Reduced Invasion and Growth of *Plasmodium falciparum* Into Elliptocytic Red Blood Cells With a Combined Deficiency of Protein 4.1, Glycophorin C, and p55

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In this investigation, we have measured the invasion and growth of the malaria parasite *Plasmodium falciparum* into elliptocytic red blood cells (RBCs) obtained from subjects with homozygous hereditary elliptocytosis. These elliptocytic RBCs have been previously characterized to possess molecular defects in protein 4.1 and glycophorin C. Our results show that the invasion of *Plasmodium falciparum* into these protein 4.1 (-) RBCs is significantly reduced. Glycophorin C (-) Leach RBCs were similarly resistant to parasite invasion in vitro. The intracellular development of parasites that invaded protein 4.1 (-) RBCs was also dramatically reduced. In contrast, no such reduction of intracellular parasite growth was observed in the glycophorin C (-) Leach RBCs. In conjunction with our recent finding that a third protein termed p55 is also deficient in protein 4.1 (-) and glycophorin C (-) RBCs, the present data underscore the importance of the membrane-associated ternary complex between protein 4.1, glycophorin C, and p55 during the invasion and growth of malaria parasites into human RBCs.

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THE INVASION OF human red blood cells (RBCs) by the merozoites of *Plasmodium falciparum* and the subsequent intraerythrocytic development of these merozoites are essential steps for the initiation of the symptomatic disease. The elucidation of the molecular basis of parasite invasion and growth in human RBCs has been a subject of considerable interest. An attractive approach is the use of abnormal RBCs from subjects carrying mutations in their RBC membrane proteins. In this context, a subset of patients with homozygous hereditary elliptocytosis has been previously characterized in which the primary defect involves a complete absence of erythroid isoforms of protein 4.1 as well as a secondary deficiency of glycophorin C, an integral membrane protein of the RBC membrane. Recently, we found that a third protein termed p55 is also deficient in protein 4.1 (-) elliptocytic RBCs. p55 is the most abundantly palmitoylated protein in the RBC membrane, and it forms a protein complex together with protein 4.1 and glycophorin C. Another form of homozygous hereditary elliptocytosis, known as the Leach phenotype, is caused by a deficiency of glycophorin C. The Leach RBCs exhibit a secondary lack of p55 and an approximate 25% deficiency of protein 4.1. The finding of combined deficiencies of protein 4.1, glycophorin C, and p55 in both the protein 4.1 (-) HE and the glycophorin C (-) HE, together with recently reported data describing the interactions of these proteins in vitro, suggest that the associations of glycophorin C, protein 4.1, and p55 may play an important role in the regulation of membrane mechanical properties and RBC shape.

In this study, we have examined the ability of protein 4.1 (-) RBCs to support malaria parasite invasion and growth in vitro. We find a significant reduction of parasite invasion into protein 4.1 (-) RBCs. The intraerythrocytic growth of the malaria parasite in the protein 4.1 (-) RBCs was also dramatically reduced. In addition, we used Leach RBCs to examine the role of glycophorin C in the invasion and growth of *Plasmodium falciparum* in vitro. Our results indicate that Leach RBCs are partially resistant to invasion by the malaria parasite, whereas intraerythrocytic growth of the malaria parasite in Leach RBCs appeared normal, in contrast with a dramatically diminished parasite growth in protein 4.1 (-) RBCs. Taken together, these results suggest an important role for the membrane-associated complex between protein 4.1, glycophorin C, and p55 in the invasion and growth of the malaria parasites into human RBCs.

MATERIALS AND METHODS

Abnormal RBCs. Homozygous 4.1 (-) hereditary elliptocytosis RBCs were obtained from a patient of African-American origin. The patient (DW) was splenectomized in childhood and does not receive blood transfusions. RBCs from this patient are completely devoid of all isoforms of protein 4.1, as examined by gel electrophoresis and Western blot analysis. Blood samples of Leach phenotype lacking glycophorin C were obtained from three members of one family (JC, SS, TL). The control blood samples were included to take into account shipment and age variables.

