Malarial merozoites invade erythrocytes; and as an essential step in this invasion process, the 42-kDa fragment of the merozoite surface protein-1 (MSP142) is further cleaved to a 33-kDa N-terminal polypeptide (MSP119) and a 19-kDa C-terminal fragment (MSP119) in a secondary processing step. Suramin was shown to inhibit both merozoite invasion and MSP142 proteolytic cleavage. This polysulfonated naphthylurea bound directly to recombinant P. falciparum MSP142 (Kd = 0.2 μM) and to Plasmodium vivax MSP142 (Kd = 0.3 μM) as measured by fluorescence enhancement in the presence of the protein and by isotermal titration calorimetry. Suramin bound only slightly less tightly to the P. vivax MSP133 (Kd = 1.5 μM) secondary processing product (fluorescence measurements), but very weakly to MSP119 (Kd > 15 mM) (NMR measurements). Several residues in MSP119 were implicated in the interaction with suramin using NMR measurements. A series of symmetrical suramin analogues that differ in the number of aromatic rings and substitution patterns of the terminal naphthylamine groups was examined in invasion and processing assays. Two classes of analogue with either two or four bridging rings were found to be active in both assays, whereas two other classes without bridging rings were inactive. We propose that suramin and related compounds inhibit erythrocyte invasion by binding to MSP1 and by preventing its cleavage by the secondary processing protease. The results indicate that enzymatic events during invasion are suitable targets for drug development and validate the novel concept of an inhibitor binding to a macromolecular substrate to prevent its proteolysis by a protease.

Plasmodium falciparum develops and replicates within erythrocytes, releasing merozoites that invade new red blood cells. This stage of the parasite’s life cycle is responsible for the disease malaria, and inhibition of merozoite invasion reduces parasitemia, with beneficial outcome for the host. Invasion is a complex process, involving interaction between parasite and host molecules; and although poorly understood at the molecular level, it may represent a new chemotherapeutic target. Current antimalarial drugs are becoming increasingly ineffective, and few new treatments have been developed in recent years (1). New drugs are required against established and novel targets, together with strategies to reverse resistance and to bypass toxicity of known antimalarial compounds (2). The development of compounds that are significantly different from those already in use or directed against new targets may delay the onset of resistance (3).

Several proteins have been identified on the surface of the merozoite (4). For example, merozoite surface protein-1 (MSP1) is synthesized as an ~200-kDa precursor and is present on the surface of the late stage parasite within the erythrocyte. At or immediately prior to merozoite release, MSP1 is cleaved (primary processing) into four fragments that form part of a protein complex on the surface of the free merozoite. One of these fragments, the 42-kDa C-terminal polypeptide (MSP142), has a glycosylphosphatidylinositol anchor holding the complex to the parasite surface. Upon erythrocyte invasion, the protein complex is released from the merozoite surface following secondary processing, which involves a single proteolytic cleavage within MSP142. The 33-kDa N-terminal part of MSP142 is shed with the complex, whereas MSP119, the C-terminal part of MSP142, remains on the surface of the invading merozoite. MSP119 contains two epidermal growth factor (EGF) domains (5). Certain MSP119-specific monoclonal antibodies inhibit both secondary processing and erythrocyte invasion (6), suggesting that inhibitors of the protease responsible for MSP1 secondary processing would inhibit invasion. Prevention of secondary processing of MSP1 may thus be a good chemotherapeutic target.

We have chosen to examine suramin, a symmetrical hexasulfonated naphthylurea, to explore its ability to bind to MSP1 and to inhibit invasion because it has been shown that this highly charged compound interferes with interaction of growth factors containing EGF domains with their receptors (7–10). Suramin has also been shown to inhibit invasion of HepG2 cells by P. falciparum sporozoites (11). Other polyanionic compounds...
such as sulfated polysaccharides are known to inhibit merozoite invasion (12). Suramin has been used to treat African trypanosomiasis (13) and filariasis (14). Although suramin is toxic, some of its analogues (15) have been found to be much less toxic than suramin itself when tested in mice (16). Here, we show that suramin binds to MSP142 and that such binding inhibits both MSP1 secondary processing and merozoite invasion of erythrocytes. Some suramin analogues are also shown to inhibit processing and invasion.

