Proteases of Malaria Parasites: New Targets for Chemotherapy

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The increasing resistance of malaria parasites to antimalarial drugs is a major contributor to the reemergence of the disease as a major public health problem and its spread in new locations and populations. Among potential targets for new modes of chemotherapy are malarial proteases, which appear to mediate processes within the erythrocytic malarial life cycle, including the rupture and invasion of infected erythrocytes and the degradation of hemoglobin by trophozoites. Cysteine and aspartic protease inhibitors are now under study as potential antimalarials. Lead compounds have blocked in vitro parasite development at nanomolar concentrations and cured malaria-infected mice. This review discusses available antimalarial agents and summarizes experimental results that support development of protease inhibitors as antimalarial drugs.

Hundreds of millions of cases of malaria occur annually, and infections with *Plasmodium falciparum*, the most virulent human malaria parasite, cause more than one million deaths per year (1). Despite extensive control efforts, the incidence of the disease is not decreasing in most malaria-endemic areas of the world, and in some it is clearly increasing (2). Malaria also remains a major risk to travelers from industrialized to developing countries. Because malaria parasites are increasingly resistant to antimalarial drugs, appropriately counseled travelers to malaria-endemic regions are more likely to contract malaria now than they were 40 years ago.

Malaria control efforts include attempts to develop an effective vaccine, eradicate mosquito vectors, and develop new drugs (2,3). However, the development of a vaccine has proven very difficult, and a highly effective vaccine will probably not be available in the near future (4). Efforts to control *Anopheles* mosquitoes have had limited success, although the use of insecticide-impregnated bed nets does appear to reduce malaria-related death rates (5). In addition, methods to replace natural vector populations with mosquitoes unable to support parasite development are under study and may contribute to malaria control in the long term (6). However, the current limitations of vaccine and vector control, as well as the increasing resistance of malaria parasites to existing drugs, highlight the continued need for new antimalarial agents.

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Established Antimalarial Drugs

Antimalarial drugs have been used for centuries. Early natural products, including the bark of the cinchona tree in South America and extracts of the wormwood plant in China, were among the first effective antimicrobial agents to be used. Cinchona bark was used in Europe beginning in the 17th century, and upon its isolation from bark in 1820, quinine became widely used. In the last 50 years, extensive efforts, including the screening of hundreds of thousands of compounds, have led to the development of a number of effective synthetic antimalarial drugs. The most important of these, chloroquine, has been the mainstay of antimalarial chemotherapy for the last 50 years. The compound eradicates parasites rapidly, has minimal toxicity, is widely available at low cost throughout the world, and needs to be taken only once a week for chemoprophylaxis. However, resistance to chloroquine has been steadily increasing since the drug's initial use in South America and Southeast Asia in the late 1950s. Chloroquine resistance is now widespread in most *P. falciparum*-endemic areas of the world (3). Thus, the use of chloroquine for presumptive treatment of falciparum malaria

or for chemoprophylaxis is usually no longer appropriate (7). Moreover, resistance to chloroquine of *P. vivax*, the second most lethal human malaria parasite, is increasing in South Asia (8).

No other antimalarial drug (9-12) is as efficacious and safe as chloroquine (Table 1). The best antimalarial drug for treating chloroquine-resistant falciparum malaria remains quinine (or intravenous quinidine), which is fairly toxic; quinine resistance is increasing in Southeast Asia, particularly in the border areas of Thailand (9). Amodiaquine, used to treat chloroquine-resistant malaria in developing countries, is also quite toxic, and resistance to it is also common (13). Mefloquine (14) is widely used for chemoprophylaxis against chloroquine-resistant *P. falciparum*, but its use is limited by toxicity (15) and (in the developing world) high cost. Mefloquine is not approved for treatment of malaria in the United States because of the neurotoxicity of doses required for the treatment. Fansidar, a combination of sulfadoxine and pyrimethamine, is no longer recommended for chemoprophylaxis because of its dermatologic toxicity (15). Fansidar is also not an ideal drug for treatment because it is slow acting, but it is increasingly important in treating chloroquine-resistant malaria in developing countries because economic constraints limit the use of other agents (16). The use of both mefloquine and Fansidar will increasingly be limited by drug resistance, already widespread in parts of Southeast Asia (9,17).

