL). The precipitate was removed by filtration and dissolved in H_2O (20 mL). The aqueous solution was decolorized using charcoal and lyophilized to give the product sodium β-cyclodextrin sulfate as a colorless solid (1.5 g)—^{1}H NMR (300 MHz, D_2O): δ 3.40–3.55 (m, 12H), 3.70–3.90 (m, 24H), 4.92 (d, *J* 3.3 Hz, 6H); ^{13}C NMR (75 MHz, D_2O): δ 60.9, 70.5, 72.3, 73.8, 81.7, 102.0; elemental analysis: C, 33.80; H, 5.19; S, 2.19; MALDI-MS $[M + Na]^+$, *m/z*: 995.3, 1097.2, 1199.1, 1301.1, corresponding to 0–3 sulfate groups.

2.1.3. *Sodium α-cyclodextrin sulfate (8.2 average degree of sulfation) (**2**)*

To a solution of α-cyclodextrin (2.0 g, 2.1 mmol) in pyridine (50 mL) was added sulfur trioxide–pyridine complex (10.0 g, 62.9 mmol, 30 equiv.) at room temperature under argon. The reaction mixture was heated at 60 °C and stirred at this temperature for 5 h. After the removal of pyridine, the residue was washed sequentially with diethyl ether (2× 30 mL) and Et_2O–$CHCl_3$ (1:1, v/v, 30 mL), and then dissolved in H_2O (30 mL). The solution was basified to pH 10 by the addition of 5N NaOH and poured into EtOH (500 mL). The precipitate was collected by filtration and dissolved in H_2O (30 mL). Sulfate ions were removed by addition of a solution of $Ba(OH)_2{\cdot}H_2O$ (6.0 g) in H_2O (30 mL). The precipitate of $BaSO_4$ was removed by filtration, and barium ions were removed by addition of Na_2CO_3 (3.4 g).

The precipitate of $BaCO_3$ was removed by filtration and the filtrate was acidified to pH 3 using Amberlite ion-exchange resin (H^+ form). The solution was basified to pH 10 using 2N NaOH, and poured into EtOH (600 mL). The precipitate was removed by filtration and dissolved in H_2O (30 mL). The aqueous solution was decolorized using charcoal and lyophilized to give the product sodium β-cyclodextrin sulfate as a colorless solid (2.1 g)—^{1}H NMR (300 MHz, D_2O): δ 3.50–4.50 (m, 36H), 4.90–5.60 (m, 6H); elemental analysis: C, 19.07; H, 3.43; S, 11.65; MALDI-MS $[M + Na]^+$, *m/z*: 995.3, 1097.2, 1199.2, 1301.1, 1403.1, 1505.0, 1606.9, 1708.9, 1810.8, corresponding to 0–8 sulfate groups.

2.1.4. *Sodium β-cyclodextrin sulfate (1.7 average degree of sulfation) (**4**)*

To a solution of β-cyclodextrin (**3**, 2.0 g, 1.8 mmol) in pyridine (40 mL) was added sulfur trioxide–pyridine complex (2.2 g, 13.8 mmol, 7.7 equiv.) at room temperature under argon. The reaction mixture was heated at 55 °C and stirred at this temperature for 2 h. After the removal of pyridine, the residue was washed sequentially with diethyl ether (2× 30 mL) and Et_2O–$CHCl_3$ (1:1, v/v, 30 mL), and then dissolved in H_2O (30 mL). The solution was basified to pH 9 by the addition of 5N NaOH and poured into EtOH (500 mL). The precipitate was collected by filtration and dissolved in H_2O (30 mL). Sulfate ions were removed by addition of a solution of $Ba(OH)_2{\cdot}H_2O$ (4.3 g) in H_2O (30 mL). The precipitate of $BaSO_4$ was removed by filtration, and barium ions were removed by addition of Na_2CO_3 (2.4 g). The precipitate of $BaCO_3$ was removed by filtration and the filtrate was acidified to pH 3 using Amberlite ion-exchange resin (H^+ form). The solution was basified to pH 9 using 2N NaOH, and poured into EtOH (600 mL). The precipitate was removed by filtration and dissolved in H_2O (30 mL). The aqueous solution was decolorized using charcoal and lyophilized to give the product sodium β-cyclodextrin sulfate as a colorless solid (1.5 g)—^{1}H NMR (300 MHz, D_2O): δ 3.40–3.60 (m, 14H), 3.70–3.90 (m, 28H), 4.94 (d, *J* 3.9 Hz, 7H); ^{13}C NMR (75 MHz, D_2O): δ 60.7, 71.0, 72.2, 72.6, 73.6, 81.6, 102.4; elemental analysis: C, 31.31; H, 5.03; S, 3.29; MALDI-MS $[M + Na]^+$, *m/z*: 1157.3, 1259.2, 1361.1, 1463.1, corresponding to 0–3 sulfate groups.

2.1.5. *Sodium β-cyclodextrin heptasulfate (**5**)*

β-Cyclodextrin (**3**) was converted into heptakis(2,3-di-*O*-acetyl)-β-cyclodextrin by the method of Takeo et al. [14]. The di-*O*-acetyl derivative was sulfated using a modified procedure of Chen et al. [15] and the product was *O*-deacetylated to afford sodium β-cyclodextrin heptasulfate (**5**) [16] as follows. To a solution of heptakis(2,3-di-*O*-acetyl)-β-cyclodextrin (500 mg, 0.29 mmol) in DMF (10 mL) was added sulfur trioxide–pyridine complex (970 mg, 6.1 mmol, 21 equiv.) at room temperature under argon. The reaction mixture was heated at 100 °C for 6 h, then at 60 °C overnight. After it was cooled to room temperature, the reaction mixture was poured into a mixed solution of EtOH (400 mL) and hexane (200 mL). A solution of the precipitate in H_2O (5 mL) was basified to pH 12 by the addition of 2N NaOH, MeOH (2 mL) was added, and the solution was stirred overnight. The solution was poured into EtOH (300 mL) and the precipitate was collected by filtration and dissolved in H_2O (5 mL). Sulfate ions were removed by addition of a solution of $Ba(OH)_2{\cdot}H_2O$ (100 mg) in H_2O (5 mL). The precipitate of $BaSO_4$ was removed by filtration, and barium ions were removed by addition of Na_2CO_3 (65 mg). The precipitate of $BaCO_3$ was removed by filtration and the filtrate was acidified to pH 3 using Amberlite ion-exchange resin (H^+ form). The solution was basified to pH 12 using 2N NaOH, and then poured into EtOH (300 mL). The precipitate was removed by filtration and dissolved in H_2O (10 mL). The aqueous solution was decolorized using charcoal and lyophilized to give the product sodium β-cyclodextrin heptasulfate (**5**) as a colorless solid (241 mg, 0.13 mmol, 45%)—^{1}H NMR (300 MHz, D_2O): δ 3.61 (dd, *J* 3.6, 9.9 Hz, 7H), 3.68 (t, *J* 9.3 Hz, 7H), 3.99 (t, *J* 9.6 Hz, 7H), 4.12 (d, *J* 9.6 Hz, 7H), 4.25–4.40 (m, 14H), 5.18 (d, *J* 3.3 Hz, 7H); ^{13}C NMR (75 MHz, D_2O): δ 67.1, 69.9, 72.3, 73.3, 79.3, 101.0. The ^{1}H NMR spectrum of **5** was identical to that shown by Vincent et al. [16].