**EXPERIMENTAL PROCEDURES**

**Materials**

Suramin (sodium salt, Antrypol, ICI) and suramin analogues (synthesized by Balaban and King in 1927 (15)) (Fig. 1) were provided by Terry Scott-Finnigan (Division of Parasitology, National Institute for Medical Research) and Dr. Roy Bicknell (Institute for Molecular Medicine, Oxford, UK). Data on the maximum tolerated doses of these compounds in mice are shown in Ref. 16. Naphthalene-1,3,6-trisulfonic acid (NTS) trisodium hydrated salt was purchased from Fluka (catalog no. 70310).

![FIG. 1. Suramin analogues (15) (numbers in brackets as used previously (16)).](image)

In Vitro Culture and Synchronization of *P. falciparum*

Asexual blood stages of *P. falciparum* (isolate FCB-1) were maintained at 37 °C in RPMI 1640/Albumax medium (Invitrogen) supplemented with 2 mM l-glutamine as previously described (17). Cultures were gassed with 7% CO2, 5% O2, and 88% N2 and maintained by routine passage in fresh human erythrocytes. Parasites were synchronized by Percoll and sorbitol treatment as described previously (18). Briefly, schizonts were purified by centrifugation over Percoll and then returned to culture in the presence of fresh erythrocytes. After 4 h, during which time released merozoites invaded erythrocytes, the cells were treated with 5% sorbitol for 10 min to lyse the residual schizonts before returning the parasites to culture.

In Vitro Invasion Inhibition Assays

Compounds were tested for their capacity to inhibit erythrocyte invasion by *P. falciparum in vitro* using two approaches: a short-term assay in which the number of newly invaded erythrocytes was counted using microscopy and an assay measuring uptake of 3H-hypoxanthine. In the short-term assay (19), compounds were incubated in triplicate with cultures containing highly synchronous *P. falciparum* schizonts at ~2% parasitemia and ~2% hematocrit. After between 6 and 24 h, thin
films were prepared, stained with Giemsa reagent, and examined by microscopy. The number of newly invaded rings was counted, and inhibition of invasion was expressed as percent invasion relative to a control untreated culture (calculated using the formula \((I_c/I_t + U_c/U_t) \times 100\), where \(I_c\) is the number of erythrocytes infected with ring stages, \(U_c\) is the number of uninfected erythrocytes in the presence of the compound, \(I_t\) is the number of erythrocytes infected with ring stages, and \(U_t\) is the number of uninfected erythrocytes in the absence of the compound).

Suramin and NTS were also assayed for *P. falciparum* invasion inhibition using \(^{3}H\)hypoxanthine uptake (20). Serial dilutions of the compounds were added in triplicate to cultures containing mature schizonts at a final parasitemia of ~0.5% and a hematocrit of ~2% in 96-well plates. Following incubation for 18 h to allow schizont rupture and erythrocyte invasion, \(^{3}H\)hypoxanthine (0.5 μCi/well) was added. After a further 18 h of culture, cells were harvested onto glass-fiber filters (Filtermat A, Wallac, Turku, Finland) using a cell harvester. Filters were wetted with scintillation mixture, and bound radioactivity was quantified in a β-counter. Control incubations without compound or without parasites were included in each experiment. The amount of radioactivity in each sample was expressed as a percentage of activity in the control wells containing no compound. Three independent experiments were performed for each compound.

**MSP1 Secondary Processing Assay**

*P. falciparum* merozoites were purified as described previously (17) after release from mature schizonts into growth medium supplemented with 0.5% DMSO to allow maturation of cultures. MSP133/MSP142 antibody, and the bands corresponding to MSP142 and MSP133 polypeptides were visualized by enhanced chemiluminescence. MSP133/MSP142 antibody, and the bands corresponding to MSP142 and MSP133 polypeptides were visualized by enhanced chemiluminescence.

**P. falciparum MSP119—**The pETAT/P-FP-MSP119 plasmid was used to express a His₆-tagged MSP119 protein (*P. falciparum* 3D7 clone, GenBank™/EBI accession number Z35327) essentially as described (21). Following bacterial growth, protein expression was induced by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside at 25 °C. After 2 h of additional growth, the bacterial cells were harvested by centrifugation, resuspended in buffer, and lysed by microfluidization. The protein was purified by binding and elution from a column of nickel-nitrotriacetic acid Superflow resin (QIAGEN Inc.), followed by passage through two ion exchange columns (SuperQ 650M and CM 650M, TosoHaas). The cell lysate was cleared by centrifugation, and the supernatant was loaded directly onto a nickel-nitrotriacetic acid column. The column was washed with 10 volumes of 50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole (pH 8.0) and with 6 column volumes of the same buffer containing 30 mM imidazole and finally eluted with 3 column volumes of the same buffer with 250 mM imidazole. The eluted protein was then loaded directly onto a Superdex 200 column (26 × 600 mm) equilibrated in 20 mM Tris-HCl and 250 mM NaCl (pH 8.0) and purified by gel filtration. The fractions containing MSP119 were pooled and dialyzed extensively against phosphate-buffered saline.

