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Malaria biology and disease pathogenesis: insights for new treatments

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Abstract

Plasmodium falciparum malaria, an infectious disease caused by a parasitic protozoan, claims the lives of nearly a million children each year in Africa alone and is a top public health concern. Evidence is accumulating that resistance to artemisinin derivatives, the frontline therapy for the asexual blood stage of the infection, is developing in southeast Asia. Renewed initiatives to eliminate malaria will benefit from an expanded repertoire of antimalarials, including new drugs that kill circulating *P. falciparum* gametocytes, thereby preventing transmission. Our current understanding of the biology of asexual blood-stage parasites and gametocytes and the ability to culture them *in vitro* lends optimism that high-throughput screenings of large chemical libraries will produce a new generation of antimalarial drugs. There is also a need for new therapies to reduce the high mortality of severe malaria. An understanding of the pathophysiology of severe disease may identify rational targets for drugs that improve survival.

Introduction

Malaria is a devastating disease, killing nearly a million children each year in Africa alone. There exists a real and urgent need for new antimalarial drugs that can meet the threat of acquired resistance to artemisinin derivatives, treat severe malaria to reduce death and complications and kill gametocytes to block transmission. Malaria is caused by Apicomplexan pathogens of the genus *Plasmodium*, the most deadly of which, *P. falciparum*, predominates in Africa. Infection by this malarial parasite usually results in an uncomplicated, mild febrile disease in which intermittent episodes of fever and peaks of parasitemia are controlled by the body's immune defenses and eventually eliminated. In some cases, however, the disease becomes severe and may lead to death. Young children are particularly susceptible to severe malaria in endemic areas of Africa, where it is estimated that nearly a quarter of all childhood deaths are caused by malaria.

There is always a need for new antimalarial drugs because we never know when *P. falciparum* will become resistant to the drugs that are presently available. *P. falciparum* resistance to the synthetic antimalarial drug chloroquine was a problem for Vietnam and US soldiers in Vietnam. The spread of chloroquine resistance heralded increased mortality and

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spurred the rush to find a replacement for chloroquine. The development of today's greatest antimalarial drug resulted from the Vietnam War when Mao Zedong assigned many institutions and over 600 scientists to search for new antimalarial drugs. In the early 1970s, the isolation of artemisinin from the ancient Chinese remedy qinghaosu brought new and urgently needed treatments in the form of artemisinin combination therapies (ACTs). Although ACTs are now highly effective, history has taught us that we must continue the search for new drugs in the event that artemisinin resistance develops.

Severe malaria in children encompasses three overlapping syndromes: cerebral malaria, metabolic acidosis and severe anemia (Fig. 1). Today's frontline antimalarial drugs target the asexual blood stage of the infection that causes the symptoms of disease. These drugs improve host survival by reducing parasite loads, disease symptoms and the risk of progression to severe disease. Despite treatment with the most rapidly acting antimalarial drugs, artemisinin derivatives, cerebral malaria and metabolic acidosis have a mortality of 15–20%, and survivors may suffer from persistent neurological sequelae. As our understanding of the underlying causes of severe malaria grows, it may be possible to develop therapies that reverse the complications of malaria infections and lessen the risk of disability or death. Therapies of such nature would modify the physiologic state of the infected human, for example, by targeting the responses of the vascular endothelium to the adhesion of infected erythrocytes with the goal of extending survival and allowing more time for antiparasitic therapies to work.

Where will new drugs to counter resistance and new therapies to improve survival come from? The involvement of Medicines for Malaria Ventures, the industry and scientists supported by governments and foundations have worked on drug discovery since the end of the Vietnam War. Antimalarial drugs will be discovered through random screening of small-molecule or natural-product libraries against either whole organisms or specific parasite molecules (Box 1). Indeed, ACT, the currently recommended treatment for malaria, was discovered by a screen of Chinese herbal medicines used to treat fevers. Antimalarial drugs may also be derived from known compounds, as exemplified by the development of atebrine, chloroquine and primaquine from efforts to find a synthetic substitute for the natural antimalarial, quinine. As our knowledge of the parasite and its life cycle expands (Fig. 1), it will be possible to target parasite molecules that are unique to it and necessary for its survival (Box 2). Particularly helpful will be drugs against gametocytes that persist after antimalarial treatment clears asexual blood-stage parasites. Antigametocyte drugs can disrupt transmission and have an important role in programs to eliminate malaria. The development of therapies to improve outcome from malarial infection may arise from a growing understanding of the molecular basis of the host-parasite interactions that cause severe disease.

Box 1

Discovery of antimalarial drugs: new methods of screening chemical compounds for antimalarial drugs available to the malaria community

High-throughput screening of large numbers of chemical compounds have identified many leads for potential antimalarial drugs[27,51,126–131]. Screens can be classified by their

use of whole-cell screening or target-based approaches. In whole-cell screening, parasite inhibition is monitored by measures of growth and proliferation, usually without knowledge of the molecular targets of the compounds that are being screened. For example, using a single concentration of 10 μM of each compound and radioactive labeling, Chong et al.[126] screened 2,687 compounds and identified 87 existing drugs that resulted in >50% inhibition; a nonsedating antihistamine, astemizole, and its principal human metabolite were found to be promising new inhibitors of chloroquine-sensitive and multidrug-resistant parasites[127]. More recently, many more promising compounds have been identified through screening large collections of small molecules using similar approaches[27,128–131], including a spiroindolone (NITD609) that kills erythrocytic-stage parasites with nanomolar potency[130].

A current focus of antimalarial drug discovery is to screen for small molecules that are active against gametocytes and can block malaria transmission. The only drugs completely effective against *P. falciparum* gametocytes are 8-aminoquinolines; these drugs cause hemolysis that is especially severe in people with Mediterranean G6PD deficiency. Artemisinin derivatives are poorly effective against mature stage V *P. falciparum* gametocytes. In addition to spiroindolones[132], other promising compounds have been screened and found to be active against gametocytes[133–135] and have the potential to block parasite transmission. A combination of quantitative high-throughput screening and genome-wide association and linkage analyses has also allowed for the identification of potential molecular targets or molecules involved in transport of the small molecules[27,131].

Another approach commonly used for drug screening involves assays that detect inhibitors of a specific parasite target, such as a kinase or cell-surface ligand for merozoite invasion of erythrocytes[136,137]. Although only a limited number of kinases or receptors have been studied in malaria parasites so far, some recent studies have shown that these molecules are promising targets for inhibitors blocking parasite development, invasion and transmission[136]. Other promising leads have emerged from target-based screens for specific inhibitors of *P. falciparum* dihydroorotate dehydrogenase[138] and enzymes of the *P. falciparum* respiratory chain, cytochrome bc_1 and PfNDH2 (ref. 139).