2.1.6. *Sodium β-cyclodextrin sulfate (12.9 average degree of sulfation) (**6**)*

To a solution of β-cyclodextrin (**3**, 2.0 g, 1.8 mmol) in DMF (100 mL) was added sulfur trioxide–pyridine complex (8.4 g, 52.8 mmol, 29 equiv.) at room temperature under argon. The reaction mixture was heated at 60 °C with stirring for 24 h. After the removal of pyridine, the residue was washed sequentially with diethyl ether (2× 40 mL) and Et_2O–$CHCl_3$ (1:1, v/v, 40 mL), and then dissolved in H_2O (30 mL). The solution was basified to pH 9 by the addition of 5N NaOH and poured into EtOH (600 mL). The precipitate was collected by filtration and dissolved in H_2O (30 mL). Sulfate ions were removed by the addition of a solution of $Ba(OH)_2{\cdot}H_2O$ (5 g) in H_2O (30 mL). The precipitate of $BaSO_4$ was removed by filtration, and barium ions were removed by the addition of Na_2CO_3 (2.8 g). The precipitate of $BaCO_3$ was removed by filtration and the filtrate was acidified to pH 3 using Amberlite ion-exchange resin (H^+ form). The solution was basified to pH 9 using 2N NaOH, and poured into EtOH (600 mL). The precipitate was removed by filtration and dissolved in H_2O (30 mL). The aqueous solution was decolorized using charcoal and lyophilized to give the product sodium β-cyclodextrin sulfate as a colorless solid (2.2 g)—^{1}H NMR (300 MHz, D_2O): δ 3.45–4.50 (m, 42H), 5.00–6.00 (m, 7H); elemental analysis: C, 16.86; H, 3.34; S, 13.68; MALDI-MS $[M + Na]^+$, *m/z*: 1157.1, 1259.1, 1361.0, 1463.0, 1564.9, 1666.8, 1768.7, 1870.6, 1972.6, 2074.5, 2176.4, 2278.3, 2380.2, 2482.1, corresponding to 0–13 sulfate groups.

2.1.7. *Sodium β-cyclodextrin sulfate (16.9 average degree of sulfation) (**7**)*

To a solution of β-cyclodextrin (**3**, 2.0 g, 1.8 mmol) in pyridine (30 mL) was added sulfur trioxide–pyridine complex (8.4 g, 52.8 mmol, 29.3 equiv.) at room temperature under argon. The reaction mixture was heated at 60 °C with stirring for 48 h. After the removal of pyridine, the residue was washed sequentially with diethyl ether (2× 40 mL) and Et_2O–$CHCl_3$ (1:1, v/v, 40 mL), and then dissolved in H_2O (30 mL). The solution was basified to pH 9 by the addition of 5N NaOH and poured into EtOH (600 mL). The precipitate was collected by filtration and dissolved in H_2O (30 mL). Sulfate ions were removed by the addition of a solution of $Ba(OH)_2{\cdot}H_2O$ (5 g) in H_2O (30 mL). The precipitate of $BaSO_4$ was removed by

filtration, and barium ions were removed by the addition of Na_2CO_3 (2.8 g). The precipitate of $BaCO_3$ was removed by filtration and the filtrate was acidified to pH 3 using Amberlite ion-exchange resin (H^+ form). The solution was concentrated to 40 mL, and then basified to pH 9 using 2N NaOH, and poured into EtOH (600 mL). The precipitate was removed by filtration and dissolved in H_2O (20 mL). The aqueous solution was decolorized using charcoal and lyophilized to give the product sodium β-cyclodextrin sulfate as a colorless solid (2.3 g)—^{1}H NMR (300 MHz, D_2O): δ 3.37–4.50 (m, 42H), 4.85–5.70 (m, 7H); elemental analysis: C, 14.18; H, 2.94; S, 15.04; MALDI-MS $[M + Na]^+$, *m/z*: 1157.4, 1259.4, 1361.3, 1463.2, 1565.2, 1667.1, 1769.1, 1871.0, 1973.0, 2074.9, 2176.9, 2278.8, 2380.7, 2482.7, 2584.6, 2686.5, 2788.5, 2890.4, 2992.5, corresponding to 0–18 sulfate groups.

2.1.8. *Sodium γ-cyclodextrin sulfate (~8.2 average degree of sulfation) (8)*

To a solution of γ-cyclodextrin (1.0 g, 0.8 mmol) in pyridine (30 mL) was added sulfur trioxide–pyridine complex (4.3 g, 27.0 mmol, 33.8 equiv.) at room temperature under argon. The reaction mixture was heated at 60 °C with stirring overnight. After the removal of pyridine, the residue was washed sequentially with diethyl ether (2× 40 mL) and Et_2O–$CHCl_3$ (1:1, v/v, 40 mL), and then dissolved in H_2O (30 mL). The solution was basified to pH 9 by the addition of 5N NaOH and poured into EtOH (600 mL). The precipitate was collected by filtration and dissolved in H_2O (30 mL). Sulfate ions were removed by the addition of a solution of $Ba(OH)_2{\cdot}H_2O$ (4.0 g) in H_2O (30 mL). The precipitate of $BaSO_4$ was removed by filtration, and barium ions were removed by the addition of Na_2CO_3 (2.2 g). The precipitate of $BaCO_3$ was removed by filtration and the filtrate was acidified to pH 3 using Amberlite ion-exchange resin (H^+ form). The solution was basified to pH 9 using 2N NaOH, and poured into EtOH (600 mL). The precipitate was removed by filtration and dissolved in H_2O (30 mL). The aqueous solution was decolorized using charcoal and lyophilized to give the product sodium γ-cyclodextrin sulfate as a colorless solid (0.44 g)—^{1}H NMR (300 MHz, D_2O): δ 3.40–4.50 (m, 48H), 5.00–5.90 (m, 8H).