**Binding of Suramin to MSP1 Assayed by Fluorometry**

The intrinsic fluorescence of suramin was used to determine its capacity to bind to MSP1-derived proteins. Fluorescence was measured using a PerkinElmer Life Sciences LS-3B or Spex Industries FluoroMax fluorometer. Suramin was excited at a wavelength of 315 or 330 nm at 2.5-nm resolution, and emission spectra were measured at wavelengths ranging from 350 to 450 nm. Suramin and the MSP1 proteins were diluted in 20 mM NaH₂PO₄ and 150 mM NaCl at pH 7.2 and measured at 10 or 20 °C. Titrations were performed by adding aliquots of the suramin solution to the MSP1 solution. In controls, suramin was titrated into buffer alone. The \(K_f\) for MSP1/suramin binding was determined from three independent experiments.

**Isothermal Titration Calorimetry**

Isothermal calorimetric titrations were performed using a Microcal Omega VP isothermal titration calorimeter. Tested proteins were dialyzed extensively against isothermal titration calorimetry buffer (phosphate-buffered saline at pH 7.4) before use. All experiments were performed at 25 °C. The calorimetric cell, which has an internal volume of 1.425 ml, contained 30 μM MSP142 or MSP133, which was titrated by injection of a total of 290 μl of 600 μM suramin. The heat of dilution of suramin into buffer alone was determined in control experiments. Data were fitted by least-squares methods using the Microcal Origin Version 5.0 evaluation software provided by the manufacturer. Each experiment was performed twice.

**NMR Studies on MSP119**

NMR experiments were carried out on Varian spectrometers operating at proton frequencies of 500, 600, and 800 MHz. Suramin (0.03–16.0 μM) \(^{1}H\) spectra were recorded at 5–35 °C. The assignments of the \(^{1}H\) signals of suramin (except for the NH signals) were made by analysis of the two-dimensional gradient selected double quantum filtered COSY spectrum at 500 MHz at 25 °C on a sample containing 1 mM suramin in 50 mM potassium phosphate and 100 mM KCl in 90% H₂O and 10% D₂O at pH 6.5. The NH assignments were made on the basis of nuclear Overhauser effect correlation spectroscopy experiments (Fig. 2). The assignments agree with those reported previously (22).
Suramin Inhibits MSP1 Secondary Processing and Invasion

min samples were examined with either unlabeled or \(^{15}N\)-labeled MSP1, in 50 mM sodium phosphate and 100 mM NaCl in 90% H\(_2\)O and 10% D\(_2\)O at pH 6.5 (pH values are pH* meter readings uncorrected for deuterium isotope effects). Titrations were carried out by mixing two samples each containing 0.1 mM MSP1, and with one also containing 16 mM suramin. One-dimensional \(^1H\) and two-dimensional \(^1H-^{15}N\) heteronuclear single quantum correlation NMR spectra were recorded for each concentration of suramin. Nuclear Overhauser effect correlation spectra were recorded on MSP1/suramin samples in D\(_2\)O (1.76 mM protein and 6 mM suramin in 50 mM sodium phosphate and 100 mM NaCl at pH 6.5); these spectra were compared with MSP1 spectra recorded in the absence of suramin to detect any suramin-induced changes in \(^1H\) chemical shifts for protein side chain resonances.

**RESULTS**

**Suramin Inhibits Erythrocyte Invasion by Merozoites and MSP1 Secondary Processing**—Suramin inhibition of erythrocyte invasion was measured both by microscopic examination of cultures following staining with Giemsa reagent and by biosynthetic incorporation of \(^{3}H\)hypoxanthine. As shown in Fig. 3, suramin inhibited invasion in a dose-dependent manner with an IC\(_{50}\) of 60 ± 9 \(\mu M\), whereas NTS did not inhibit invasion at 200 \(\mu M\), the highest concentration tested. Similar results were obtained in both assays. Free merozoites were observed in the stained samples, suggesting that suramin does not inhibit merozoite release from schizonts, but does prevent invasion, confirming our earlier findings. At these concentrations, suramin had no effect on the intracellular growth of the parasite (data not shown).