Box 2

Examples of specific drug targets: the apicoplast and the nutrient channel

The apicoplast is an organelle in Apicomplexa derived from the chloroplast and from photosynthesis in its ancient ancestor. Recent studies demonstrated that the apicoplast has only one crucial function during the asexual blood stage, namely isoprenoid precursor synthesis. Yeh and DeRisi[140] showed that the elimination of the apicoplast of *P. falciparum* by treatment with fosmidomycin, which blocks the conversion of 1-deoxy-D-xylulose-5-phosphate (DOXP) to methylerythritol phosphate, killed *P. falciparum*. The parasites without an apicoplast survived in media supplemented with isopentenyl pyrophosphate (IPP). Although both IPP and dimethylallyl diphosphate (DMAPP) are required for isoprenoid synthesis, IPP alone was sufficient to supplement parasites

lacking apicoplasts, indicating that there must be an IPP isomerase to convert IPP to DMAPP, although this enzyme has not yet been identified. The elimination of the apicoplast in fosmidomycin-treated IPP-supplemented *P. falciparum* has been proven by the absence of the apicoplast genome and by the absence of the four-membrane apicoplast organelle. This study demonstrates that the pathway from DOXP to IPP is the crucial function of the apicoplast during the asexual blood stage and that enzymes in this pathway[141] would be excellent targets for small-molecule inhibitors and should be synergistic with fosmidomycin.

Infected erythrocyte membranes have nutrient channels with increased permeability to a range of solutes, including anions, organic cations, sugars, amino acids, purines and vitamins. A recent study demonstrated that a single parasite-encoded molecule was responsible in part for the permeability changes in the infected erythrocyte[142]. One inhibitor identified by high-throughput screening was highly specific for solute uptake by the *P falciparum* clone Dd2 and had little or no activity against the *P. falciparum* clone HB3. The progeny clones from a Dd2 × HB3 genetic cross were used to map the locus to a single region on chromosome 3 of the parasite; complementation and allelic exchange implicated two related genes, *clag3.1* and *clag3.2*. These findings are strong evidence for a parasite-encoded nutrient uptake channel. In addition, other parasite molecules and perhaps host erythrocyte molecules may be involved in channel activity. Although parasite clone–specific inhibitors were crucial for gene identification, high-throughput screening of them showed that most inhibitors target conserved parts of the channel; some inhibitors have preserved activity against the channel from divergent *P. falciparum* clones. The unusual nature of the parasite channel has no function or molecular homology to channels in humans. Different parasite channel inhibitors show a correlation of blocking the channel and parasite growth inhibition. Thus, blocking the channel would lead to parasite death.

In this review, we will discuss recent developments in studies of the molecular basis of disease, parasite development and host-cell invasion and point to potential parasite targets that may be explored for drug development and improved treatment of children with severe disease. We will focus on the discovery of drugs for use against *P. falciparum* in endemic countries and not on the development of drugs for tourists or the military. Readers who are interested in the broader aspects of antimalarial drug development, current drugs in the pipeline, efforts to develop drugs against relapses of *Plasmodium vivax* and malaria elimination are referred to some excellent review articles on the subjects[1–3].

The digestive vacuole and continuing drug discovery

About two-thirds of the hemoglobin content of *P. falciparum*–infected erythrocytes is consumed in the digestive vacuole to provide the maturing parasite with amino acids and guard against premature rupture. *Plasmodium* parasites digest hemoglobin by an array of functionally redundant peptidases, making proteases a poor drug target[4]. A consequence of hemoglobin digestion is the release of heme as Fe(II)–protoporphyrin IX (Fe(II)PPIX) that quickly oxidizes to Fe(III)PPIX (hematin), a product that poisons the parasite by membrane damage and lysis (Fig. 2)[5]. Malaria parasites avoid heme toxicity by converting dimers of

hematin into inert crystals (called hemozoin) and perhaps also by heme degradation through peroxidative or glutathione-mediated pathways[6]. Drugs that interfere with crystal formation have been some of the most successful in malaria therapy. Among these is chloroquine, an antimalarial drug that was used for hundreds of millions of treatments annually before chloroquine resistance developed.

Resistance is conferred by mutations in a conserved molecule termed the *P. falciparum* chloroquine resistance transporter (PfCRT) that is expressed on the digestive vacuole membrane[7]. Drug efflux by the mutated molecule represents an expansion of the normal physiological function of PfCRT, which may include transport of amino acids or short polypeptides from the digested hemoglobin[8]. Mutations or changes in the expression of a *P. falciparum* P-glycoprotein homolog (PfPgh1) encoded by the *P. falciparum* multidrug resistance 1 gene (*pfmdr1*) that also resides on the digestive vacuole membrane[9] can modulate chloroquine resistance in PfCRT-mutant parasites and, along with PfCRT and other transport molecules, alter the responses to other antimalarial drugs, including mefloquine, quinine, lumefantrine, halofantrine and artemisinin[10].

The concentration of chloroquine that kills chloroquine-resistant *P. falciparum* parasites *in vitro* can be reduced by verapamil and other pharmacologically and structurally diverse agents, including tricyclic antidepressants, antihistamines, phenothiazine, some plant alkaloids and primaquine. This effect on chloroquine-resistant but not chloroquine-sensitive parasites is referred to as chemosensitization. These agents generally include a pharmacophore of one or more aromatic rings plus a protonated nitrogen that competes with chloroquine in the PfCRT pore, with the extent of chemosensitization depending on the mutations present[11]. Differential degrees of resistance to the active metabolites of chloroquine (monodesethylchloroquine), amodiaquine (monodesethylamodiaquine) and other quinoline drugs are similarly linked to variations in the mutations of PfCRT[12,13]. The structural specificity implied by these observations coincides with reports that a number of compounds related to chloroquine but not efficiently effluxed by PfCRT can be remarkably effective against chloroquine-resistant as well as chloroquine-sensitive *P. falciparum*. Examples include such well-known antimalarial drugs as pyronaridine and piperaquine (Fig. 1), compounds with 4-amino side chain variations on the 7-chloro-4-aminoquinoline moiety of chloroquine and certain other 3- or 7-substituted 4-aminoquinolines[14–18]. The risk of arrhythmia, as indicated by hERG (human ether-a-go-go related gene product) channel assays, remains a concern for the 4-aminoquinoline compounds. These risks must be considered in light of the safety record of chloroquine, evidence that the ratio of hERG half-maximum inhibitory concentration (IC_{50}) to free drug concentration may not be a reliable predictor of human risk and the suggestion that 4-aminoquinoline candidates with higher hERG IC_{50} values have lower risk of arrhythmia in comparison to chloroquine[18,19].

Although chemosensitizing agents are not sufficiently active at concentrations that can be safely and effectively used in humans[20], their resistance-reversing activity has inspired the synthesis of chloroquine derivatives that incorporate various chemosensitizer moieties at the 4-amino position. Examples of side chains in these ‘reversed chloroquine’ compounds include dibemethins, imipramine and other pharmacophores predicted to confer reversal activity[21,22]. Ferroquine, a potent new organometallic chloroquine analog that carries a

ferrocenyl group in the side chain[23], also seems to fall within this theoretical framework. Successful phase 1 trials of ferroquine[24] and AQ-13, a short-chain analog of chloroquine[25], have raised hope that these and other 4-aminoquinolines will be successful in clinical development and join pyronaridine and piperaquine as alternative drugs for use against chloroquine-resistant malaria.