2.2. *Biological assays*

2.2.1. *Parasite culture and viability assays*

P. falciparum cultures were grown in O+ blood obtained by venipuncture of volunteers. Cultures of the laboratory line ItG were maintained by the method of Trager and Jensen [17] using RPMI 1640 supplemented with 10% human serum and 50 μM hypoxanthine (RPMI-A). The effects of the test compounds on the viability of *P. falciparum* cultures were evaluated using a lactate dehydrogenase (LDH) enzyme assay specific to the enzyme found in *P. falciparum* (pLDH) [18,19]. Briefly, compounds to be tested were dissolved in RPMI-A to achieve a concentration of 10 mg/mL and were then filter-sterilized by passing them through a 0.22-μm polycarbonate filter. Fifty microliters of RPMI-A were added to every well in a 96-well plate, then 50 μL of the 10 mg/mL compound solution were added to the first well, and then serially diluted across the plate to produce a compound gradient with two-fold dilutions. Fifty microliters of parasite culture (2% hematocrit, 2% parasitemia) were added to each well and the plates were then incubated at 37 °C in 95% N_2, 3% CO_2, and 2% O_2 for 72 h. The contents of the wells were then re-suspended and 15-μL samples were removed and added to 100 μL of pLDH enzyme assay mixture in the corresponding well of a second 96-well plate [18]. After 1 h the absorbance of the wells at 540 nm was determined using a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA.). The IC_{50} values of individual compounds were determined using a non-linear regression analysis of the data [13] using the computer program SigmaPlot (Jandel Scientific). The IC_{50} values represent the mean ± S.D., $N = 4$. The determination of the activity of compound **7** in *P. falciparum* and *P. berghei* cultures was performed using the SYBR-Green method [20] as previously described. *P. berghei* was obtained by cardiac puncture of an infected mouse (parasitemia of 5%); the blood was washed three times in RPMI and was then plated at a 2% hematocrit in a gradient of **7** as above. After 24 h the contents of the well were agitated to effect merozoite release [21] and the plate was incubated for a further 24 h before being processed as described in [22]. Relative fluorescence was determined using a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany).

2.2.2. *Invasion assays*

Parasite cultures were adjusted to 2% hematocrit and 2% parasitemia and 1 mL was added to each well of a 12-well plate. Groups of four wells were either left untreated (control) or were made up to a final concentration of 150 nM mefloquine or 200 μg/mL of **7** prior to the incubation (pre-treated) or 1 h prior to the end of the incubation period (post-treated). At the end of the incubation period the contents of each well were transferred to a 1.5-mL tube. Three 50-μL samples were then transferred to a 96-well assay plate to determine total relative fluorescence and the tubes were then centrifuged at 500 × *g* for 10 min. The supernatants of the tubes were placed in a new tube and centrifuged at 22,000 × *g* for 10 min before the volume present was reduced to 150 μL by aspirating the supernatant. After vortexing, three 50-μL samples per tube were transferred to a 96-well assay plate. The erythrocyte pellets were re-suspended in 500 μL of Tris-buffered saline (50 mM Tris–HCl, 150 mM NaCl, pH 7.4, TBS) and three 50-μL samples per tube were transferred to a 96-well assay plate. The relative fluorescence in the plates was then determined using a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany). Parallel assays were performed using equal volumes and concentrations of non-parasitized erythrocytes and the results obtained were subtracted from the values obtained from the corresponding *P. falciparum* culture samples.

2.2.3. *Determination of staining enhancement*

Fifty microliters samples of erythrocyte suspension (10^5 cells/mL in TBS) were added to a 96-well plate and mixed with 50-μL aliquots of test compound dissolved in TBS at a concentration of 200 μg/mL. The plate was then placed in an incubator (5% CO_2, 37 °C) for 2 h, after which the supernatant was removed by gentle aspiration; the blood film at the bottom of the well was allowed to air dry. The blood film was then fixed

by the addition of 100 μL of dry methanol for 2 min. The methanol was removed and 100 μL of a 0.5 mg/mL Methylene Blue solution (Sigma, St. Louis) were added. After 30 min at room temperature the stain solution was removed, the wells were rinsed twice with 200 μL of water, and 100 μL of DMSO were added to dissolve the contents of the wells. The optical density at 650 nm of each well was determined using a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA.). Treatment of erythrocytes with SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid, Molecular Probes) was performed by washing erythrocytes (suspended at 10^5 cells/mL) three times with phosphate buffered saline (50 mM PO_4^{3-}, 120 mM NaCl, pH 7.4, PBS) before incubating them in the presence of SITS dissolved in PBS for 30 min in the dark at 20 °C (total volume 100 μL). Compound **7** or **3** was then added to achieve a final concentration of 200 μg/mL in a volume of 150 μL. The plate was incubated at 37 °C for 2 h and then processed as above.

2.2.4. *Determination of compound activity in mice*

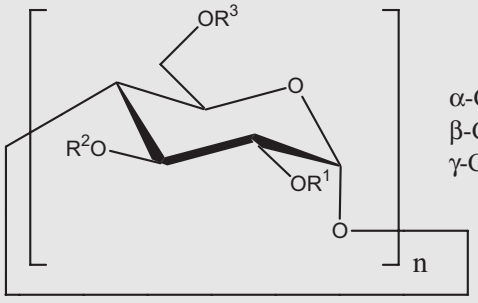
Female BALB/c mice (age, 6–8 weeks, Charles River, Montreal, Quebec, Canada) were used in all experiments. For each experiment the mice were divided into four groups of eight mice each. Each group was infected with *P. berghei* as described previously [13], and 24 h after infection either **3** or **7** was administered by intraperitoneal (i.p.) injection every 12 h (9 am and 9 pm) for 8 days. The quantities administered were based on the effective concentration determined in *P. falciparum* cultures, based on the assumptions that the compound was distributed evenly within the animal, and that a mouse weighed 25 g (i.e., an animal volume of 25 mL). At the end of the experiment the animals were killed by CO_2 narcosis. The compounds were dissolved in distilled water and administered by injection in a volume of 0.5 mL [13]. The progression of the parasitemia in each mouse was monitored as described previously [13]. Briefly, 10–15 μL of blood were collected from the tip of the tail once every 24 h; a blood smear was prepared and stained with Giemsa. The number of intracellular parasites per 100 RBCs was determined using an image analysis program (MC-5, version 4.0, Beta 2.0) from MCID Imaging Research Inc. (Brock University, St. Catharines, Canada). A minimum of 500 RBCs were counted per sample.