Suramin was shown to inhibit secondary processing of MSP1, in extracts of merozoites (Fig. 4A). Following a 1-h incubation of merozoites in the presence of suramin or control compounds, no MSP1 protein product was detected in the sample incubated with 1 mM PMSF (a potent inhibitor of processing) or 200 \(\mu M\) suramin. In contrast, 200 \(\mu M\) NTS had no effect on MSP1 secondary processing, with clear formation of MSP1 occurring in the NTS-treated sample. Significant MSP1 cleavage was detected only in samples incubated with 12.5 \(\mu M\) or lower concentrations of suramin (Fig. 4B).

**Suramin Binds to MSP1 and MSP1**—The intrinsic fluorescence of suramin is low when excited at 315 nm; but when it bound to MSP1, it exhibited a pronounced increase in emission intensity, with the maximum emission wavelength shifted slightly from 408 to 411 nm (Fig. 5, inset). This fluorescence enhancement was used to measure binding of suramin to MSP1 from *P. falciparum*. Following titration of a solution of MSP1 with suramin, the marked increase in the fluorescence intensity was measured as a function of suramin concentration (Fig. 5). Analysis of the binding data revealed that suramin bound to *P. falciparum* MSP1 with a \(K_d\) of 0.22 ± 0.04 \(\mu M\).

**Fig. 2. Structure of suramin and its \(^1H\) chemical shifts at 25 °C and 3.3 \(\mu M\).** The shifts are strongly dependent on temperature and concentration, and this was taken into account in assessing the suramin shifts observed upon binding to MSP1.

**Fig. 3. Suramin inhibits *P. falciparum* merozoite invasion in vitro.** Cultures containing mature schizonts (~0.5% parasitemia, ~2% hematocrit) were incubated at 37 °C overnight with serial dilutions of either suramin (open symbols) or NTS (closed symbols) and then cultured for an additional 18 h in the presence of \(^{3}H\)hypoxanthine. The amount of radioactivity incorporated is proportional to the number of parasites that have invaded erythrocytes, and the effect of the compound is expressed relative to incorporation in the absence of compound (percent relative parasite invasion). Data are the results of three experiments, each performed in triplicate.

**Fig. 4. Suramin (but not NTS) inhibits secondary processing of MSP1 as detected by Western blotting.** A, purified *P. falciparum* merozoites were incubated for 1 h in reaction buffer containing SDS (lane 1), no further addition (lane 2), 1 mM PMSF (lane 3), 200 \(\mu M\) NTS (lane 4), or 200 \(\mu M\) suramin (lane 5). The presence of both MSP1 (substrate for the processing enzyme) and MSP1 (the product) was detected by Western blotting; the positions of these bands are indicated to the right. The absence of an MSP1 band is indicative of inhibition of processing. B, inhibition of MSP1 secondary processing by suramin is concentration-dependent. Using the standard processing assay (A), merozoites were incubated in reaction buffer containing SDS (lane 1), no further addition (lane 2), 1 mM PMSF (lane 3), 200 \(\mu M\) suramin (lane 4), 100 \(\mu M\) suramin (lane 5), 50 \(\mu M\) suramin (lane 6), 12.5 \(\mu M\) suramin (lane 7), 3.1 \(\mu M\) suramin (lane 8), or 1.25 \(\mu M\) suramin (lane 9).
concentrations, preventing measurements of suramin binding in vitro. However MSP133 from *P. vivax* is soluble to at least 20 mg/ml and hence amenable to in vitro methods of measuring suramin binding. When analyzed by fluorometry, *P. vivax* MSP142 and MSP133 showed large binding-induced enhancements of the intrinsic suramin fluorescence similar to that observed with *P. falciparum* MSP142. *P. vivax* MSP142 bound suramin with a $K_d$ of 0.3 $\pm$ 0.1 M compared with 0.22 $\pm$ 0.04 M for *P. falciparum* MSP142. MSP133 from *P. vivax* showed a high affinity site that was 5-fold weaker compared with MSP142 ($K_d = 1.5 \pm 0.5 \mu M$). The $\Delta H$ values analyzed by isothermal titration calorimetry (11 $\pm$ 1 kcal/mol for MSP142 and 12 $\pm$ 1 kcal/mol for MSP133) are also very similar. At the high concentrations (>30 $\mu M$) required for isothermal titration calorimetric analysis, there was evidence for some nonspecific binding. The suramin binding for MSP133 was only 5-fold weaker than that for MSP142, and this, taken together with the similar large induced fluorescence enhancements, indicates that there is a similar hydrophobic suramin-binding pocket in the two proteins.