Various studies have identified a number of antimalarial compounds that show greater activity against chloroquine-resistant than chloroquine-sensitive *P. falciparum* and have IC_{50} values that are linked to PfCRT isotype[26,27]. Combinations of various antimalarial pairs may also act in synergy or in antagonism, depending on the exact mutations encoded by the *PfCRT* allele[12,27]. These observations raise the possibility of drug combinations that together may combat malaria drug resistance by selection pressure against mutant forms of PfCRT. Examples of suppressive drug combinations have been reported in studies of *Escherichia coli* and *Staphylococcus aureus* populations in which strongly antagonistic pairs of drugs reduced the potential for evolving resistance far more efficiently than pairs of synergistic drugs[28,29].

Products of hemoglobin digestion are also involved in the action of artemisinin-class antimalarials on the erythrocytic stages of *Plasmodium* spp.[30]. The artemisinins, developed from the ancient Chinese herbal remedy qinghaosu, carry a superoxide pharmacophore with a peroxide bridge that is cleaved and activated by ferrous heme or free ferrous iron. Highly reactive carbon-centered radicals from this activation kill the parasite by processes that may include damage to cellular lipids and digestive vacuole membranes[31], inactivation of *Plasmodium* proteins, alkylation of heme and interference with heme sequestration into crystals[32]. The effect on intraerythrocytic stages is broad, including strong action on rings stages as well as trophozoites and early schizonts[33].

Artemisinin-based treatments with artesunate, artemether or dihydroartemisinin are now the first-line recommendation for *P. falciparum* malaria in most endemic areas and are increasingly used against the problem of chloroquine-resistant *P. vivax* malaria[34,35]. Artemisinin treatment reduces parasitemia very rapidly, with a parasite reduction of about 10,000-fold per cycle *in vitro*. When given as monotherapy, a 7-d regimen was found to be required to achieve acceptable cure rates, but some recrudescences by day 28 still occurred[36]. These recrudescences, the difficulty of patient adherence to the dosing regimen and the short *in vivo* half-life of the active dihydroartemisinin metabolite ($t_{1/2} < 1$ h) led to early realization of the importance of artemisinin therapy being partnered with a more slowly eliminated antimalarial drug. Because combinations can fail in regions where malaria parasites are resistant to the partner drugs, artemisinin-combination therapies require continuous monitoring for efficacy, and the appropriate choices of alternative drug combinations must be available when failure rates become unacceptable.

Prolonged parasite clearance times (PCTs) have recently been reported from western Cambodia[37,38], raising concerns that *P. falciparum* parasites in the region are becoming resistant to artemisinin and its derivatives[39]. However, the parasites that show a prolonged PCT do not show correlated increases of IC_{50} values to dihydroartemisinin *in vitro*[37,40]. Multiple host and parasite factors are involved in PCT. Recrudescences in association with

prolonged PCTs have been documented after 7-d treatment courses of artesunate[41] (Box 3). Evaluations of patients in Cambodia infected with identical or nonidentical parasite genotypes showed that prolonged PCTs could be attributed to heritable parasite factors (~60% of PCT variation)[42]. Evidence from DNA microarray studies suggests that prolonged PCTs may be associated with reduced activity of basic metabolic and cellular maturation pathways in the parasites[43]. More recently, genome surveys have suggested that differences in a number of genome regions may be associated with prolonged PCT after artemisinin treatment, including the possibility of a selective sweep on *P. falciparum* chromosome 13 (ref. 44).

Box 3

Is recrudescence after artemisinin treatment linked to a dormancy phenomenon?

Dormancy, a quiescent state in which a small fraction of inactive parasites are unaffected by drug treatment, is proposed to account for some observed discrepancies between *in vivo* and *in vitro* responses to drugs. Although dormancy during chloroquine treatment was not demonstrated in laboratory studies, results from sorbitol-treated, pyrimethamine-treated or mefloquine-treated parasite cultures suggested that small numbers of inactive parasites, perhaps lag-phase ring forms, could survive these exposures and later reactivate with the same characteristics of drug sensitivity as the original population[143,144]. Other reports have described possible cell-cycle delays and recrudescence without innate changes in drug sensitivity after treatment with atovaquone, atovaquone plus proguanil or mefloquine[145,146]. Dormancy has also been proposed from mathematical modeling of artesunate treatment results[147], and several studies have identified dormancy and a ring-stage quiescence mechanism of survival during exposure to artemisinin drugs[148–151]. These and additional observations[152–154] have raised important questions regarding the nature of the relationship between a dormancy phenomenon by which no more than 1–2% of parasites recover[147,149] and a mechanism that reduces clearance rates by doubling or tripling the PCTs to over 100 h after the initiation of artemisinin drug treatment[38]. Evidence from DNA microarray studies suggests that prolonged PCTs are associated with reduced activity of basic metabolic and cellular maturation pathways[43]; data implicating these same pathways in quiescent parasites would provide evidence for common underlying mechanisms in prolonged PCTs and dormancy.

Because the artemisinin derivatives act for only a short time and require frequent dosing, searches for other artemisinin derivatives and synthetic analogs with improved pharmaceutical properties over artemisinin have received considerable attention (Fig. 2). Among these are completely synthetic, low-cost peroxides, including 1,2,4-trioxanes, 1,2,4-trioxolanes and 1,2,4,5-tetraoxanes, some of which are currently in clinical trials or are new candidates for preclinical development[45,46]. A particularly promising series of ozonide compounds from spiroadamantine-shielded 1,2,4-trioxolanes has yielded compounds that are well tolerated with good oral bioavailability, outstanding efficacy and pharmacokinetic half-lives that are much longer than those of artemisinin derivatives[47]. OZ439, a particularly

encouraging candidate currently in phase 2 trials supported by Medicines for Malaria Ventures, showed a half-life of ~30 h in preclinical tests and produced full cures after one-time oral administration in mice infected with *Plasmodium berghei*, raising the prospect that OZ439 (in combination with a suitable partner) may be effective for single-dose treatment for malaria[48]. As OZ439 moves into phase 3 trials, it will be important to establish the effect of its much longer half-life compared to that of dihydroartemisinin on parasites with prolonged PCTs and evaluate the impact of this longer period of continuous drug exposure on recrudescences after treatment.

Inhibitors targeting parasite kinases

Kinases are traditionally the targets of drug screens because these molecules are involved in various important biological pathways, and their activities can be readily assayed. Additionally, large numbers of known or potential kinase inhibitors are available in the small-molecule libraries of pharmaceutical companies. This principle can be applied to screening for antimalarial drugs, although malaria parasites have a much smaller number of kinases in their genomes[49,50]. The divergences between *Plasmodium* protein kinases and those of their mammalian hosts suggest that the parasite kinases can be attractive targets for new antimalarials[50]. Examples include *P. falciparum* MAPK2 (PfMAPK2), which is member of MAPK family but has unique characteristics, the FIKK kinases that are found only in Apicomplexa and the calcium-dependent protein kinases (CDPKs) that are present exclusively in plants and alveolates[49]. Additionally, different kinases are expressed at different parasite developmental stages, which can be explored for drugs of different purposes, such as transmission blocking.