Parasite culture. Three “knobby” lines of *Plasmodium falciparum* were interchangeably used in the experiments reported here: (1) 7901 Palo Alto, Uganda strain; (2) A-2, a clonal derivative of the nonclonal Gambian FcR-3 line; and (3) ItG-2 selected for its increased adhesion to ICAM-1. The in vitro parasite culture was performed in the presence of 15% human serum, as described previously, and ring-stage parasites were synchronized in 5% Sorbitol.

Parasite invasion and growth assay. Normal and mutant RBCs were transported under identical conditions. Invasion assays were started within 24 hours after the blood was drawn in the ACD anticoagulant. The intracellular ATP content of normal, Leach, and protein 4.1 (-) RBCs was comparable (~0.12 μmol/10⁹ RBCs). Parasites in normal RBCs were grown to 6% parasitemia. Trophozoite/schizont infected RBCs were concentrated to about 98% parasitemia by Percoll-Sorbitol sedimentation. Purified trophozoite/schizont...
containing normal RBCs were mixed with either control or mutant RBCs to attain a hematocrit of 2%. Parasitemia was determined by counting either rings or trophozoites per 5,000 RBCs on Giemsa-stained smears. All values are the average of triplicate determinations.

**[1H]-hypoxanthine invasion assay.** Malaria parasite invasion assay by [3H]-hypoxanthine incorporation was carried out as described before. [12] [H]-hypoxanthine was added to the parasites at the ring stage of parasite development in a 96-well enzyme-linked immunosorbent assay (ELISA) plate. After incubation, parasites were lysed in distilled water and the parasite-bound radioactivity was measured using a Millipore filter manifold.

**Fluorescent labeling.** Control RBCs were washed with phosphate-buffered saline (PBS). One milliliter of packed RBCs was diluted with 3.0 mL of PBS and mixed with 3.0 mL of fluorescein isothiocyanate (FITC; 0.5 mg/mL) solution in PBS. After incubation for 1 hour on ice, fluorescently labeled RBCs were extensively washed with PBS followed by two washes with the RPMI culture solution. The FITC-labeled RBCs have been previously characterized with no measurable effects on cell morphology. [19]

**Western blot analysis.** Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed in the buffer system of Laemmli. [21] After transfer, proteins on the nitrocellulose blots were detected by either BCIP/Nitroblue tetrazolium or the ECL chemiluminescence system (Amersham, Arlington Heights, IL). Rabbit polyclonal antibodies against human protein 4.1 and p55 were purified by affinity chromatography on immobilized antigens. The intracellular ATP content was determined using an enzymatic kit purchased from Sigma (St Louis, MO).

**Parasite adherence assay.** The attachment of trophozoite/schizont infected RBCs was examined by an in vitro cytoadherence assay. Recombinant chimeric ICAM-1 (a gift from Dr D.E. Staunton, Center for Blood Research, Boston, MA) was adsorbed onto plastic plates (Falcon 1007) overnight at 4°C. Rabbit polyclonal antibodies against human protein 4.1 and p55 were purified by affinity chromatography on immobilized antigens. The plate was blocked for 1 hour with the RPMI 1640 culture medium containing 1% human serum albumin. In vitro cultured trophozoite/schizont-infected RBCs obtained from the Leach and protein 4.1 (-) subjects were incubated in the ICAM-1–coated plate for 2 hours at 37°C. Unbound RBCs were removed by a gentle rinsing of the plates. Adherent cells were fixed with 2% glutaricid/ethanol and stained with Giemsa.

**RESULTS**

**Biochemical characterization of protein 4.1 and glycophorin C-deficient RBCs.** We recently reported that a major palmitoylated protein termed p55 is missing in the RBCs of individuals with either homozygous protein 4.1 (-) HE or glycophorin C (-) HE. [10] In the previous study, two unrelated patients were examined with the homozygous protein 4.1 (-) deficiency. The RBCs of both patients were completely devoid of any intact p55 when examined by Western blot analysis. [12] In the present study, we examined a third unrelated individual with homozogueous protein 4.1 (-) hereditary elliptocytosis. Again, no intact p55 was detected by Western blot analysis of the RBC membranes (Fig 1, lane 4). The RBCs of this individual with protein 4.1 (-) HE are completely devoid of all isoforms of protein 4.1 (Fig 1, lane 6) and contain only 10% of normal glycophorin C (data not shown).

