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Review

Antimalarial drug discovery: old and new approaches

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Summary

New drugs against malaria are greatly needed. Many approaches to antimalarial drug discovery are available. These approaches must take into account specific concerns, in particular the requirement for very inexpensive and simple to use new therapies and the need to limit the cost of drug discovery. Among important efforts that are currently ongoing are the optimization of therapy with available drugs, including the use of combination therapy, the development of analogs of existing agents, the discovery of natural products, the use of compounds that were originally developed against other diseases, the evaluation of drug resistance reversers, and the consideration of new chemotherapeutic targets. The last category benefits from recent advances in malaria research technologies and genomics and is most likely to identify new classes of drugs. A number of new antimalarial therapies will likely be needed over the coming years, so it is important to pursue multiple strategies for drug discovery.

Key words: malaria, *Plasmodium falciparum*, drugs, chemotherapy, drug discovery, resistance.

Introduction

Malaria is one of the most important infectious diseases in the world (Breman, 2001). Unfortunately, mortality from malaria appears to be increasing in the highest risk group, African children (Snow et al., 2001). A major contributor to malarial morbidity and mortality is almost certainly the increasing resistance of malaria parasites to available drugs (Olliaro and Bloland, 2001). Resistance is primarily seen in *Plasmodium falciparum*, the most virulent human malaria parasite. Antimalarial drug resistance is discussed in detail in other reviews in this volume.

Considering increasing resistance to available agents, there is broad consensus that there is a need to develop new antimalarial drugs (Ridley, 2002). Antimalarial drug development can follow several strategies, ranging from minor modifications of existing agents to the design of novel agents that act against new targets. Increasingly, available agents are being combined to improve antimalarial regimens. This review will discuss multiple approaches to antimalarial drug discovery, emphasizing the varied strategies that have led to available drugs and that are likely to provide important new drugs in the future. Additional detailed reviews of antimalarial chemotherapy and potential new targets for drug discovery have been published recently (Olliaro and Yuthavong, 1999; Ridley, 2002; Rosenthal, 2001a).

Unique aspects of antimalarial drug discovery

Antimalarial drug development is constrained by the same factors as any drug development program in that new agents must demonstrate efficacy, be safe and have additional properties important for the specific disease indication. In the case of malaria, the major need is for widespread treatment of malaria in developing countries. Considering resource limitations in this setting, it is generally agreed that new antimalarials should be dosed orally and be effective with single-daily dosing, and that curative regimens should be short, ideally 1–3 days in length. The critical consideration in antimalarial drug development is economic. Financial constraints are relevant in two key regards. First, to be widely useful, antimalarial drugs must be very inexpensive so that they are routinely available to populations in need in developing countries. Indeed, even a price of $1 per treatment is probably unacceptable in many regions, considering severe poverty in most of the malarious world and familiarity with available drugs, especially chloroquine, that are very inexpensive (less than $0.1 per treatment), albeit increasingly ineffective. Second, since malaria markets are primarily in poor countries, marketing opportunities have generally been considered to be limited, and so investment in antimalarial drug discovery and development has been small. Thus, drug discovery directed against malaria is particularly reliant upon shortcuts that may obviate excess cost. A number of such approaches will be discussed below. Antimalarial drug discovery is also dependent on support outside of large pharmaceutical companies. Such support includes grants to academic and industry groups from research agencies and new

Table 1. *Approaches to antimalarial drug discovery and development*

Approach	Examples	References
Optimize therapy with existing agents	Amodiaquine/sulfadoxine/pyrimethamine	Staedke et al., 2001; Dorsey et al., 2002; Schellenberg et al., 2002
	Amodiaquine/artesunate	Adjuik et al., 2002
	Artesunate/sulfadoxine/pyrimethamine	von Seidlein et al., 2000; Dorsey et al., 2002
	Artesunate/mefloquine	Price et al., 1997
	Artemether/lumefantrine	Lefevre et al., 2001
	Chlorproguanil/dapsone	Nzila et al., 2000; Mutabingwa et al., 2001; Kublin et al., 2002
	Chlorproguanil/dapsone/artesunate	–
	Atovaquone/proguanil	Vaidya, 2001
Develop analogs of existing agents	New aminoquinolines	Raynes et al., 1999; Kaschula et al., 2002
	New endoperoxides	Vennerstrom et al., 2000; Posner et al., 2003
	New folate antagonists	Tarnchompoo et al., 2002
Natural products	New natural products	Tagboto and Townson, 2001
Compounds active against other diseases	Folate antagonists	Plowe, 2001
	Antibiotics	Clough and Wilson, 2001
	Atovaquone	Vaidya, 2001
	Iron chelators	Loyevsky and Gordeuk, 2001
Drug resistance reversers	Verapamil, desipramine, trifluoperazine	van Schalkwyk et al., 2001
	Chlorpheniramine	Sowunmi et al., 1997
Compounds active against new targets	See Table 2	–

public–private partnerships formed to specifically address the imbalance in funding between developed and developing world diseases. However, this imbalance remains large, it is unclear if some recent improvement in funding for malaria research will be sustainable and, in any event, cost remains a primary concern in assessing different approaches toward antimalarial drug development.