3. Results

3.1. *Chemical synthesis of cyclodextrin derivatives*

A series (**1**, **2**, **4**, **6**–**8**) of sulfated cyclodextrin derivatives (see Table 1) were synthesized by treatment of the respective cyclodextrin with sulfur trioxide–pyridine complex under varying conditions (see Section 2). In each case, the resulting products were heterogeneous mixtures of cyclodextrin sulfates (possible degrees of sulfation: 0–18 for α, 0–21 for β, and 0–24 for γ). The sulfated compounds synthesized were analyzed initially by MALDI-mass spectrometry ($[M + Na]^+$ peaks detected) to obtain an estimate of the range of the degree of sulfation in each sample mixture. As a typical example, the MALDI-MS spectra for compound **6** is shown in Fig. 1. It should be noted that the number of peaks observed corresponding to the sodium-adduct species was dependent upon the matrix employed in the MALDI experiment; the various matrices examined are described in Section 2. Consequently, elemental analysis were employed to determine the average degree of sulfation (see Table 2).

Table 1 – Structural and activity data for sulfated cyclodextrins



Compound	*n*	R^1	R^2	R^3	Average degree of sulfation[a]	Average molecular formula and weight	IC_{50} *Plasmodium falciparum* (μM)
1	6	H, SO_3Na	H, SO_3Na	H, SO_3Na	0.8	$C_{36}H_{59.2}Na_{0.8}O_{32.4}S_{0.8}$, M_W = 1054.48	>1000
2	6	H, SO_3Na	H, SO_3Na	H, SO_3Na	8.2	$C_{36}H_{51.8}Na_{8.2}O_{54.6}S_{8.2}$, M_W = 1809.62	44.4 ± 5.4
3	7	H	H	H	0	$C_{42}H_{70}O_{35}$, M_W = 1134.98	>1000
4	7	H, SO_3Na	H, SO_3Na	H, SO_3Na	1.7	$C_{42}H_{68.3}Na_{1.7}O_{40.1}S_{1.7}$, M_W = 1308.46	>1000
5	7	H	H	SO_3Na	7	$C_{42}H_{63}Na_7O_{56}S_7$, M_W = 1849.31	63.5 ± 6.4
6	7	H, SO_3Na	H, SO_3Na	H, SO_3Na	12.9	$C_{42}H_{57.1}Na_{12.9}O_{73.7}S_{12.9}$, M_W = 2451.38	12.9 ± 0.6
7	7	H, SO_3Na	H, SO_3Na	H, SO_3Na	16.9	$C_{42}H_{53.1}Na_{16.9}O_{85.7}S_{16.9}$, M_W = 2859.56	2.4 ± 0.3
8	8	H, SO_3Na	H, SO_3Na	H, SO_3Na	~8.2[b]	$C_{48}H_{71.8}Na_{8.2}O_{64.6}S_{8.2}$, M_W = 2133.89	45.6 ± 1.2

[a] Determined by elemental analysis.
[b] γ-CD was sulfated using essentially the same procedure as for **2**, which afforded an average degree of sulfation of 8.2; the product γ-CD sulfate is presumed to have an analogous degree of sulfation.

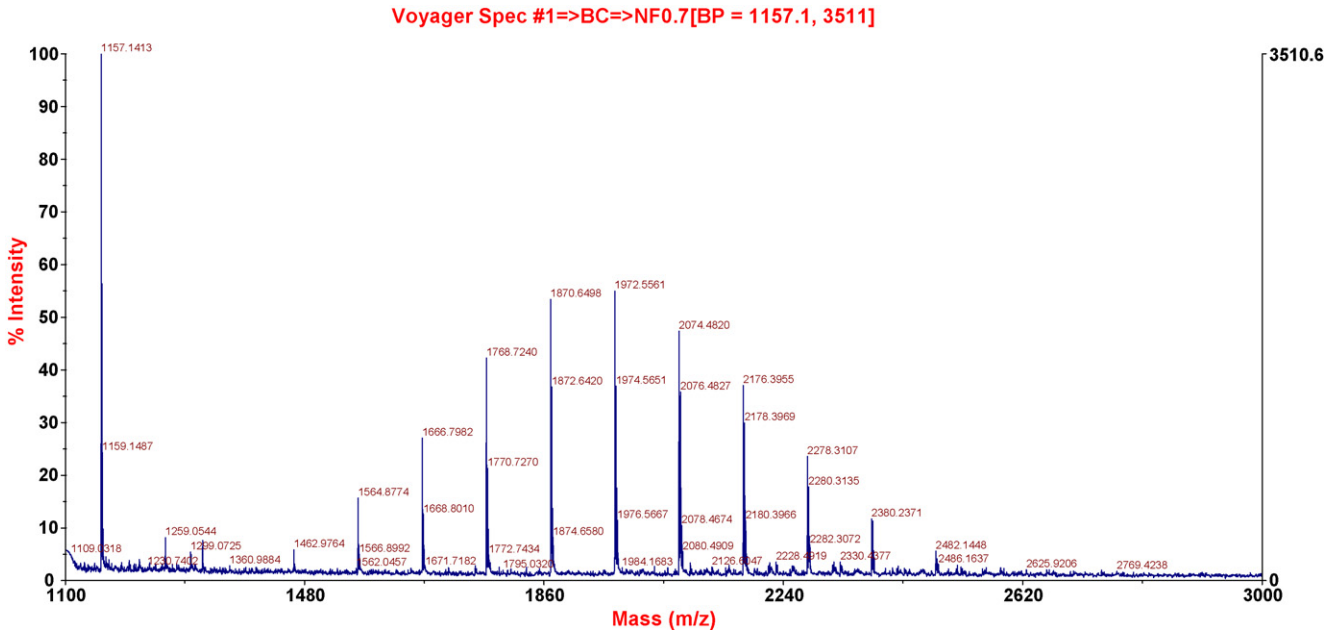


Fig. 1 – MALDI mass spectrum of *6* (see Section 2 for matrix employed).