One example of parasite kinases used in screening of small molecules for inhibitors is *P. falciparum* CDPK1 (PfCDPK1). In a recent study, ~20,000 compounds from a kinase-directed heterocyclic library were screened for their ability to inhibit recombinant PfCDPK1, which seems to be essential for the asexual stages[51]. Recombinant PfCDPK1 was expressed as a GST fusion protein, and biotinylated casein kinase II peptide was used as a substrate in an assay measuring the ability of PfCDPK1 to catalyze the transfer of the γ-phosphate group from (γ-33P)ATP to the biotinylated substrate peptide. Approximately 50 compounds that could inhibit the kinase activity by >80% at a concentration of 1 μM were identified[51], including a compound called purfalcamine that was found to bind PfCDPK1 in parasite lysates and block the development of late schizonts. In another study, histidine-tagged PfCDPK1 was expressed in *E. coli* and used in screens against 54,733 compounds, leading to the identification of 70 compounds with submicromolar activities[52]. Cyclin-dependent kinases (CDKs) that control cell-cycle progression have also been investigated as the targets of antimalarial drugs. Several homologs of human CDKs have been characterized from *P. falciparum*, including PfPK5 (a homolog of human CDK1) and Pfmrk (a homolog of human CDK7)[49,53,54]. Both PfPK5 and Pfmrk were identified as potential targets for the development of antimalarial agents[53–55]. Screening of extracts from marine sponges for inhibitors of a *P. falciparum* never in mitosis *Aspergillus* (NIMA)-related protein kinase (Pfnek-1) also led to the identification of several molecules with activities against *Plasmodium* parasites *in vitro* and *in vivo*[56,57]. Another potential kinase target is the *P. falciparum* cyclic GMP (cGMP)-dependent protein kinase (PKG), which has been shown to

be essential for xanthurenic acid–induced and zaprinast-induced gametogenesis[58]. PKG, xanthurenic acid and cGMP were all found to be components of the gametocyte activation pathway in *P. falciparum*. In a follow-up study, the same group examined the role of PfPKG in the asexual blood stage of the parasite life cycle using a PKG inhibitor, trisubstituted pyrrole. Addition of this compound prevented synchronized schizonts-infected erythrocytes from releasing merozoites. Using genetically manipulated *P. falciparum* parasites expressing a *PfPKG* allele that was insensitive to the compound, they also showed that the mutant parasites were able to complete schizogony in the presence of the compound but not in the presence of the broad-spectrum protein kinase inhibitor staurosporine, suggesting that PfPKG is the primary target of the compound during schizogony. These results showed that cGMP signaling is a key regulator of both the sexual and asexual stages, which can be explored for potential drug targets for both therapeutic and transmission-blocking activities.

Merozoite invasion of erythrocytes: in search of drug targets

Mature merozoites are released from infected erythrocytes in the blood stream by rupturing the erythrocyte membrane. The freed merozoites then rapidly invade uninfected erythrocytes. There is extensive redundancy of the use of ligands by *P. falciparum* for invasion, and, consequently, no erythrocyte type has been identified in endemic areas refractory to invasion as a result of the loss of a receptor for *P. falciparum*. This is in contrast to *P. vivax*, where the loss of an erythrocyte receptor, the Duffy blood group system, led to refractoriness.

Invasion of erythrocytes by merozoites involves five discrete steps (Fig. 3). The ligands in the first step, the binding of any part of the merozoite to erythrocytes, are unknown and are thus not currently drug targets. In the second step in invasion, the merozoite orients its apical end to the erythrocyte in a process that involves two families of *P. falciparum* ligands that bind receptors on the erythrocyte surface, namely the four Duffy binding-like (DBL) ligands and the five reticulocyte homology ligands (Fig. 3; step 2, reorientation). Possibly because of the redundancy in *P. falciparum* DBL and reticulocyte homology ligands, all these ligands can be knocked out with one exception, reticulocyte homology 5 (RH5), making it a possible drug target[59,60]. RH5 has no transmembrane domain and binds another soluble protein with ten epidermal growth factor-like domains (PFC1045c) that also has no transmembrane-like domains[61]. In addition, the erythrocyte receptor for RH5 was found to be CD147 (also known as Ok^a or basigin)[62]. In this study, soluble basigin and antibodies to basigin were found to block parasite invasion of erythrocytes. The essential nature of RH5 in the face of so much redundancy in ligand-receptor interactions raises the possibility that the interactions of RH5 could be blocked using small molecules.

In the third step in invasion, apical membrane antigen 1 (AMA1) moves from micronemes to the merozoite surface and binds a segment of rhoptry neck protein 2 (RON2). AMA1 has a groove formed by two PAN/apple domains in AMA1 (ref. 63). The RON proteins are secreted into the erythrocyte membrane and accumulate on the cytoplasmic face, whereas a segment of RON2 remains outside the erythrocyte membrane to bind AMA1 (refs. 64,65). The junction that must be formed to bring the merozoite into the erythrocyte requires the binding of AMA1 to RON2 (Fig. 3; step 3, junction formation). No junction is formed and

no invasion occurs if the AMA1 binding to RON2 is blocked by soluble RON2 peptide or antibodies to the AMA1 groove[66]. The RON2-AMA1 junction would be an ideal target for small-molecule inhibitors because RON2 and AMA1 are both parasite molecules that must fit together for effective junction formation. Small-molecule inhibitors of protein-protein interactions are potential targets for drug development where there is a pocket (groove) for binding small molecules[67].

The internal signals for the release of microneme and rhoptry contents are still not completely understood. Binding of glycophorin A to its ligand, erythrocyte-binding antigen 175 (EBA-175), on the merozoite surface leads to the release of rhoptry proteins[68]. However, the EBA-175–glycophorin A interaction cannot be an absolute requirement, as parasites lacking EBA-175 still are able to invade erythrocytes.

AMA1 has a crucial serine on its cytoplasmic tail (Ser610) that if mutated to an alanine (S610A) cannot function in invasion. Ser610 is phosphorylated by the parasite protein kinase A (PKA, also known as PFI1685w), a cyclic AMP–activated kinase[69]. Although crucial for invasion, the function of this phosphorylation event and the timing of changes in the level of phosphorylation are unknown. One speculation is that it may signal the actin-myosin motor to begin bringing the merozoite into the erythrocyte. The binding of aldolase to the cytoplasmic tail of AMA1 that connects it to the actin-myosin motor is unaffected by the S610A mutation. Although the role of this phosphorylation in invasion is unknown, PKA might be a target for small-molecule inhibition.

In the fourth step of invasion after the formation of the junction, unknown factors stimulate the actin-myosin motor to power the junction to move from the anterior to the posterior end of the merozoite, bringing the merozoite into the erythrocyte (Fig. 3; step 4, invasion). Simultaneously with the movement of the junction, a vacuole forms around the merozoites derived from the erythrocyte membrane, flowing past the junction as the parasite moves into the erythrocyte[70]. Little is known of the signals for the release of parasite proteins from organelles and the factors involved in activating the motor. It has been observed that one of the rhoptry proteins, rhoptry associated protein 1 (RAP1), is located within the invaginated membrane[71], but proof that it is the cause of the invagination is lacking. The fifth step of invasion, the resealing of the vacuole and the erythrocyte membrane (Fig. 3; step 5, resealing), is crucial for parasite survival, as anything that blocks resealing, such as a residual body attached to the merozoite, leads to erythrocyte lysis. The remnants of the moving junction remain on the outside of the vacuolar membrane[70].