In the previous study, we also examined the RBCs of two unrelated subjects with homozygous glycophorin C (-) HE. [12] The RBC membranes of one Leach subject were completely devoid of intact p55, whereas the RBC membranes of the second subject contained a residual amount (<2%) of intact p55 as detected by Western blot analysis. [12] In the present study, we have examined three related members of a family with homozygous glycophorin C (-) HE. RBC membranes of all three subjects showed a residual amount (<2%) of intact p55 by Western blot analysis (Fig 1, lane 10). In addition, two degradation products of p55 corresponding to 37 kD and 29 kD were also detected by Western blot analysis (Fig 1, lane 10). It should be noted that, although the Leach RBCs examined here lack ~98% of intact p55, the p55 mRNA is present in the reticulocytes (data not shown). The RBC membranes of all three subjects with the Leach phenotype exhibited a 25% deficiency of protein 4.1 (Fig 1, lane 12). The results described below were obtained from the subjects DW (protein 4.1-deficient RBCs) and JC (glycophorin C-deficient Leach RBCs).

**Invasion and growth of Plasmodium falciparum into protein 4.1 (-) RBCs.** The susceptibility of protein 4.1 (-) RBCs to invasion by Plasmodium falciparum was examined using two established techniques: (1) microscopic counting of the ring-infected elliptocytic RBCs after Giemsa staining of blood smears and (2) incorporation of [3H]-hypoxanthine during maturation of ring-stage parasites to trophozoites. Protein 4.1 (-) RBCs were incubated with infected normal RBCs containing trophozoite/schizont stage malaria parasites (Fig 2). Normal RBCs containing mature parasites were first enriched to ~98% parasitemia by Percoll/Sorbitol sedimentation. This prior enrichment of parasites was necessary to quantify the invasion of protein 4.1 (-) elliptocytes by the merozoites released from normal RBCs. We first measured the invasion of protein 4.1 (-) RBCs by Plasmodium falciparum by counting the number of parasite-infected elliptocytes (Fig 2A). As compared with normal RBCs, the invasion of merozoites into protein 4.1 (-) elliptocytes was reduced by ~60% in the first cycle of parasite invasion (Fig 2A, 24 hours). The percentage of parasitemia did not change as the ring-stage parasites matured to trophozoites in the protein 4.1 (-) elliptocytes (Fig 2A, 48 hours). The reinvasion of merozoites that matured in the protein 4.1 (-) elliptocytes was reduced even further, as shown in Fig 2A (72 hours). In the second and third cycles of invasion, the merozoite invasion into protein 4.1 (-) RBCs was reduced by 70% and 88%, respectively (Fig 2A). The reduced invasion of protein 4.1 (-) RBCs by merozoites released from normal RBCs was also measured by [3H]-hypoxanthine incorporation (Fig 2B). The invasion of merozoites into protein 4.1 (-) elliptocytes was reduced by ~75% to 80% as compared with normal RBCs (Fig 2B, 24 to 48 hours). Based on the results of these two independent techniques, we conclude that the invasion of merozoites into protein 4.1 (-) RBCs is significantly reduced.

To further support the conclusion that the protein 4.1 (-) RBCs exhibit a resistance to parasite invasion, we have determined whether the normal merozoites preferentially invade normal RBCs as compared with the protein 4.1 (-) elliptocytes. We examined this possibility by mixing an
equal number of normal and protein 4.1 (-) RBCs before their exposure to the malaria parasite. The normal RBCs in the mixture were labeled with FITC to distinguish them from the unlabeled protein 4.1 (-) elliptocytes. It should be noted that the labeling of normal RBCs with FITC does not affect merozoite invasion and intraerythrocytic growth as compared with unlabeled normal RBCs. The invasion of merozoites was visualized by fluorescence microscopy after staining the ring-stage parasite DNA by ethidium bromide. As shown in Fig 3, the normal merozoites preferentially invaded normal RBCs in a mixture containing equal proportions of normal RBCs and protein 4.1 (-) elliptocytes. Although the invasion of merozoites into protein 4.1 (-) RBCs was reduced, the morphology of intracellular parasites that successfully invaded and matured in protein 4.1 (-) elliptocytes appeared normal as examined by light microscopy (Fig 4). However, the dramatic reduction in parasitemia during the second and third invasion cycles in the protein 4.1 (-) RBCs strongly suggests that the parasite has been greatly weakened during its intracellular growth in the protein 4.1 (-) RBCs. As compared with normal RBCs, the level of parasitemia in protein 4.1 (-) elliptocytes during the second and third invasion cycles was reduced by 94% and 99.3%, respectively (Fig 2).