Other antimalarial drugs have specialized uses. Tetracyclines and some other antibiotics (clindamycin, sulfas) are slow acting and generally best used as an adjunct to quinine therapy in treating falciparum malaria (9). Doxycycline is also used for chemoprophylaxis in regions with high levels of drug resistance, especially Southeast Asia (10,17). Other drugs for chemoprophylaxis include proguanil, which remains effective in combination with chloroquine in many areas other than Southeast Asia, and Maloprim, a combination of dapsone and pyrimethamine (10,17). Resistance to these drugs is fairly common, however. Primaquine has a well-defined specific role: eradicating chronic liver stages of *P. vivax* and *P. ovale* after treating the acute blood infection with chloroquine.

New Antimalarial Drugs

Relatively few new antimalarial drugs are undergoing clinical testing (Table 2). Halofantrine, identified in the 1940s, was not developed until

Table 1. Established antimalarial drugs[a]

Drug	Role	Best Feature(s)	Limitations
Chloroquine	TX of and CP against non-Pf and sensitive Pf parasites	Very safe; low cost; long half-life	Widespread R
Quinine/quinidine	Best TX for Pf malaria; low cost	Limited R; rapidly acting	Fairly toxic (cinchonism, cardiac)
Amodiaquine[b]	TX of R Pf malaria	Low cost	Toxicity (bone marrow, liver); R common
Mefloquine	CP against R Pf malaria; not approved for TX in United States	Relatively little R, though increasing; long half-life	Moderately toxic (mostly CNS); high cost; R in SE Asia
Fansidar	TX of Pf malaria; no longer recommended for CP	Relatively low cost; long half-life	Skin toxicity (can be fatal); increasing R
Primaquine	Eradication of chronic liver stage Pv, Po malaria	Only drug for this indication	Hemolysis with G6PD deficiency; increasing R
Proguanil[b]	CP only (often with chloroquine)	Low cost; nontoxic	R common
Maloprim[b]	CP only (often with chloroquine)	Low cost	R common; skin rashes
Tetracyclines	CP; TX of Pf malaria in combination with quinine	Low cost	Skin and gastrointestinal toxicity

[a]TX, therapy; CP, chemoprophylaxis; R, resistance/resistant; Pf, *Plasmodium falciparum;* Pv, *P. vivax;* Po, *P. ovale;* CNS, central nervous system; G6PD, glucose 6-phosphate dehydrogenase.
[b]Not available in the United States.

Table 2. New antimalarial drugs

Drug	Role	Best Feature(s)	Limitations
Halofantrine	TX of Pf malaria; not approved for CP	Usually effective against R Pf malaria	Variable bioavailability, cardiac toxicity
Artemisinin and related compounds[a]	TX of Pf malaria	Rapidly acting; effective against multidrug-R strains	Recurrence after TX fairly common
Atovaquone	? TX of Pf malaria; ? CP (probably in combination with proguanil)	Limited toxicity	Limited studies so far show frequent recurrence after TX
Pyronaridine[a]	? TX of Pf malaria	Effective against R strains	Studies limited to date
Desferrioxamine	? TX of severe Pf malaria	Well tolerated when used for iron overload	Studies limited to date
Azithromycin	? CP	Limited toxicity	Studies limited to date

For abbreviations, see Table 1, footnote a.
[a]Not available in the United States.

the 1980s; its use has been limited by variable oral absorption and cardiac toxicity (12,18). The drug is approved in the United States for treatment of chloroquine-resistant *P. falciparum* infection, although in most cases quinine (or intravenous quinidine) is preferable. The most effective new drugs are artemisinin and related compounds. Artemisinin was isolated in 1972 from *Artemisia annua*, a plant used in China for centuries to treat fever (19). Artemisinin derivatives (artesunate, artelinate, artemether, arteether, dihydroartemisinin) have been synthesized and are undergoing extensive clinical testing. These compounds, which are already widely used in some areas, are potent, rapidly acting antimalarials that are effective against chloroquine-resistant *P. falciparum* (20). Because recrudescences of infection after treatment are common, however, artemisinin and related compounds might best be used in combination with another drug.

Other compounds are under evaluation. Atovaqone (21), which is approved for treating patients with *Pneumocystis* infections, appears to be effective against malaria in combination with proguanil (22), but its use has been limited by recrudescence after treatment. Pyronaridine, an acridine derivative used to treat malaria in China, has shown efficacy against falciparum malaria (23). The iron chelator desferrioxamine enhances the clearance of parasites in mild malaria (24) and, in conjunction with quinine and Fansidar, hastens recovery from deep coma in severe falciparum malaria (25). Azithromycin, a quinolone antibiotic, appears efficacious in malaria chemoprophylaxis (26).