**Suramin Binds to MSP119—**No fluorescence enhancement was observed when suramin was added to MSP119 from *P. falciparum*; and thus, suramin binding to MSP119 could not be determined by fluorescence measurements. However, using NMR, it was possible to follow changes in chemical shifts of the $^1H$ and $^{15}N$ signals in $^1H$-$^{15}N$ heteronuclear single quantum correlation spectroscopy experiments when titrating $^{15}N$-labeled MSP119 with suramin. At the maximum concentration of suramin used (16 mM), ~50% of MSP119 was complexed with the ligand. The residues that showed the largest shifts upon addition of suramin were Ile5, His5, Phe19, His21, Leu22, and Arg25. The shifts were fitted by nonlinear regression analysis to a single binding curve and gave an average $K_d$ of $-15 \pm 5 \text{ m}\mu\text{M}$ (Fig. 6). Suramin seems to bind weakly to a site or sites near the N terminus of the protein, as indicated in Fig. 7, which shows the positions of the affected MSP119 residues in a three-dimensional structure. All of the observed shifts were very small, <0.15 ppm for the NH $^1H$ signals. The chemical shift of the protein aromatic ring protons showed almost no change, and the protons on suramin itself showed shifts of <0.05 ppm. These small changes indicate that suramin does not bind to MSP119 in a hydrophobic pocket (see “Discussion”).

**Suramin Analogues Inhibit Erythrocyte Invasion and MSP1 Secondary Processing—**A number of suramin analogues were examined to probe the features of the molecule necessary for binding to MSP1 and for inhibiting *P. falciparum* MSP1 processing. Four series of symmetrical compounds (Groups A–D)
(Fig. 1) differing in the number of central aminobenzoylurea units and having various substitutions on the terminal naphthyl rings were examined. None of the Group A compounds or the single compound (D1) in Group D inhibited invasion in vitro at 200 μM, the highest concentration tested (Fig. 8, A and D). However, all Group B and C compounds inhibited invasion in vitro (Fig. 8, B and C), with IC₅₀ values similar to that of suramin. The suramin analogues differ in toxicity (16), with the least toxic inhibitory compound (B1) being ~10 times less toxic than suramin. All of the compounds that inhibited invasion also inhibited MSP1₄₂ processing (Fig. 9, B and C), whereas those that did not inhibit invasion also did not inhibit processing (Fig. 9, A and D) when tested at a concentration of 200 μM. Similar parasite invasion and MSP1 processing assays could not be carried out for P. vivax because this species cannot be cultured in vitro.

**DISCUSSION**

Sulfated polyanions have been shown to have a number of inhibitory effects on malaria parasites in vitro. For example, merozoite invasion of erythrocytes and sporozoite invasion of hepatocytes are inhibited by sulfated glycans (11, 12, 23–25), and suramin has been shown to inhibit invasion of HepG2 cells by P. falciparum sporozoites with an IC₅₀ of 50 μM (11). The molecular targets of these agents are unclear, although the 135-kDa Duffy-binding ligand of Plasmodium knowlesi has been shown to bind to fucoidan (26), and TRAP (thrombospondin-related anonymous protein) has been implicated in the effects of suramin on sporozoite invasion (11).

Here, we have demonstrated that suramin prevented merozoite invasion in a dose-dependent manner with an IC₅₀ (~60 μM), which is similar to that at which the compound inhibits sporozoite invasion (11). In addition, suramin bound to MSP1₄₂ and inhibited secondary processing of MSP1 on the merozoite surface at the time of invasion. Previous studies have also

**FIG. 8.** Some analogues of suramin inhibit P. falciparum merozoite invasion in vitro. Purified schizonts were incubated at 37 °C with serial dilutions of compounds in Groups A–D; results with individual compounds are indicated with the compound reference number (see Fig. 1). Erythrocyte invasion was assessed using microscopy by counting the number of ring stage parasites in triplicate cultures. Inhibition is expressed as invasion in the presence of drug as a percentage of invasion in the absence of drug (±S.D.). Individual compounds in Groups B and C inhibited invasion, whereas compounds in Groups A and D did not.