We next examined whether the malaria parasites that matured in protein 4.1 (-) RBCs could resume their normal growth when transferred to normal RBCs. Equal number of normal and protein 4.1 (-) RBCs containing the trophozoite/schizont stage parasites were isolated by Percoll-Sorbitol density gradient centrifugation. The malaria parasite infected normal and elliptocytic RBCs were then cultured in the presence of uninfected normal RBCs. In contrast to normal parasites in which the parasitemia increased at the expected rate, the parasites derived from protein 4.1 (-) RBCs did not resume growth in normal RBCs (Fig 5).

Invasion and growth of Plasmodium falciparum into Leach RBCs. During the first cycle of parasite invasion, the invasion of Plasmodium falciparum into glycophorin C (-) RBCs was reduced by 55% to 60% as compared with the normal RBCs (Fig 6). Morphologically, the growth of the ring-stage parasites appeared to be normal during subsequent cycles of parasite invasion/growth in Leach RBCs (data not shown). As compared with normal RBCs, the reduced level of parasitemia in the Leach RBCs (55% to 60% reduction) was maintained during the three consecutive cycles of parasite invasion and growth examined in this study (Fig 6). These results are in a striking contrast to the dramatic reduction of parasitemia in protein 4.1 (-) RBCs during the second and third cycles of parasite invasion and growth (Fig 2). Based on these observations, protein 4.1 emerges as an important determinant that regulates parasite growth in human RBCs.

In vitro adherence of parasite infected RBCs. A functionally unique property of RBCs infected with the trophozoite/schizont stage of Plasmodium falciparum is their ability to adhere to receptors on the endothelial cells, thus contributing to the development and pathology of cerebral malaria.

![Fig 1. Biochemical analysis of the elliptocytic RBCs. Protein 4.1 (-) and glycophorin C (-) RBCs were obtained from subjects DW and JC, respectively. Note that protein 4.1 was completely missing in protein 4.1 (-) HE RBCs (lanes 2 and 6), whereas only 25% of protein 4.1 was lost in the membranes of glycophorin C (-) RBCs (lanes 8 and 12). The membrane proteins were purposely overloaded to highlight the region of p55. The arrows also highlight the presence of two degradation products of p55 corresponding to the mass of 37 and 29 kD (lane 10).](image-url)
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This process is likely to be mediated by RBC surface protrusions called knobs. To determine whether the protein 4.1 (-) and glycophorin C (-) RBCs infected with mature-stage parasites can attach to the surface receptors on the endothelial cells, adhesion assays were performed using ICAM-1, one of the important molecules representing the attachment sites on endothelial cells for parasitized RBCs. The recombinant ICAM-1 (rICAM-1) that contains only the D1 and D2 domains was adsorbed onto the plastic plates. The D1 and D2 domains of ICAM-1 have been previously characterized as binding sites for Plasmodium falciparum-infected RBCs. Both protein 4.1 (-) and glycophorin C (-) RBCs containing trophozoite/schizont stage parasites bound to the immobilized rICAM-1 on plastic plates (data not shown). These results indicate that the lack of protein 4.1 and glycophorin C as well as the elliptocytic morphology of infected RBCs do not directly influence the binding of infected cells to recombinant ICAM-1 in vitro.

DISCUSSION

The aim of this study was to determine the functional role of erythroid membrane proteins during the invasion and growth of Plasmodium falciparum into human RBCs. The rationale for this investigation was based on the availability of genetically abnormal RBCs from subjects with homozygous hereditary elliptocytosis (HE). These elliptocytic RBCs have been divided into two categories based on their primary deficiency of either protein 4.1 or glycophorin C. RBCs from protein 4.1 (-) HE have been previously characterized with genetic defects in protein 4.1, leading to the total deficiency of all isoforms of erythroid protein 4.1 as well as a secondary deficiency (70% to 90%) of glycophorin C.