Malarial Proteases: New Targets for Chemotherapy

The limitations of antimalarial chemotherapy underscore the need for new drugs, ideally directed against new targets. Potential targets for chemotherapy include malarial proteases (27). The erythrocytic life cycle, which is responsible for all clinical manifestations of malaria, begins when free merozoites invade erythrocytes. The intraerythrocytic parasites develop from small ring-stage organisms to larger, more metabolically active trophozoites and then to multinucleated schizonts. The erythrocytic cycle is completed when mature schizonts rupture erythrocytes, releasing numerous invasive merozoites. Proteases appear to be required for the rupture and subsequent reinvasion of erythrocytes by merozoite-stage parasites and for the degradation of hemoglobin by intraerythrocytic trophozoites (Figure).

Proteases and Erythrocyte Rupture and Invasion

The rupture of erythrocytes by mature schizonts and the subsequent invasion of erythrocytes by free merozoites appear to require malarial protease activity, possibly to breach the erythrocyte cytoskeleton, a complex network of proteins. In addition, a number of malarial proteins are proteolytically processed during the late schizont and merozoite life-cycle stages; for example, merozoite surface protein-1 is processed in a manner inhibited by serine protease inhibitors (28), presumably to facilitate the complex series of events involved in erythrocyte

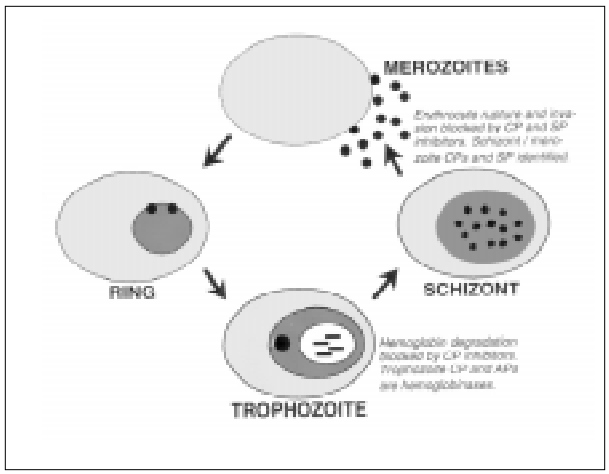

Figure. Protease targets in erythrocytic malaria parasites. The *Plasmodium falciparum* erythrocytic life cycle is shown schematically, and data supporting cysteine (CP), serine (SP), and aspartic (AP) proteases of the different parasite stages as chemotherapeutic targets are provided in italics.

rupture and invasion (29). Although the specific roles of different classes of proteases are not completely clear, inhibitors of cysteine and serine proteases have consistently blocked erythrocyte rupture and invasion (27).

Candidate *P. falciparum* rupture/invasion proteases have been identified, but none has been fully characterized biochemically or molecularly: 1) a 68 kD cysteine protease was identified in schizonts and merozoites and localized to the merozoite apex, suggesting that it may be released from the rhoptry organelle during invasion (30); 2) a cysteine protease of mature schizonts and a serine protease of merozoites were identified in highly synchronized parasites (31); 3) a serine protease was shown to be bound to the schizont/merozoite membrane by a glycosylphosphatidylinositol anchor, to be activated by phosphatidylinositol-specific phospholipase C during the merozoite stage, and to be capable of cleaving the erythrocyte cytoskeletal protein band 3 (32,33); 4) another protease, inhibited by both cysteine and serine protease inhibitors, hydrolyzed the erythrocyte cytoskeletal proteins spectrin and band 4.1 (34); and 5) the serine repeat antigen (35,36) and the related protein SERP H (37), both expressed in mature schizonts, have important similarities in their sequences with cysteine proteases. Further research should identify the specific biologic roles of the proteases mentioned and better characterize these enzymes, thus fostering the development of specific inhibitors.

Host proteases may also play a role in erythrocyte rupture by *P. falciparum*. In recent studies, host urokinase was shown to bind to the surface of *P. falciparum*-infected erythrocytes, and the depletion of urokinase from parasite culture medium inhibited erythrocyte rupture by mature schizonts (38). This inhibition was reversed by exogenous urokinase.

Drug Development Efforts

Synthetic peptide inhibitors of the *P. falciparum* schizont cysteine protease Pf 68 inhibited erythrocyte invasion by cultured parasites (39,40). The most effective peptide, GlcA-Val-Leu-Gly-Lys-NHC_2H_5, inhibited the protease and blocked parasite development at high micromolar concentrations (40; Table 3). Although these results do not demonstrate levels of inhibition expected to be therapeutically relevant, they suggest that a specific protease activity is required for erythrocyte invasion by malaria parasites and thus is a potential target for antimalarial drugs.