**FIG. 9.** Some analogues of suramin inhibit secondary processing of MSP1 as detected by Western blotting. Purified P. falciparum merozoites were incubated for 1 h in reaction buffer containing the compounds in Groups A–D (see Fig. 1) at a concentration of 200 μM. For each panel, the following controls were included: SDS (lane 1), no further addition (lane 2), or 1 mM PMSF (lane 3). A, Group A compounds: A1 (lane 4), A2 (lane 5), A4 (lane 6), A5 (lane 7), and A6 (lane 8). B, Group B compounds: B1 (lane 4), B2 (lane 5), B3 (lane 6), B4 (lane 7), B5 (lane 8). C, Group C compounds: C1 (lane 4), C7 (lane 5), C2 (lane 6), C3 (lane 7), C4 (lane 8), and C5 (lane 9). D, Group D compound: D1 (lane 4). The presence of both MSP1₄₂ (substrate for the processing enzyme) and MSP1₃₃ (the product) was detected by Western blotting; the positions of these bands are indicated to the right. The absence of an MSP1₃₃ band is indicative of inhibition of processing. All of the compounds in Groups B and C inhibited processing, whereas those in Groups A and D did not.

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3 J. Walker, unpublished data.
shown that inhibition of MSP1 secondary processing results in inhibition of erythrocyte invasion. For example, the protease responsible for MSP1 secondary processing is inhibited by calcium-chelating agents such as ECGT and EDTA and by PMSF (a broad-range serine protease inhibitor) (27, 28) and by a specific peptidyl chloromethyl ketone based on the cleavage site within MSP142.4 All of these agents also inhibit erythrocyte invasion. Furthermore, certain monoclonal antibodies that bind to the C-terminal region of MSP142 inhibit both processing and erythrocyte invasion (17, 19). To date, all of the data obtained using either small inhibitor molecules or antibodies support the proposition that MSP1 secondary processing is essential for invasion, validating the processing as a chemotherapeutic target. The secondary processing protease has not yet been identified, but has been characterized as a calcium-dependent, membrane-bound serine protease (28) with candidates including members of the subtilisin family (29–31).

There is no direct evidence that suramin can act as an inhibitor of the specific protease, but suramin is known to inhibit other proteases, e.g. neutrophil serine protease (32). It is unlikely that suramin acted by chelating calcium, as calcium was present in the processing assays at a concentration at least 10 times that of the compounds. In the absence of any direct information about protease inhibition, our characterization of the direct binding of suramin to MSP142, inhibition of secondary processing, and erythrocyte invasion offers the most plausible mechanism for the observed antimalarial action.

To investigate the mechanism by which suramin inhibits processing and invasion, it was necessary to examine two species of plasmodia, P. falciparum and P. vivax, because all of the relevant experiments could not be done with either species alone. Fluorescence measurements were used to examine suramin binding to MSP142 and to the two polypeptide fragments (MSP119 and MSP133) that result from its secondary processing. Binding experiments using MSP142 from both P. falciparum and P. vivax indicated that suramin has a similar binding constant for the two species. Binding studies were also carried out with P. vivax MSP133 (aggregation problems prevented such studies with P. falciparum MSP133). Suramin bound to MSP142 with a $K_d$ of $2 \times 10^{-7}$ M and only five times more weakly to the MSP119 fragment ($K_d = 1.5 \mu M$), but bound $10^5$ times more weakly to the MSP133 fragment ($K_d = 15 \pm 5$ nM). The estimate of binding to MSP119 was from NMR studies, as it was not possible to use the fluorescence approach.

The NMR spectral assignments and solution structure of MSP119 have been determined previously (5). The structure contains two EGF domains folded into a U-shaped structure such that the N and C termini are close to each other. Some of the residues affected by suramin binding, notably His$^5$, Phe$^{19}$, and Leu$^{22}$, are located in the region between the two EGF domains and near the closely apposed N and C termini of MSP119 (5). Suramin contains many aromatic rings, and these would be expected to cause large ring current chemical shifts of the signals from protein residues in any hydrophobic binding site. Such shifts were not observed. The very small changes in chemical shifts observed both for the NH signals from the protein as well as the suramin signals themselves indicate that suramin does not bind to a hydrophobic pocket in MSP119. The most likely mechanism for suramin binding involves electrostatic interaction of its negatively charged groups with positively charged residues on MSP119 (Fig. 7). Such electrostatic interactions could be affected without the naphthyl aromatic rings approaching protein nuclei too closely. Candidate residues for such electrostatic interaction are His$^5$, His$^{21}$, and

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4 I. Wells, M. J. Blackman, and A. A. Holder, unpublished data.