The RBCs of the subject with protein 4.1 (-) HE that we have examined in this study have not been characterized at the molecular level, but the total absence of protein 4.1 in RBCs of two unrelated subjects with homozygous protein 4.1 (-) HE. In addition, deficiency (~98%) of intact p55 was also observed in the RBC membranes of three Leach subjects used in this study, which is again consistent with...
then incubated with a mixture of uninfected RBCs from normal and cently labeled to distinguish them from elliptocytic RBCs. Although protein 4.1 (-) HE subjects. Note that the normal RBCs were fluores-

The results obtained from counting ring-stage parasites as described in the Materials and Methods. Parasites were

protein 4.1 (-) HE exhibit 100% elliptocytosis, whereas the Leach RBCs are known to show varying degrees of elliptocytosis. The molecular basis of these differences is not known.

We used two independent techniques to measure the invasion of Plasmodium falciparum into protein 4.1 (-) RBCs. The results obtained from counting ring-stage parasites as well as from incorporation of 3H-hypoxanthine show that the parasite invasion into RBCs lacking protein 4.1 (4.1-) was reduced by 60% to 80% as compared with normal RBCs (Fig 2A and B). This diminution in parasite invasion into protein 4.1 (-) RBCs became even more significant during the subsequent cycles of parasite invasion and growth in protein 4.1 (-) elliptocytes (Fig 2). More significant was the dramatic reduction in parasitemia during the second and third cycle of parasite growth in protein 4.1 (-) RBCs (Fig 2A).

The reduced parasite invasion and growth in protein 4.1 (-) RBCs was observed with three different geographical isolates of Plasmodium falciparum (data not shown).

A previous study by Schulman et al has measured the invasion and growth of Plasmodium falciparum into protein 4.1 (-) RBCs from the same individual examined in the present study. Using incorporation of 3H-hypoxanthine as a measure of parasite invasion, their data show normal invasion of Plasmodium falciparum into protein 4.1 (-) RBCs, which is in contrast to our finding showing significant reduc-

The reason of this discrepancy is not known at present. The conditions for the in vitro parasite culture and invasion assay used in this study are similar to those used by Schulman et al, except that we have used synchronized parasite cultures to study parasite invasion. Schulman et al also reported that the growth of intracellular parasite became abnormal after the first cycle of parasite invasion and growth in protein 4.1 (-) RBCs. Our results showing a dramatic reduction in parasitemia in protein 4.1 (-) cells are consistent with their finding of abnor-

mal parasite growth in protein 4.1 (-) RBCs. In addition, the data presented in this study indicate that the parasite growth abnormality persists even when the mature parasites are transferred from protein 4.1 (-) RBCs to the normal RBCs (Fig 5). These results suggest that the growth abnormality inflicted on the intracellular parasites during their develop-

ment in protein 4.1 (-) RBCs may be of permanent nature and cannot be corrected even when the abnormal parasites are cultured in normal RBCs. The molecular nature of this abnormality remains to be determined.

The invasion of Plasmodium falciparum into glycoporphin C (-) Leach RBCs was reduced by 60% as compared with the normal RBCs (Fig 6). These results are in agreement with the previously reported observations for the reduced invasion of Plasmodium falciparum into Leach RBCs. However, the subsequent invasion and growth of Plasmodium falciparum in the Leach RBCs is in contrast to the dramatically reduced invasion and growth of parasites in protein 4.1 (-) RBCs (compare Figs 6 and 2). In this study, we have also found that both protein 4.1 (-) and glycoporphin C (-) Leach RBCs infected with mature parasites retain their adherence properties towards recombinant ICAM-1. These results suggest that the complete deficiency of either protein 4.1 or glycoporphin C does not interfere with the expression of putative "adherence receptors" on the surface of infected RBCs.