Proteases and Malarial Hemoglobin Degradation

Extensive evidence suggests that the degradation of hemoglobin is necessary for the growth of erythrocytic malaria parasites, apparently to provide free amino acids for parasite protein synthesis (27,50). In *P. falciparum*, hemoglobin degradation occurs predominantly in trophozoites and early schizonts, the stages at which the parasites are most metabolically active. Trophozoites ingest erythrocyte cytoplasm and transport it to a large central food vacuole. In the food vacuole, hemoglobin is broken down into heme, a major component of malarial pigment (51), and globin, which is hydrolyzed to its constituent amino acids. The food vacuole is an acidic organelle analogous to lysosomes. Several lysosomal proteases are well characterized, including cysteine (cathepsins B, H, and L) and aspartic (cathepsin D) proteases (52), and malaria parasites contain analogous food vacuole proteases that degrade hemoglobin. At least two aspartic proteases and one cysteine protease have been isolated from purified *P. falciparum* food vacuoles (53).

Malarial aspartic protease activities have been identified (54-60). Two recently characterized aspartic proteases (plasmepsin I and

Table 3. Protease targets for chemotherapy

Protease	Biologic role	Effective inhibitors[a] Compound (Reference)	In vitro[b] (IC_{50}; µM)	In vivo[c] (mg/kg/day)
Pf68	Erythrocyte invasion	GlcA-Val-Leu-Gly-Lys-NHC_2H_5 (40)	900	
Plasmepsin I	Hemoglobin degradation	SC-50083 (41)	2-5	
		Ro 40-4388 (42)	0.25	
Plasmepsin II	Hemoglobin degradation	Compound 7 (43)	20	
Falcipain	Hemoglobin degradation	Z-Phe-Arg-CH_2F (44)	0.064	
		Mu-Phe-HPh-CH_2F (45)	~0.03	400
		Mu-Leu-HPh-VSPh (46)	0.01	
		Oxalic bis ((2-hydroxy-1-naphthylmethylene)hydrazide) (47)	7	
		1-(2,5-dichlorophenyl)-3-(4-quinolinyl)-2-propen-1-one (48)	0.23	
		7-chloro-1,2-dihydro-2-(2,3-dimethoxy-phenyl)-5,5-dioxide-4-(1H,10H)-phenothiazinone (49)	2	

[a]The structures of these compounds and details of the described studies are in the references noted.
[b]Assays compared the development of new ring-form parasites or the uptake of [^{3}H]hypoxanthine by treated and control parasites.
[c]Cure of *Plasmodium vinckei*-infected mice.

plasmepsin II) are located in the food vacuole, have acid pH optima, and share sequence homology with other aspartic proteases (41,53,61,62). Furthermore, the aspartic proteases can cleave hemoglobin. One of the enzymes, plasmepsin I, cleaves native hemoglobin (53,59). Plasmepsin II appears to prefer denatured globin as a substrate (53). On the basis of these data, plasmepsin I is thought to be responsible for initial cleavages of hemoglobin after the molecule is transported to the food vacuole (53).

Incubation of cultured *P. falciparum* parasites with the protease inhibitor leupeptin caused trophozoite food vacuoles to fill with apparently undegraded erythrocyte cytoplasm (63-65). Analysis of the leupeptin-treated parasites showed that they contained large quantities of undegraded globin, while minimal globin was detectable in control parasites (64,66). Leupeptin inhibits both cysteine and some serine proteases, but the highly specific cysteine protease inhibitor E-64 also caused undegraded globin to accumulate. After parasites were incubated with inhibitors of other classes of proteases including the aspartic protease inhibitor pepstatin (63-67), globin did not accumulate. More recent studies that used nondenaturing electrophoretic methods demonstrated that cysteine protease inhibitors not only blocked malarial globin hydrolysis, but also inhibited earlier steps in hemoglobin degradation, including denaturation of the hemoglobin tetramer and the release of heme from globin (68). Another study showed that E-64, but not pepstatin, inhibited the production of hemozoin (the malarial end product of heme) by cultured parasites (69). These results suggest that a cysteine protease is required for initial steps in hemoglobin degradation by *P. falciparum*.