Compound **5**, which has the same substitution pattern on each residue, namely a sulfate group at C-6 of each glucose unit of β-cyclodextrin, was prepared by a directed synthesis. The method involved the conversion of β-cyclodextrin into the heptakis(6-*O*-*tert*-butyldimethylsilyl) derivative, treatment of the derivative with acetic anhydride–pyridine to afford heptakis(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin, and removal of the silyl groups using boron trifluoride etherate to give heptakis(2,3-di-*O*-acetyl)-β-cyclodextrin, as described by Takeo et al. [14]; this product was sulfated using the procedure of Chen et al. [15] to give the corresponding heptasulfate which was *O*-deacetylated to afford sodium β-cyclodextrin heptasulfate (**5**) [16].

3.2. *Activity of cyclodextrin derivatives*

When the series of compounds were assayed for activity in *P. falciparum* cultures, it was evident that a high degree of sulfate groups was a requisite for activity, since **1**, **3**, and **4** were essentially inactive in our assays (Table 1). As observed in the β-cyclodextrin series, increasing the number of sulfates, to an average of 7–17, increased the potency (**7** > **6** > **5** > **4** or **3**). Thus, higher degrees of sulfation resulted in more potent compounds. The most potent compound against *P. falciparum* was the sulfated β-cyclodextrin compound **7** (IC_{50} = 2.4 ± 0.3 μM) which was found to have the highest average degree of sulfation (16.9) by elemental analysis.

It is noteworthy that the sulfated α-, β-, and γ-cyclodextrins **2**, **5**–**7**, and **8**, respectively, all inhibited parasite replication, a result suggesting that ring size was not a critical factor in the interaction of the sulfated cyclodextrins with their receptor(s); however, it appears that for each cyclodextrin ring system, a threshold degree of sulfation must be reached in order to attain inhibitory activity against *P. falciparum*. To determine if sulfated cyclodextrins inhibited parasite cultures in a manner similar to short-chain aliphatic polysulfonates (see [13]), samples from treated and untreated wells were transferred to a microscope slide, fixed with methanol, and stained with

Table 2 – Determination of degree of sulfation by elemental analysis and mass spectrometry

Compound	%C	%H	%S	%S/%C	Average degree of sulfation[a]	Range of degree of sulfation[b]
1	33.80	5.19	2.19	0.06	0.8	0–3
2	19.07	3.43	11.65	0.61	8.2	0–8
3	38.96	6.14	<0.3	0	0	0
4	31.31	5.03	3.29	0.11	1.7	0–3
6	16.86	3.34	13.68	0.81	12.9	0–13
7	14.18	2.94	15.04	1.06	16.9	1–18[c]

[a] Determined by elemental analysis; calculated by dividing the %S/%C ratio by 0.0741 for α-CDs, by 0.0629 for β-CDs, and by 0.0556 for the γ-CD.
[b] Determined by MALDI-MS analysis.
[c] Matrix: mixture of D, E, and F (see Section 2); all other analyses utilized the matrix mixture composed of A, B, and C.

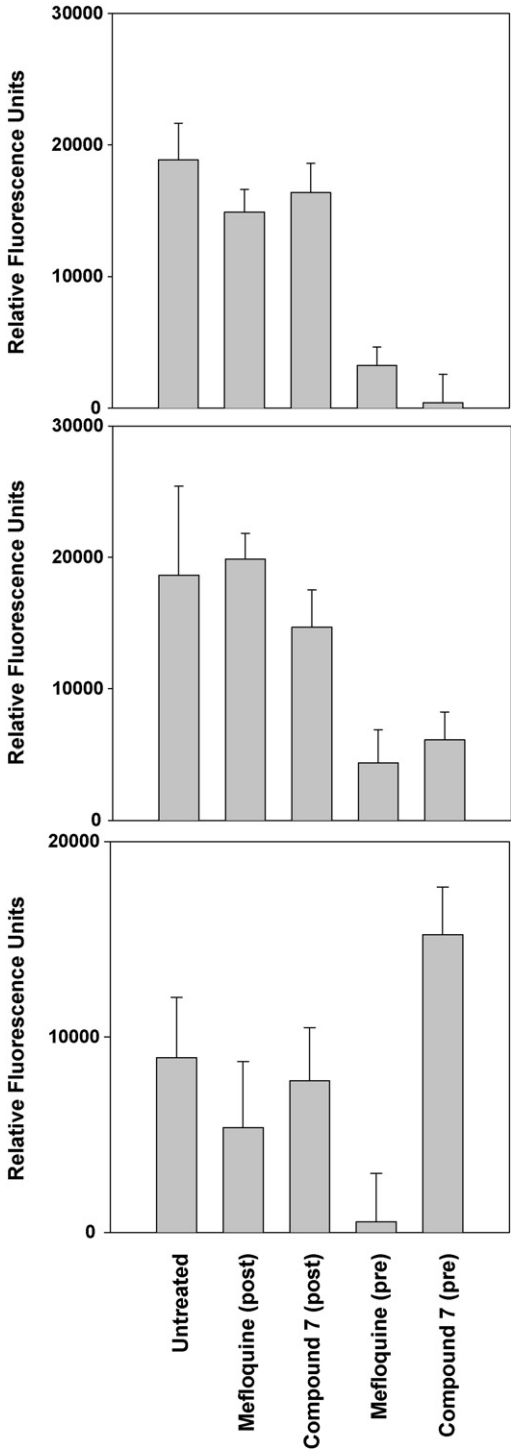


Fig. 2 – The effect of adding mefloquine and 7 to *Plasmodium falciparum* cultures. Culture samples and samples of non-parasitized erythrocytes with equal hematocrits and volumes were left untreated, exposed to mefloquine during the incubation period (mefloquine (pre)), exposed to mefloquine 1 h prior to final processing (mefloquine (post)), exposed to 7 during the incubation period (compound 7 (pre)), or exposed to compound 7 1 h prior to final processing (compound 7 (post)). SYBR-Green was then added to facilitate the quantitation of parasite DNA [20] and the relative fluorescence of the whole cultures (top panel), the pelleted erythrocytes (middle panel), and the culture supernatants (bottom panel) were determined. The results obtained from parasite culture samples were then subtracted from the corresponding values obtained from non-parasitized erythrocyte samples and the results were plotted as the mean ± S.E.M. Duplicate readings from four independent determinations were used to reduce reader variation. As expected, the addition of mefloquine and 7 after the incubation period ended produced results similar to the untreated control; however, the presence of 7 during the incubation period resulted in parasite DNA, in the form of merozoites, in the supernatants of the cultures.