Besides invasion, release from the erythrocyte is an essential process and, consequently, a potential drug target. The process of erythrocyte membrane rupture is less well understood at a molecular level than is erythrocyte invasion. Nonetheless, several factors required for merozoite release have been identified, including a calcium-dependent protein kinase, PfCDPK5. PfCDPK5 conditionally deficient parasites mature into fully viable merozoites in the infected erythrocyte but are unable to leave the vacuole and thus are not released from the infected erythrocyte[72]. PfCDPK5 evolved from a plant kinase and may be selectively targeted by small-molecule inhibitors that may be less likely to bind human kinases. A *Plasmodium* membrane attack complex (MAC)/perforin domain-may be another potential

target, as a MAC/perforin domain has been shown to be involved in the release of *Toxoplasma gondii* from its vacuole and host cell[73] and may have a similar role in *Plasmodium* escape from the infected erythrocyte.

Endothelium-targeted strategies to improve survival

Antiparasitic drugs are the cornerstone of malaria treatment; however, even the most efficacious parasite-killing drug, artesunate, could not prevent deaths among children with severe complications of malaria, such as severe anemia (1% mortality)[74], acidosis (15% mortality) or coma (18% mortality)[35]. Thus, even rapid and effective parasite killing is insufficient to prevent death in severe cases. Although blood transfusion reduces mortality in children with severe anemia and respiratory distress, there are no specific therapies for acidosis or coma (Fig. 1b).

Metabolic acidosis is present in 40–60% of cases of severe malaria, often presenting as respiratory distress. The blood concentration of lactic acid, a product of anaerobic glycolysis from inadequately perfused host tissues, predicts death from severe malaria. Unlike septic shock, where lactic acid production is associated with hypotension, hypovolemia or both, most patients with malaria-associated lactic acidosis are not substantially volume depleted[75], and intravenous fluid bolus may be harmful[76].

Impaired consciousness, measured by the Blantyre coma score, is consistently associated with increased risk of lasting neurological injury and death. The prognostic value of the coma score is enhanced when combined with visual examination of the retina. Papilledema and retinal hemorrhage are each associated with increased risk of death[77], and retinopathy is associated with postmortem counts of adherent infected erythrocytes, thrombosis and hemorrhage in cerebral microvessels[78]. Microvascular obstruction may be a pathogenic mechanism shared by cerebral malaria and acidosis, the two severe malarial syndromes that cause the most deaths.

Disruption of microvascular blood flow has been directly observed in human clinical studies of severe malaria[79]. Beyond mechanically obstructing small vessels, adherent infected erythrocytes transduce pathologic endothelial activation signals that promote adhesion, coagulation and inflammation, disrupt endothelial barrier integrity and impair vasoregulation (Fig. 4)[80–82]. Endothelial dysfunction is exacerbated by malaria-induced decreases in nitric oxide, a signaling molecule that normally maintains endothelial homeostasis[83]. To improve survival from severe malaria, therapeutics that can modulate key endothelial functions need to be tested in combination with parasite-killing drugs.

Endothelium-targeted strategies to improve survival

Targeting parasite adhesion to the vascular endothelium

P. falciparum expresses highly variable proteins on the surface of erythrocytes called *P. falciparum* erythrocyte membrane proteins 1 (PfEMP1s). PfEMP1s bind to protein and carbohydrate ligands on the surfaces of endothelial cells (including CD36, intercellular adhesion molecule 1 (ICAM1), platelet/endothelial cell adhesion molecule (PECAM), CR1, heparan sulfate and chondroitin sulfate A), allowing infected erythrocytes to attach,

sequester and avoid clearance in the spleen. Clinical episodes of severe malaria have recently been associated with expression of *var* genes that encode specific PfEMP1s[84]. The same *var* genes were independently observed to bind brain endothelium *in vitro*[85,86]. Once the crucial *var* ligand and its endothelial receptor have been identified, high-throughput screening could be used to identify small molecules that block infected erythrocytes from binding to or activating microvascular endothelium through this pathway. An existing drug, levamisole, interrupts CD36-dependent binding by inhibiting the dephosphorylation of CD36 that is required for high-affinity binding.

Interrupting malaria-mediated blood coagulation and thrombosis

Systematic postmortem examinations have demonstrated thrombosis and hemorrhage where infected erythrocytes adhere to cerebral microvascular endothelium[78]. Adherent infected erythrocytes trigger coagulation by inducing tissue factor and activating thrombin on the apical surface of endothelial cells[80]. Thrombin not only catalyzes fibrin deposition in blood vessels but also amplifies inflammation and disrupts endothelial barrier integrity through protease-activated receptor signaling[87]. Heparin, which activates antithrombin III, was tested in several small studies of severe malaria but had no effect on outcome[88]. Activated protein C, an endogenous anticoagulant that is diminished in severe malaria[89], has been administered therapeutically but has never been evaluated in a controlled study. Defibrotide, a DNA aptamer, blocks endothelial tissue factor induction by infected erythrocytes *in vitro* and shows favorable anti-inflammatory properties *in vivo*[90] but has yet to be tested in human malaria.

Microvascular obstruction can also be caused by deficiency of the von Willebrand Factor (vWF) protease ADAMTS13 (ref. 91), resulting in long vWF multimers that tether platelets and infected erythrocytes to endothelium[92]. The number and length of vWF multimers could be decreased by restoring the deficient activity of the vWF protease ADAMTS13 (ref. 91) with plasma transfusion or by inhibiting exocytosis of the endothelial Weibel-Palade bodies that release vWF multimers[93].

Preserving endothelial barrier integrity

Loss of endothelial barrier integrity may be an important step in the progression to fatal cerebral malaria. The angiopoietin (Ang) proteins regulate endothelial barrier integrity and are associated with retinopathy and death from cerebral malaria[94,95]. In the setting of tumor necrosis factor (TNF) stimulation, Ang2 permits breakdown of endothelial barrier integrity and expression of endothelial adhesion molecules such as ICAM1. The exocytosis of Ang2 from endothelial Weibel-Palade bodies might contribute to the vascular leak, inflammation and cerebral edema that are associated with cerebral malaria, whereas upregulation of ICAM1 would increase the adhesion of infected erythrocytes. Endothelium-targeted therapies that suppress Weibel-Palade body exocytosis might interrupt the pathogenic autocrine activity of Ang2.

Erythropoietin is secreted primarily by renal peritubular cells in response to hypoxia. In addition to promoting erythropoiesis, erythropoietin exerts neuroprotective effects through the heterodimer of the erythropoietin receptor and CD131. Erythropoietin decreases the

expression of inflammatory cytokines, limits astrocyte water uptake and prevents neuronal apoptosis. Among Kenyan children with cerebral malaria, high amounts of erythropoietin were associated with protection from neurological sequelae and death[96]. In a mouse model of cerebral malaria, erythropoietin decreased the expression of inflammatory cytokines in the brain, reduced the number of perivascular hemorrhages and improved neurological function and survival[97]. Erythropoietin was recently shown to be safe in children with malaria, but efficacy studies have not been performed.