A *P. falciparum* trophozoite cysteine protease with biochemical features expected for a food vacuole hemoglobinase has been identified (31) and biochemically (70-72) and molecularly (73) characterized. This protease, called falcipain, degraded denatured and native hemoglobin in vitro; its acid pH optimum, substrate specificity, and inhibitor sensitivity indicated that it was a papain family cysteine protease (64,70,71). Specific inhibitors of falcipain blocked hemoglobin degradation and prevented parasite development. The degree of inhibition of falcipain by fluoromethyl ketones (44) and vinyl sulfones (46) correlated with their inhibition of hemoglobin degradation and parasite development, supporting the hypothesis that falcipain is the cysteine protease required for hemoglobin degradation.

The specific mechanism for hemoglobin degradation in the malarial food vacuole remains unclear. As noted above, both the aspartic protease plasmepsin I and the cysteine protease falcipain have been identified in parasite food vacuoles and shown to cleave denatured and native hemoglobin in vitro (53,71). Results showing that only cysteine protease inhibitors block hemoglobin processing and globin hydrolysis in cultured parasites suggest that falcipain is required for initial steps of hemoglobin degradation (66-68,74). However, other studies have

shown that native hemoglobin is cleaved by plasmepsin I, but not falcipain, in nonreducing conditions that may be present in the food vacuole (53,59,72). In any event, regardless of the exact sequence of hemoglobin processing, multiple enzymes, including at least the three proteases already identified, appear to participate in the degradation of hemoglobin. These proteases are thus logical targets for antimalarial drug development.

Aminopeptidase activity has also been described in malaria parasites (75-77). This activity, with a neutral pH optimum, was not found in food vacuole lysates (77). When these lysates were incubated with hemoglobin, discrete peptide fragments, but not free amino acids, were identified (77). These results suggest that hemoglobin is degraded to small peptides in the food vacuole, that these peptides are transported to the parasite cytosol, and that additional processing of hemoglobin peptides is mediated by cytosolic aminopeptidase activity (77).

Drug Development Efforts

Both the cysteine protease inhibitor E-64 and the aspartic protease inhibitor pepstatin blocked *P. falciparum* development (63-67). Administered together, the two inhibitors acted synergistically (67). However, only E-64 blocked globin hydrolysis (64-67). Numerous peptide-based cysteine protease inhibitors, including fluoromethyl ketones (44,70,78) and vinyl sulfones (46), inhibited falcipain at low nanomolar concentrations and inhibited *P. falciparum* development and hemoglobin degradation at concentrations below 100 nanomolar (Table 3). In a malaria animal model, a fluoromethyl ketone that inhibited falcipain at low nanomolar concentrations blocked *P. vinckei* protease activity in vivo after a single subcutaneous dose, and, when administered for 4 days, cured 80% of murine malaria infections (45). Thus, despite the theoretical limitations of potentially rapid degradation in vivo and inhibition of host proteases, peptide protease inhibitors show promise as candidate antimalarial drugs. Fluoromethyl ketones have subsequently shown toxicity in animal studies, but evaluations of related, apparently nontoxic inhibitors of falcipain as antimalarial drugs are under way.

A computer model for the structure of falcipain was used to identify nonpeptide inhibitors (47). Screening of potential nonpeptide inhibitors identified a low micromolar lead compound (47; Table 3). Subsequent synthesis and testing of small molecules based on the structure of the lead compound have identified biologically active falcipain inhibitors, including chalcones that block parasite metabolism at submicromolar concentrations (48) and phenothiazines that block parasite metabolism and development at low micromolar concentrations (49).

Peptidelike aspartic protease inhibitors are potent inhibitors of plasmepsins I and II. In independent studies SC-50083 (41), Ro 40-4388 (42), and "compound 7" (43) inhibited plasmepsin I or II at nanomolar concentrations and blocked parasite development at high nanomolar to micromolar concentrations (Table 3). Drug development efforts should be assisted by the recent determination of the structure of plasmepsin II (43). Inhibitors of aspartic and cysteine proteases have synergistic effects in inhibiting the growth of cultured malaria parasites (67), and these proteases also act synergistically to degrade hemoglobin in vitro (41). Therefore, the combination of inhibitors of malarial cysteine and aspartic proteases may provide the most effective chemotherapeutic regimen and best limit the development of parasite resistance to protease inhibitors. Ultimately, a better understanding of the biochemical properties and biologic roles of malarial proteases will foster the development of protease inhibitors that specifically inhibit parasite enzymes and thus are the most suitable candidates for chemotherapy.

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