Fig. 10. Working model of the structure for the complex of suramin with MSP142. Suramin is proposed to bind strongly to a hydrophobic binding site on the MSP133 fragment and weakly via electrostatic interaction to the MSP119 fragment close to the secondary processing cleavage site.

Arg$^{25}$. They exhibit perturbed chemical shifts in the complex and are located near the other affected residues. All of the perturbed residues are from the first domain of MSP119 and lie close to the processing site at the N terminus (Fig. 7). Two monoclonal antibodies that inhibit parasite invasion and secondary processing in vitro also interact with binding sites, including the suramin-binding region of MSP119 (33).

It is unlikely that the binding of suramin to MSP1 depends exclusively upon electrostatic interactions because other ligands with negatively charged sulfonate groups (such as NTS and the Group A and D compounds) are not effective inhibitors of MSP1 processing. The large fluorescence enhancements seen upon suramin binding to P. falciparum MSP142 and P. vivax MSP142 and MSP133 suggest that suramin binds to a hydrophobic region of MSP142 and MSP133. The similar $K_d$ and $\Delta H$ values obtained for MSP142 and MSP133 suggest that the hydrophobic binding site for suramin is similar in the two proteins. Although suramin binding to MSP142 requires residues from both regions of MSP142, it is clear that the EGF domains of MSP142 make only a small contribution to the overall strength of the interaction. A working model of the structure for the complex of suramin with MSP142 is shown in Fig. 10, which indicates the general features of a strong hydrophobic binding site on the MSP133 fragment and a weaker electrostatic interaction with MSP119.

Although suramin and some related compounds inhibit secondary processing and invasion, several related molecules do not. For example, whereas all Group B and C compounds inhibited both MSP1 secondary processing and merozoite invasion, NTS as well as the Group A and D compounds did not. We observed no examples of compounds that inhibited processing but not invasion or vice versa. This direct correlation between the two activities provides further evidence that MSP1 secondary processing is an essential requirement for successful erythrocyte invasion.

The most active suramin-like compounds are those in Groups B and C with two or four bridging aminobenzoyleurea groups. These molecules have rigid symmetrical structures, and it is possible that either the presence or length of these aminobenzoyleurea groups is essential for binding to MSP1. The position of the sulfonate groups and other substitutions of the naphthyl rings have little effect on the activity relationships of the compounds. A study of basic fibroblast growth factor inhibition in vitro and anti-angiogenic activity in vivo using a similar series of compounds (16) concluded that the extended multiple ring structure is important for activity and that the substitution pattern of the naphthyl rings is irrelevant. From the series of
19 compounds we examined, only suramin, NTS, and compound A6 possess three sulfonate groups/naphthyl ring, whereas all of the other compounds have two; therefore, it is unlikely that the exact number of sulfonate groups is important for activity. The methyl groups of suramin are absent in the analogues we examined, suggesting that they are not important for inhibiting processing or invasion.

In vivo studies with suramin have shown that >99% of suramin is bound to plasma proteins such as albumin, which reduces the effective free suramin in circulation; its slow release from these complexes accounts for its prolonged half-life as 300 μg/ml (~200 μM) are clinically achievable without significant toxicity (35) This concentration is only 3–4-fold higher than the IC50 for suramin inhibition of merozoite invasion in vitro; and therefore, achievable levels of suramin are unlikely to be sufficient to completely prevent merozoite invasion in humans. However, some of the suramin analogues examined here are 10 times less toxic than suramin, and further analysis of structure-activity relationships may identify more active compounds with a more favorable therapeutic index.

In summary, this work provides evidence that MSP1 secondary processing is essential for invasion and can be inhibited by suramin and its analogues. Secondary processing has the potential to be a new target area for antimalarial drug development. Our results also exemplify the novel concept of an inhibitor binding to the substrate of a protease to prevent proteolysis. This may be exploited to develop a fluorescence-based competitive high throughput screen to identify other potent inhibitors of MSP1 processing.

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