Endothelial nitric oxide is diminished in severe malaria

Vascular endothelium regulates adhesion, coagulation, inflammation, barrier integrity and smooth muscle tone through nitric oxide. Diminished nitric oxide bioavailability may exacerbate endothelial dysfunction and contribute to the pathogenesis of severe malaria. Indonesian adults with severe malaria had decreased endothelial nitric oxide bioavailability, as demonstrated by impaired vasodilation after 5 minutes of brachial artery occlusion[83]. This vasodilation index correlated with the plasma concentration of arginine and the nitric oxide synthase (NOS) substrate and inversely correlated with the concentration of methylarginine, an endogenous NOS inhibitor[98]. Malian children with severe malarial anemia showed evidence of pulmonary vasoconstriction, consistent with low nitric oxide bioavailability[99]. In both studies, the functional activity of nitric oxide was inversely correlated with the concentration of plasma hemoglobin, a nitric oxide scavenger.

Intravascular hemolysis releases hemoglobin into the plasma, where it can interrupt endothelial nitric oxide signaling. Haptoglobin binds to hemoglobin in plasma, and the haptoglobin-hemoglobin complex is removed from circulation by CD163 on macrophages and monocytes. Intracellularly, heme oxygenase-1 (HO1) metabolizes heme into biliverdin, releasing carbon monoxide and ferrous iron. Although carbon monoxide has anti-inflammatory properties, the net effect of HO1 activity in humans may be proinflammatory because free iron potentiates the neutrophil oxidative burst. In Gambian children, *HMOX1* alleles with high inducible expression conferred susceptibility to severe malaria[100].

In addition to nitric oxide scavenging and inflammation, hemolysis causes depletion of the NOS substrate arginine by releasing arginase from erythrocytes and inducing expression of host arginase[101]. Arginine depletion not only diminishes nitric oxide synthesis but also causes NOS to function as an oxidase, generating superoxide radicals instead of nitric oxide from molecular oxygen.

Intravascular hemolysis followed by proteolysis of erythrocyte proteins releases asymmetric dimethylarginine (ADMA) into plasma that can overwhelm host mechanisms of ADMA clearance and cause endothelial dysfunction[102,103]. By inhibiting NOS, ADMA activates endothelial Rho kinase[104] (a molecular switch also activated by the adherence of infected erythrocytes[81]), which increases expression of NF-κB, secretion of TNF, display of procoagulant tissue factor and expression of the infected erythrocyte receptor, ICAM1. ADMA decreases endothelial cell motility, disrupts endothelial barrier integrity and inhibits vascular repair of endothelial disruptions. Dimethylarginine dimethylaminohydrolase (DDAH) indirectly regulates nitric oxide synthesis by metabolizing ADMA[105]. DNA

sequence polymorphism in the gene *DDAH1* was associated with increased susceptibility to severe malaria in a recent genome-wide association study of Gambian children[106].

Restoring endothelial nitric oxide bioavailability

The emerging evidence for decreased nitric oxide bioavailability in the pathogenesis of severe malaria has stimulated interest in restoring nitric oxide signaling as an endothelium-targeted adjunctive therapy. Arginine supplementation resulted in increased exhaled nitric oxide and nitric oxide–mediated vasodilation in adult patients with moderately severe malaria; however, these patients had normal nitric oxide production before treatment[83]. Arginine supplementation might be less effective at restoring NOS-mediated nitric oxide production in severely ill patients in which arginase, ADMA, NOS glutathionylation or tetrahydrobiopterin deficiency may limit NOS activity. Furthermore, plasma hemoglobin[99,107,108] or reactive oxygen species[109] limit the half-life of nitric oxide.

Restoration of nitric oxide bioavailability may require a NOS-independent approach. Nitric oxide gas can be delivered directly to the pulmonary circulation by inhalation, where it exerts local pulmonary arterial vasodilator effects. Inhaled nitric oxide can be transported in blood as S-nitrosothiols, nitrosylhemoglobin or nitrite, where it can act at distant sites. Administration of exogenous nitric oxide inhibits NF-κB–mediated expression of inflammatory cytokines, adhesion molecules and tissue factor[110,111] and decreases platelet activation and adhesion of leukocytes and infected erythrocytes[93,112,113]. Furthermore, nitric oxide preserves endothelial barrier integrity and prevents pathologic thrombosis by inhibiting Weibel-Palade body exocytosis of Ang2 and vWF[93]. There are currently two controlled trials of inhaled nitric oxide ongoing in African children with malaria.

An alternative is to generate nitric oxide in blood and tissues by the nitrite-heme–nitric oxide pathway, a physiologic mechanism that regulates hypoxia-mediated vasodilation in humans[114]. Nitrite can be reduced to nitric oxide by deoxyhemoglobin in blood[115], as well as by hemeglobins[116], xanthine oxidase and aldehyde oxidase[117] in tissues. Nitrite improves nitric oxide bioavailability in animal models of hemolysis[118], hemoglobin-based oxygen carrier transfusion[119], ischemia-reperfusion injury[120], lipopolysaccharide-induced inflammation[121] and argininosuccinate lyase deficiency[122], conditions that have features in common with malaria. Nitrite improved survival in a mouse model of severe malaria[123]. In humans, nitrite has been safely administered to healthy volunteers[114] and patients with sickle cell–induced hemolysis in which nitrite improves regional blood flow[124]. The low cost, room-temperature stability and oral bioavailability of nitrite would make it feasible for use in resource-limited health care settings.

The way forward

Each sick child represents our failure to prevent malaria, and each death represents our failure to treat soon enough. Despite having the ‘wonder drug’, artemisinin derivatives, we must always have new drugs in reserve. We have outlined promising targets for drug discovery, such as parasite metabolism, parasite signaling and host responses. These and other targets will require the expertise of industry or public and private collaboration to see them to completion, and whole-cell screens (infected erythrocytes and gametocytes) for drug

discovery are important. New drugs against stage V *P. falciparum* gametocytes and *P. vivax* hypnozoites are needed immediately for malarial elimination. Vaccines may ultimately offer the most cost-effective tool for disease prevention, but they are not currently available. Likewise, vector research will be essential for the development of new insecticides to replace the failing synthetic pyrethroids[125] and to create mosquitoes refractory to malaria transmission. Ultimately, elimination of malaria will require advancements in drug development, vaccines and vector biology. Until malaria elimination has been achieved, drug treatment will remain crucial to prevent complications and death from malaria. Drugs in the development pipeline will always be needed as parasites acquire resistance to each new generation of antimalarial drugs.

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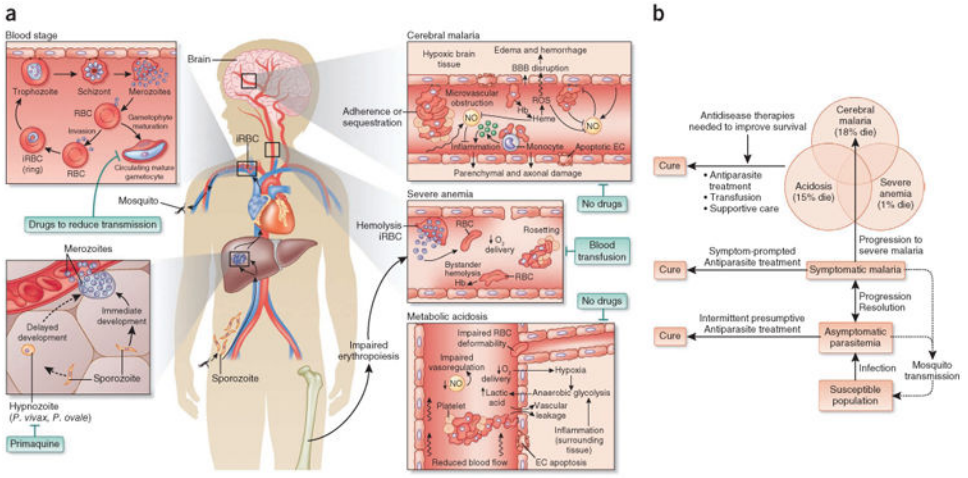

Figure 1.