Giemsa. It was observed that: (1) wells with low parasite viability contained unusually large numbers of extracellular merozoites (e.g. >95% of the parasites present in the culture were outside erythrocytes) and (2) the presence of large numbers of extracellular merozoites was correlated with an enhanced staining of the surface of all of the erythrocytes present in the sample. The observations suggested that the viability of the cultures might be compromised because the inhibitory compounds occupied a receptor(s) on the surface of the erythrocyte and thereby preventing the merozoite invasion process.

3.3. *Merozoite invasion*

In *P. falciparum* cultures the efficiency of merozoite invasion can be variable, resulting in the presence of extracellular merozoites. To confirm that a high degree of inhibition of merozoite entry into erythrocytes was taking place in wells that contained **7**, cultures were: (1) left untreated; or (2) were treated with mefloquine, which inhibits intercellular forms of the parasite [22]; or (3) were treated with **7**. As expected, pre-treatment with mefloquine inhibited parasite replication, however, it did not result in an increase in the number of extracellular merozoites when compared to the untreated control (Fig. 2). Pre-treatment with **7** had the expected result of reducing the number of viable parasites in the culture, however, it also caused a dramatic increase in the number of extracellular merozoites (Fig. 2). When the degree of stain enhancement of erythrocytes was quantified, there appeared to be a direct correlation between stain enhancement and inhibitory activity (Fig. 3). Furthermore, pre-treatment of erythrocytes with the stilbene disulfonate SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid) could be used to reduce staining enhancement by >70% (Fig. 3).

3.4. *Stilbene disulfonate inhibition of sulfated cyclodextrin binding*

SITS is a specific inhibitor of the erythrocyte anion-exchange protein AE1, or band 3 [23]. To determine if **7** interacted with AE1 on intact erythrocytes, SITS-Affigel beads (synthetic beads with covalently attached SITS [24]) were incubated with erythrocytes either in the presence of **3** or **7**. It was observed that, in the presence of **3**, erythrocytes bound avidly to the SITS beads. However, in the presence of **7**, few erythrocytes adhered to the SITS beads (Fig. 4).

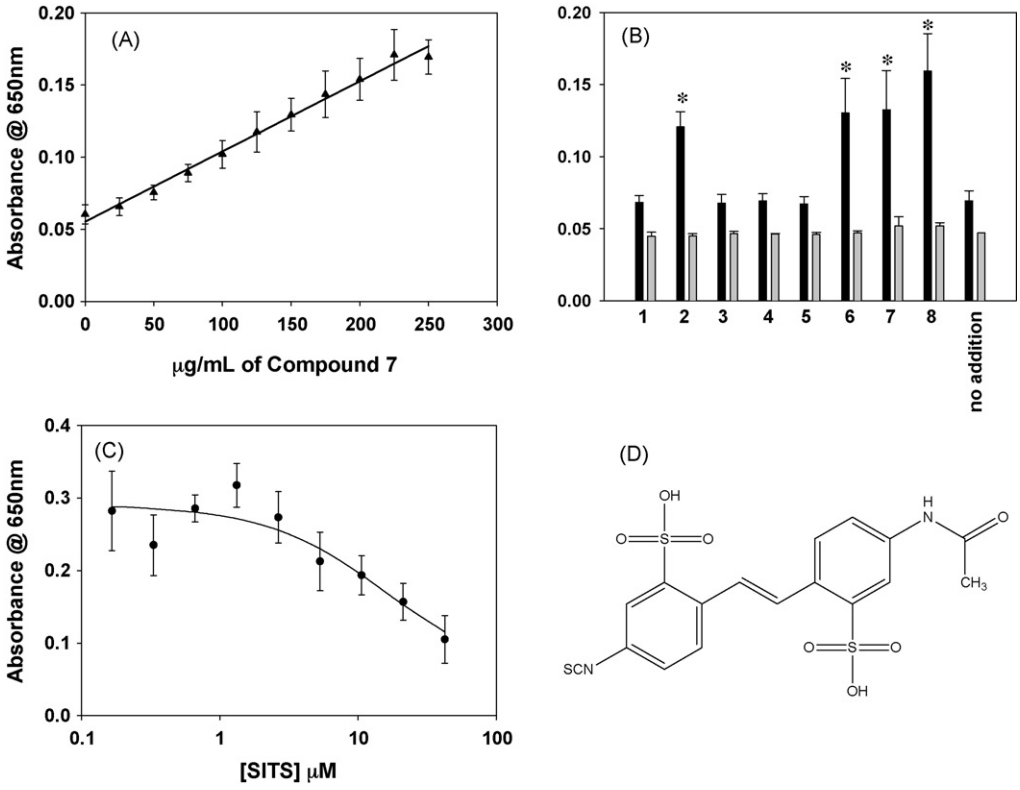


Fig. 3 – Determination of enhanced Methylene Blue uptake. Panel A: erythrocytes were incubated with increasing concentrations of *7* as indicated on the *x*-axis and were then fixed, stained with a 0.5 mg/mL solution of Methylene Blue in water, solubilized with DMSO, and the absorbance at 650 nm determined. Results represent the mean ± S.E.M. of eight determinations with a best-fit line indicated. Panel B: RBCs were incubated in the presence of TBS alone (no addition) or 200 μg/mL of compound (as indicated) before being fixed, stained, and solubilized (solid bars). Parallel assays were performed omitting the erythrocytes (gray bars). Results represent the average of four determinations with the standard deviation indicated by error bars. Results with a $p < 0.01$ compared to the matching "no addition" sample are indicated by *. Panel C: erythrocytes were washed three times with phosphate buffer saline (50 mM PO_4^{3-}, 120 mM NaCl, pH 7.4), then incubated with the indicated concentration of SITS for 30 min in the dark at 20 °C (total volume 100 μL). Compound 7 or 3 was then added to every well to achieve a final concentration of 200 μg/mL in a volume of 150 μL. The plate was then incubated at 37 °C for 2 h and processed. Results represent the mean ± S.E.M. of four determinations with a best-fit line indicated. The presence of SITS resulted in >70% inhibition of the binding of 7 with an IC_{50} of 19 μM. This compares with a previously published K_i value of SITS binding to erythrocytes of 10 μM [24]. Panel D: the chemical structure of SITS.