Approaches to antimalarial chemotherapy

Many different approaches to the identification of new antimalarials are being pursued at this time. This review will describe strategies that take advantage of specific aspects of the biology of malaria parasites and/or utilize shortcuts to generate new compounds for study at relatively small cost. Many examples of different approaches are listed in Table 1, and additional detail on some promising approaches is provided below.

Optimization of therapy with existing agents

A first approach is to optimize therapy with existing agents. New dosing regimens or formulations may optimize activity. Combination therapies, including newer agents (e.g. artemisinin derivatives, atovaquone) and new combinations of older agents (e.g. amodiaquine/sulfadoxine/pyrimethamine, chlorproguanil/dapsone), are under study as first-line therapies for Africa and other areas with widespread drug resistance. The use of combination antimalarial therapy offers two important potential advantages. First, the combination should improve antimalarial efficacy, providing additive or, ideally, synergistic antiparasitic activity. In the case of both the artemisinin derivatives and atovaquone, the new agents have had unacceptable failure rates when used as single agents to treat falciparum malaria but they have been highly effective in combination with other established antimalarials. Second, and probably most important, the use of combination therapy should slow the progression of parasite resistance to the new agents. This latter factor is a key consideration as we attempt to develop new therapies that will retain activity for a long period. Ideally, a combination regimen that prevents resistance development should include at least two agents against which parasite resistance has not yet developed and which have similar pharmacokinetics, so that low blood levels of a single agent will not be present. No such ideal regimen is currently available, although chlorproguanil/dapsone/artesunate may prove to fit this description. Alternatively, the combination of a short-acting, highly potent compound and a longer-acting agent may prove effective, if the initial decrease in parasite burden is so great as to limit subsequent resistance development to the long-acting agent (e.g. artesunate/mefloquine). As another alternative, two drugs with similar pharmacokinetics may prove effective even if resistance to each agent is present in the community (e.g. amodiaquine/sulfadoxine/pyrimethamine). Considerations of

combination therapy generally impact on drug development only late in the process, when individual drugs of proven efficacy are considered as components of combinations. However, it may be appropriate to consider combination therapy earlier in the drug discovery process. For example, relatively slow-acting antimalarials (e.g. antibiotics) may seem to be poorly suited therapeutic agents but they may work well in combination regimens (e.g. quinine and doxycycline).

Some older agents, which are now significantly limited by drug resistance, may nonetheless remain effective in combination. Presumably, the prevalence of resistance to each agent is low enough such that resistance to both drugs is unlikely. Although a combination regimen, sulfadoxine/pyrimethamine loses efficacy quickly once resistance is seen; in this case, efficacy is dependent on synergism, so is lost once resistance develops to either drug. However, the combination of sulfadoxine/pyrimethamine with amodiaquine, a chloroquine analog that remains active against many chloroquine-resistant parasites, provides two effective long-acting drugs. Importantly, this combination regimen uniquely provides two available and inexpensive drugs, although both components are already limited by drug resistance in many areas and safety concerns (with rare but severe toxicity with long-term prophylactic use). Despite these concerns, the combination of amodiaquine and sulfadoxine/pyrimethamine showed excellent antimalarial efficacy in regions of East Africa, with fairly high levels of resistance to each individual agent (Dorsey et al., 2002; Schellenberg et al., 2002; Staedke et al., 2001). Another intriguing possibility is the reuse of chloroquine, ideally in combination regimens, in areas where it has not been used for an extended period; chloroquine sensitivity was recently shown to reemerge in Malawi after its use was curtailed for about a decade (Kublin et al., 2003).

Artemisinin analogs, in particular artesunate and artemether, have recently shown great promise as rapidly acting and potent antimalarials, but the short half-lives of these compounds lead to many late recrudescences after therapy, suggesting that combination therapies are necessary to fully exploit the potency of this class. Artesunate has been studied in combination with both sulfadoxine/pyrimethamine (von Seidlein et al., 2000) and amodiaquine (Adjuik et al., 2002) in Africa, with good efficacy, although underlying resistance to the two artesunate partners may lead to unacceptable rates of late recrudescence in many areas, as seen with artesunate/sulfadoxine/pyrimethamine in Uganda (Dorsey et al., 2002). Combinations of artemisinins with longer-acting drugs without underlying resistance may prove to be optimal antimalarial agents. In Thailand, where drug resistance is particularly severe, the combination of artesunate and mefloquine has proven to be highly effective, even in areas where mefloquine resistance was previously seen to be quite common (Price et al., 1997). Artemether has been combined with lumefantrine, a new agent related to halofantrine, to provide a highly effective therapy (Lefevre et al., 2001). Atovaquone, an agent first marketed for *Pneumocystis* pneumonia, has been combined with proguanil, an old dihydrofolate reductase (DHFR) inhibitor, to provide synergistic antimalarial activity (Canfield et al., 1995) and action even against parasites resistant to individual agents in the combination (Vaidya, 2001). Artesunate/mefloquine, artemether/lumefantrine and atovaquone/proguanil are all quite expensive, do not include components with similar pharmacokinetics, may have toxicity concerns (especially for mefloquine) and, in some cases, do not have ideal dosing regimens (for artemether/lumefantrine, twice-daily dosing with a fatty meal). Thus, these combination regimens offer promise for some indications but may not be ideal for widespread use in many areas, in particular Africa.