Based on the data presented in this study, erythrocyte protein 4.1 emerges as a critical host skeletal protein that modulates the intracellular growth of malaria parasite. Indeed, we and others have recently shown that the phosphory-
lation of host protein 4.1 correlates with the intracellular development of Plasmodium falciparum in normal RBCs. The dramatic reduction of parasitemia in subsequent cycles of parasite development in protein 4.1 (-) RBCs (Fig 2) may suggest a novel mechanism by which perturbations in the host membrane cytoskeleton may influence the viability of intracellular parasites. Although the molecular nature of par-

![Fig 3. Preferential invasion of Plasmodium falciparum into protein 4.1 (-) elliptocytes. Mature stage parasites were enriched to 98% parasitemia as described in the Materials and Methods. Parasites were then incubated with a mixture of uninfected RBCs from normal and protein 4.1 (-) HE subjects. Note that the normal RBCs were fluores-

cently labeled to distinguish them from elliptocytic RBCs. Although both normal (N) and elliptocytic (E) RBCs were present in equal proportions, parasites preferentially invaded normal RBCs (N*).](www.bloodjournal.org)
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Fig 4. Morphology of mature parasites in normal, protein 4.1 (-), and Leach elliptocytes. The morphology of parasites at the late trophozoite stage appears to be indistinguishable in normal and mutant erythrocytes.

Parasite abnormality in protein 4.1 (-) RBCs is not yet known, one possible mechanism may involve alterations in the release of intracellular parasites from host RBCs. The appearance of morphologically normal parasites in protein 4.1 (-) elliptocytes (Fig 4) may provide further credence to this hypothesis.

The partial resistance of both protein 4.1 (-) and glycophorin C (-) RBCs to parasite invasion suggests that alterations in the RBC membrane skeleton may influence the susceptibility of the RBCs to invading malaria merozoites. Glycophorin C is directly linked to the underlying membrane skeleton via protein 4.1 and p55,12 and its absence results in a decrease in membrane deformability as well as a significant reduction in membrane mechanical stability.15,30 Indeed, a reduction in RBC membrane deformability has been previously correlated with the inhibition of malaria parasite invasion.31 It is noteworthy that the high incidence of Gerbich-negative phenotypes in Melanesians may confer a selective advantage against malaria.32,33 The Gerbich antigens present on the surface of human RBCs are carried by glycophorin C and D.34 In contrast, partial deficiency of protein 4.1 in heterozygous individuals does not appear to provide any protection against parasite invasion in vitro.35 At present, it is not known whether the prevalence of homozygous deficiency of protein 4.1 correlates with selective pressure of malaria in the endemic areas. Upon completion of the review of this manuscript, Magowan et al36 reported that the presence of parasite-derived protein, MESA, markedly influences the rate of parasite invasion and development in pro-

Fig 5. Transfer of mature parasites from the protein 4.1 (-) RBCs to normal RBCs. The numbers on the X-axis refer to the trophozoite stage at each cycle of parasite invasion and growth. Mature parasites were cultured separately in normal and protein 4.1 (-) RBCs. After their enrichment to 98% parasitemia,16 both parasites were then separately transferred to uninfected normal RBCs. Note that the parasites that matured in protein 4.1 (-) RBCs could not resume growth even when transferred to normal RBCs. The starting parasitemia was low because of the lower number of mature parasites available from protein 4.1 (-) RBCs.

Fig 6. Invasion and growth of *Plasmodium falciparum* into the glycophorin C (-) RBCs (Leach phenotype). Mutant RBCs were obtained from three related individuals with the Leach phenotype. Although the results from one subject (JC) are shown here, virtually identical results were obtained from the other two blood samples. The conditions of the invasion assays were the same as described in Fig 2. The intracellular ATP content of normal, Leach, and protein 4.1 (-) RBCs was comparable, as shown in the Materials and Methods.
tein 4.1-deficient erythrocytes. Because the parasite strains used in our study are MESA positive, the results reported in this manuscript are fully consistent with the reduced invasion observed by Magowan et al in using MESA-positive parasite lines.

Our finding showing that the major palmitoylated protein of the RBC membrane termed p55 is also missing in both protein 4.1 (-) and glycophorin C (-) RBCs suggests that a ternary complex between p55, protein 4.1, and glycophorin C may constitute the biochemical basis of elliptocytosis in these genetically abnormal RBCs. More recent reconstitution assays have shown direct associations between protein 4.1, glycophorin C, and p55, suggesting the existence of a ternary complex at the erythrocyte plasma membrane. The availability of naturally occurring elliptocytes provides a unique opportunity to examine the functional role of this protein complex during the invasion and growth of malaria parasite in vitro.

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