3.5. *In vivo inhibition*

The site at which SITS binds to AE1 is highly conserved across a number of species, including mice; therefore, we determined if **7** was active against a murine malaria. The activity of compound **7** was assayed by the SYBR-Green method [20] and was found to inhibit the replication of *P. berghei* cultures at a concentration that was similar to that observed for *P. falciparum* cultures assayed by the same method with IC_{50} values of 13 ± 4 and 4 ± 2 μM, respectively (Fig. 5, panel A). Infusion of the inactive compound **3** into mice at four doses resulted in no significant change in the course of the infection (Fig. 5, panel B). However, infusion of **7** produced a marked decrease in the parasitemia in the mice, furthermore, an increased dose was correlated with a decrease in parasitemia (Fig. 5, panel C). In contrast to our previous studies with linear polysulfonated aliphatics [13], no toxicity was observed using the doses of the compounds described in the present communication.

4. Discussion

Sulfated glycans have been shown to inhibit a number of processes performed by malaria parasites including the invasion of erythrocytes by merozoites [8,9], the invasion of hepatic cells by sporozoites [10,11], the formation of rosettes with unparasitized erythrocytes [12], and the adherence of parasitized erythrocytes to chondroitin sulfate A [9]. It has been suggested that sulfated saccharide polymers were inhibitory because they increased the negative repulsive charge and sterically interfered with ligand/receptor interactions [9]. In the present work relatively small cyclic saccharide oligomers having varying degrees of sulfation have been used

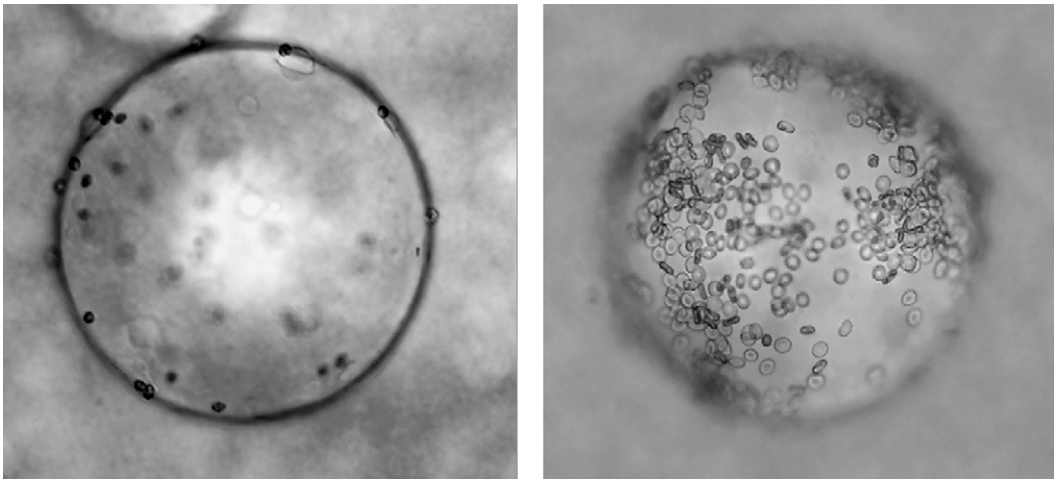


Fig. 4 – The adherence of erythrocytes to SITS-Affigel beads in the presence of 3 and 7. Erythrocytes were incubated with 200 μg/mL of 3 (left panel) or 7 (right panel) prior to the addition of SITS-Affigel beads. After an incubation period of 20 min, the beads were placed on a microscope slide and the degree of adherence of the erythrocytes to the beads was assessed by microscopy. Compound 3 did not interfere with the adhesion of erythrocytes to SITS beads; however, significant disruption was observed with 7.

to inhibit the entry of merozoites into erythrocytes. The use of smaller molecules, instead of naturally occurring polymers of 5–500 kDa, combined with some control over the degree of sulfation, has allowed us to partially define what makes the structures inhibitory to the merozoite invasion process. Our data suggest that the sulfated cyclodextrins are able to mimic known inhibitors of AE1, namely the stilbene disulfonates (Fig. 3). This conclusion is based on the observations that sulfated cyclodextrins having the appropriate degree of sulfation are able to prevent SITS from interacting with AE1 (Fig. 4), and, conversely, occupancy of the stilbene disulfonate site in AE1 prevents inhibitory compounds from binding (Fig. 3). The ability of stilbene disulfonates to prevent merozoite invasion has been previously noted [25]; however, it was attributed to a stiffening of the red-cell surface by the presence of these compounds. Evidence that active compounds are interacting with the erythrocyte membrane comes primarily from the correlation between a compound's activity and its capacity to enhance surface staining of all erythrocytes (Fig. 3). The enhanced staining of erythrocytes in the presence of inhibitory compounds may occur when the sulfated cyclodextrin ring binds to the erythrocyte surface and exposes exofacial sulfate groups to the surrounding media, thereby ''decorating'' the erythrocyte surface with anions that then attract cationic dyes. A similar assay has been used to detect phosphomannans on the surface of *C. albicans* [26] and we observed that the cationic dyes Methylene Blue (Fig. 3), Alcian Blue, and Azure A (data not shown) gave identical results. The ability of **7** to prevent the tethering of erythrocytes to SITS-coated beads is consistent with the stilbene disulfonate site being occupied by this compound and demonstrates that the interaction of **7** and AE1 is not an artifact of methanol fixation, or other steps in the stain enhancement assay.

The entry of *P. falciparum* merozoites into human erythrocytes is an essential step in the parasite's reproductive cycle. The presence of compounds such as **7** leads to disruption of this process; however, the mechanism by which it does this is unclear. Compound **7** may induce a conformational change in AE1 that prevents further interactions with the *Plasmodium* ligand at a site that is distinct from the stilbene disulfonate site. Alternatively, it is possible that the presence of **7** affects the interaction between AE1 proteins and reduces oligomerization [27]. It is also notable that AE1 is the most abundant protein on the erythrocyte surface with 10^6 copies of this protein per erythrocyte [28]. Therefore, the association of compounds such as **7** at high densities on the surface of the erythrocyte may provide a repulsive negatively charged coat to the cell. Finally, it is possible that compounds such as **7** share structural features with the parasite ligand and therefore directly compete for a critical receptor in the merozoite invasion process.