Severe malaria in children. (**a**) Life cycle and pathogenesis of malaria. Malaria infections begin with the injection of parasite sporozoites by infected mosquitoes during a blood meal. Sporozoites invade hepatocytes and proliferate into merozoites. One *P. falciparum* sporozoite develops into 40,000 merozoites per liver cell over 6 d. During *P. vivax* and *Plasmodium ovale* infection, some sporozoites also differentiate into hypnozoites that remain dormant in the liver for months to years before undergoing division and development into merozoites. Only one drug family, the 8-aminoquinolines such as primaquine, kills hypnozoites. However, the 8-aminoquinolines are toxic in glucose-6-phosphate dehydrogenase (G6PD)-deficient humans, a common deficiency in malaria-endemic regions of the world. Consequently, elimination of *P. vivax* and *P. ovale* may require new antihypnozoite drugs that can be safely administered to a population in which G6PD deficiency is prevalent. The blood stage of malaria begins when hepatic merozoites invade erythrocytes. Within 12 h of invasion, the parasite remodels the red blood cell (RBC), facilitating the growth of the parasite and transporting PfEMP1 to the erythrocyte membrane. Infected RBCs (iRBCs) bind to endothelium through PfEMP1 primarily to avoid clearance by the spleen. Sequestration of infected RBCs injures endothelial cells (ECs) and disrupts blood flow, causing tissue hypoxia and lactic acidosis. These mechanisms contribute to organ-specific syndromes such as cerebral malaria and placental malaria when sequestration occurs in the brain or placenta. Hemolysis of infected and bystander (uninfected) RBCs causes anemia that may be exacerbated by impaired erythropoiesis. Hemolysis also contributes to endothelial injury and dysfunction as free hemoglobin (Hb) catalyzes oxidative damage and consumes nitric oxide (NO), a regulator of endothelial cells. Merozoites develop in the sequestered RBCs, and the rupture of infected erythrocytes causes fever and rigors. Most merozoites invade uninfected RBCs and circulate as ring-stage parasites, but a small fraction of merozoites develop into male and female gametocytes that infect mosquitoes when taken up during a blood meal. Gametocytes continue to circulate after treatment at the asexual blood stages; therefore, safe drugs to kill circulating gametocytes would help in *P. falciparum* elimination. ROS, reactive oxygen species; BBB, blood-brain barrier. (**b**) Progression of malaria in a susceptible population and opportunities for treatment. Approximately 2 billion people live in areas where malaria is transmitted. In

regions where malaria is endemic, asymptomatic parasitemia is common and contributes to transmission. Intermittent presumptive treatment given to a population helps to eliminate parasites from asymptomatic carriers. Of the ∼500 million symptomatic cases of malaria globally each year, only about 1% progress to severe malaria. The major severe malaria syndromes are cerebral malaria, acidosis (respiratory distress) and severe anemia. Effective antidisease therapies that can be combined with parasite-killing drugs are needed to improve survival from severe malaria.

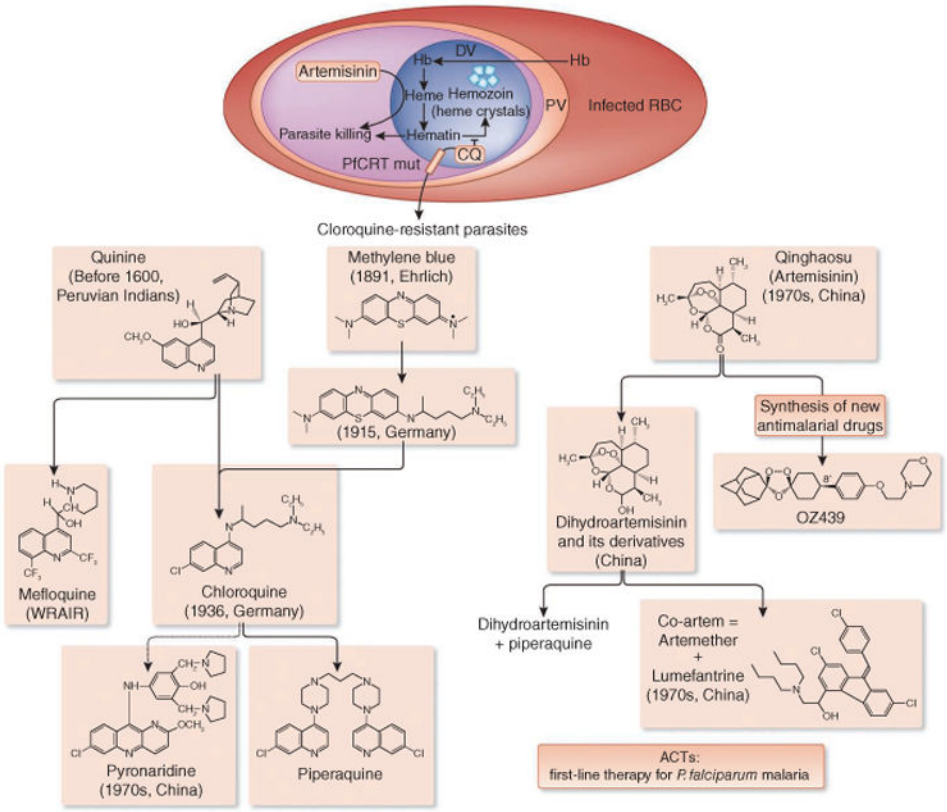

Figure 2.

Quinine and artemisinin discoveries led to the development of many synthetic antimalarial drugs. In the digestive vacuole (DV) of the intraerythrocytic forms of the parasite, hemoglobin (Hb) is digested, and hematin is released, which is detrimental to the parasite. The parasite can reduce the harmful effects of hematin by converting it into hemozoin; however, this reaction is inhibited by chloroquine (CQ). Heme activates artemisinin activity, resulting in parasite killing. RBC, red blood cell; Mut, mutant; PV, parasitophorous vacuole.