The results from the merozoite invasion inhibition assay agree with microscopic observations that the presence of mefloquine in cultures results in intracellular parasite death, while the presence of **7** results in a large number of free merozoites that are poorly attached to erythrocytes. The relatively mild procedure of resuspending the contents of the culture followed by differential centrifugation resulted in a significant number of merozoites being recovered in the non-erythrocyte fraction (Fig. 2). Furthermore, our implication of AE1 as a *Plasmodium* invasion receptor is in agreement with the work of Goel et al. [29] and Li et al. [30] who have demonstrated that peptides patterned on regions of band 3, that are predicted to be exofacial, inhibit the invasion of *P. falciparum* in cultures. Also, Kushwaha et al. [31] have reported that AE1 is a receptor for the parasite ABRA protein, which may be involved in the invasion process. Previous work has implicated AE1 in tight junction formation; our work neither directly supports nor refutes this hypothesis.

Compound **7** was found to have similar efficacy in both human and murine *Plasmodium* cultures, an observation that is consistent with its interaction with a conserved receptor on

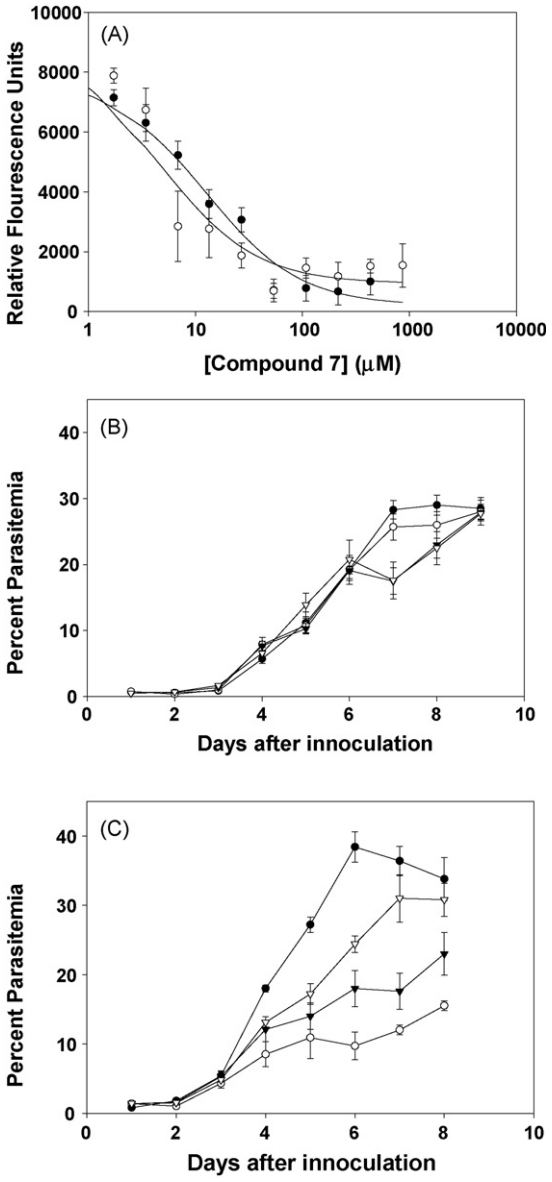


Fig. 5 – The effect of 7 on *P. berghei*. Panel A: the effects of varying amounts of 7 on *P. falciparum* (○) and *P. berghei* cultures (●) were determined by using the SYBR-Green method [20]. Compound 7 was found to inhibit *P. berghei in vitro* at a concentration that was similar to that observed for *P. falciparum* cultures with IC_{50} values of 13 ± 4 and 4 ± 2 μM, respectively. Each point represents the mean ± S.E.M. for four independent determinations. The effects of different doses of compound 3 (panel B), and 7 (panel C), on the progression of *P. berghei* parasitemia *in vivo* were determined. Doses correspond to control (no compound, ●), 700 μg/dose q12h i.p. (○), 175 μg/dose q12h i.p. (▼), and 87.5 μg/dose q12h i.p. (△). In contrast to the case of compound 3, where there was no difference in the progression of the parasitemia in treated vs. non-treated animals, compound 7 significantly ($p < 0.001$, as determined by a two-way analysis of variance) inhibited the progression of the parasitemia over the 8 days of the experiment in a dose responsive manner. Each point represents the mean ± S.E.M. with eight animals per group. Compound infusion studies were performed using two groups of 32 mice infected with the same parasite line.

the erythrocyte of both species. The stilbene disulfonate binding site is highly conserved in a wide range of band 3 orthologues [27], including humans and mice. The use of inhibitors that form a covalent bond with AE1, such as SITS, in a murine malaria model would confirm the role of this site in merozoite invasion, however, it could be lethal since anion exchange is an essential function of erythrocytes; sulfated cyclodextrins appear to be tolerated at levels that result in significant inhibition of *P. berghei* infections without compromising the animal's health. The relative lack of toxicity of **7** *in vivo* may be attributable to the lack of the isothiocyanato group that is present in SITS and which forms a covalent bond with AE1; therefore, compounds such as **7** would form a reversible complex which may limit problems associated with AE1 inhibition.

Other reports have noted that sulfated dextrans inhibit the invasion of both merozoites of *P. falciparum* and *P. berghei* [9]; however, the dextran sulfates used in those studies were considerably larger in molecular weight than the compounds employed in the present work, and their action was attributed to increasing negative repulsive charge. Our use of smaller molecules that lack serious side effects allowed us to perform *in vivo* experiments. Interestingly, the lack of "breakthrough" in the *P. berghei* experiments over the period of the experiment suggests that the parasite line did not contain parasite sub-populations [32] that were refractory to the presence of **7**. Attempts to produce resistant *P. falciparum* cultures were also unsuccessful (data not shown) suggesting that **7** inhibited a process that could not be compensated for with redundant ligand/receptor combinations. Clearly, further work is required to confirm that **7** targets a non-redundant interaction in the merozoite invasion pathway and whether knowledge of this process can be used to formulate antimalarial agents that inhibit merorzoite entry.

Acknowledgements

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