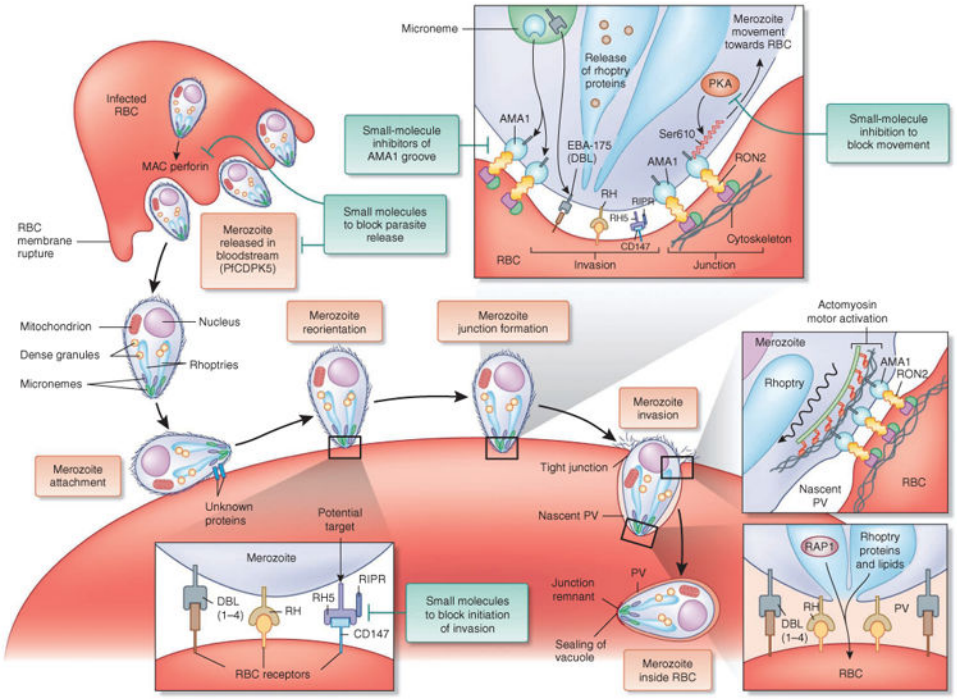

Figure 3.

Merozoite invasion of the erythrocyte involves five steps, including the movement of erythrocyte membrane past the junction to form the parasitophorous vacuole (PV). RBC, red blood cell; RH, reticulocyte homology ligand; RIPR, RH5 interacting protein.

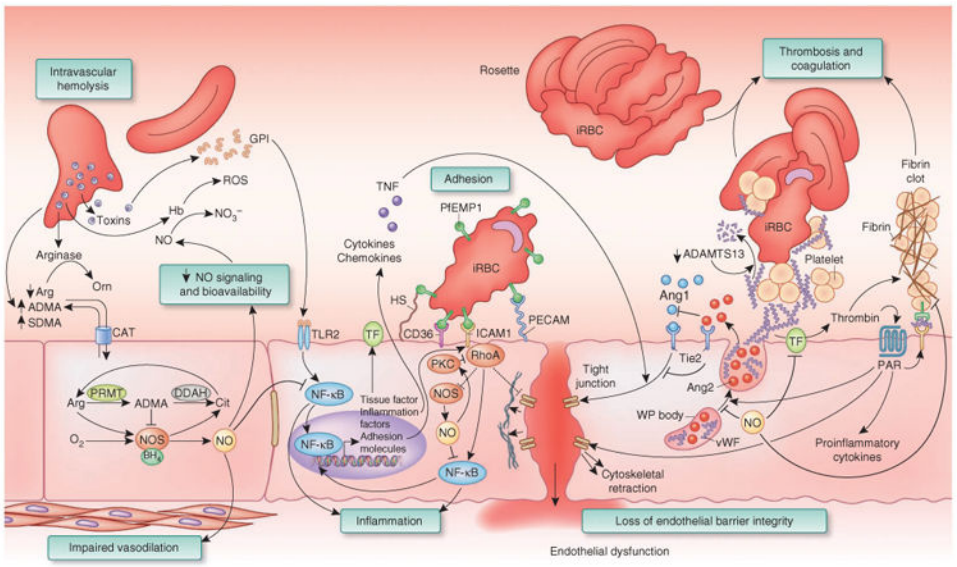

Figure 4.

Malaria infection disrupts nitric oxide metabolism and causes harmful endothelial activation. **Intravascular hemolysis limits nitric oxide (NO) bioavailability**: destruction of the erythrocyte releases hemoglobin, arginase and ADMA into plasma. Hemoglobin (Hb) reacts with endothelial nitric oxide and converts it to biologically inactive nitrate (NO_3^-), diminishing nitric oxide signaling. Hemoglobin can also catalyze the production of reactive oxygen species (ROS). Arginase is released from erythrocytes and metabolizes arginine to ornithine (Orn), limiting the arginine that is available to NOS. Erythrocytes have high concentrations of ADMA incorporated in proteins; hemolysis and proteolysis releases free ADMA into plasma. **Impaired nitric oxide synthesis:** NOS catalyzes the generation of nitric oxide from the substrates oxygen (O_2), Arg and NADPH. Tetrahydrobiopterin (BH_4) is an essential NOS cofactor. In the absence of either arginine or BH_4, NOS functions as an oxidase, generating superoxide from NADPH and molecular oxygen. The product citrulline (Cit) can be recycled to arginine by argininosuccinate synthase (ASS1) and argininosuccinate lyase (ASL). ADMA is a NOS inhibitor that is generated by the methylation of arginine in polypeptides by protein arginine methyltransferase (PRMT) followed by proteolysis to release free ADMA. DDAH regulates nitric oxide synthesis by metabolizing ADMA. Arginine, ADMA and symmetrical dimethylarginine (SDMA) cross the endothelial cell membrane by a cationic amino acid transporter (CAT). **Inflammation**: glycosylphosphatidylinositol (GPI) from malaria parasites can trigger inflammation through Toll-like receptor 2 (TLR2) signaling, leading to NF-κB activation and transcription of inflammatory cytokines (such as TNF), adhesion molecules (such as ICAM1) and procoagulant molecules (such as tissue factor, TF). Nitric oxide can downregulate NF-κB to exert anti-inflammatory effects. **Adhesion:** infected erythrocytes (iRBCs) bind to endothelial cells through parasite-encoded PfEMP1 and endothelial receptors such as ICAM1 and heparan sulfate (HS). Binding to ICAM1 triggers RhoA and Rho kinase, leading to cytoskeletal rearrangements that cause cell retraction and disrupt cell-cell junctions. Rho kinase also activates NF-κB to exert proinflammatory, proadhesive and procoagulant effects. Rho kinase downregulates NOS activity, but nitric oxide can suppress

RhoA activation by nitrosylation of protein kinase C (PKC). Loss of barrier integrity: tight junctions between endothelial cells are maintained by signaling through the Ang1–endothelial-specific receptor tyrosine kinase (Tie2) axis. Ang2 binds to Tie2 but does not transduce a signal, thereby interrupting constitutive Ang1-Tie2 signaling. In the setting of proinflammatory cytokines such as TNF, this leads to loss of integrity of the endothelial cell layer. **Weibel-Palade (WP) body exocytosis:** Weibel-Palade bodies contain vWF multimers that can bind to circulating platelets and trigger thrombosis. vWF multimers are cleaved by the vWF protease ADAMTS13 to limit the extent of thrombosis. Patients with severe malaria have reduced ADAMTS13 activity and an overabundance of vWF multimers. Tethered vWF multimers can bind to platelets and form a bridge to infected erythrocytes. **Thrombosis and coagulation:** adherent infected erythrocytes trigger a display of tissue factor on the endothelial cell apical surface and recruit circulating coagulation factors to activate thrombin. Thrombin generates fibrin to form a blood clot that can block the lumen of small vessels and stop blood flow. Thrombin also activates protease-activated receptors (PARs) that couple with multiple G proteins to cause cytoskeletal retraction and expression of inflammatory cytokines and adhesion molecules.