An interesting new approach to antimalarial drug development is chlorproguanil/dapsone, which combines a close analog of proguanil with dapsone, an old dihydropteroate synthase (DHPS) inhibitor that has been widely used to treat leprosy. Chlorproguanil/dapsone has been specifically devised for the treatment of malaria in Africa, where resistance to chloroquine is very common and resistance to sulfadoxine/pyrimethamine is increasing. Although the new regimen shares the targets of sulfadoxine/pyrimethamine, it is generally effective against sulfadoxine/pyrimethamine-resistant parasites, as the common DHFR and DHPS mutations that mediate this resistance do not lead to clinical resistance to chlorproguanil/dapsone, and additional mutations that lead to higher level antifolate resistance (and resistance to chlorproguanil/dapsone) are rare in Africa (Kublin et al., 2002; Mutabingwa et al., 2001; Nzila et al., 2000). Another key advantage of chlorproguanil/dapsone is a relatively short half-life, which appears to be long enough to provide effective therapy with 3-day daily dosing but is not so long as to readily select for resistance. Chlorproguanil/dapsone will probably be available for the treatment of malaria very soon. However, its optimal use may be as a three-drug combination. The combination of chlorproguanil/dapsone and artesunate may be ideal, combining the rapid potency of artesunate with the slower curative efficacy of chlorproguanil/dapsone, but definitive studies of this combination are still needed.

Development of analogs of existing agents

Another approach to antimalarial chemotherapy is to improve upon existing antimalarials by chemical modifications of these compounds. This approach does not require knowledge of the mechanism of action or the biological target of the parent compound. Indeed, this approach was responsible for the development of many existing antimalarials. For example, chloroquine, primaquine and mefloquine were discovered through chemical strategies to improve upon quinine (Stocks et al., 2001). More recently, 4-aminoquinolines that are closely related to chloroquine appear to offer the antimalarial potency of the parent drug, even against chloroquine-resistant parasites (Kaschula et al., 2002; Raynes et al., 1999). A related compound, pyronaridine, was developed in China and is now undergoing extensive clinical trials in other areas (Ringwald et al., 1996). An 8-

aminoquinoline, tafenoquine, offers improved activity against hepatic-stage parasites over that of the parent compound, primaquine (Walsh et al., 1999), and is effective for antimalarial chemoprophylaxis (Lell et al., 2000). Since halofantrine use is limited by toxicity, the analog lumefantrine was developed and is now a component of the new combination co-artemether (artemether/lumefantrine; van Vugt et al., 2000). New folate antagonists (Tarnchompoo et al., 2002) and new endoperoxides related to artemisinin (Posner et al., 2003; Vennerstrom et al., 2000) are also under study.

Natural products

Plant-derived compounds offer a third approach to chemotherapy. Importantly, this approach can benefit from knowledge of medicinal plants among natives of malarious regions, where the appreciation of the use of plant products to treat febrile illnesses has grown over many generations. Therefore, as a great improvement over random screening, a plant product with specific clinical activity can be the starting point for a medicinal chemistry effort. Natural products are the sources of the two most important drugs currently available to treat severe falciparum malaria, quinine and derivatives of artemisinin. In the case of artemisinin, relatively simple chemical modifications of the natural product parent compound have led to a series of highly potent antimalarials that are playing an increasingly important role in the treatment of malaria (Meshnick, 2001). However, the cost of these compounds may be limiting, and so efforts to design fully synthetic endoperoxides that are less expensive to produce are an important priority (Posner et al., 2003; Vennerstrom et al., 2000). Extensive evaluations of natural products as potential new therapies for many human diseases are underway (Tagboto and Townson, 2001). It is important that such trials include the evaluation of the antimalarial activity of plant extracts and potential drugs purified from these extracts. As with both the quinolines and artemisinins, it is likely that antimalarial natural products will be the parent compounds for the semi-synthetic or fully synthetic production of new drugs.

Compounds active against other diseases

A fourth approach to antimalarial chemotherapy is to identify agents that are developed or marketed as treatments for other diseases. These compounds might act against orthologs of their targets in other systems or by different mechanisms against malaria parasites. Considering the difficulties of funding antimalarial drug discovery, the advantage of these compounds is that, whatever their mechanism, they have already been developed for a human indication, so will be quite inexpensive to develop as antimalarials. However, costs of production for drugs vary greatly, and some new agents, especially those developed for diseases of wealthy nations such as cancer, may be too expensive to produce as antimalarials, even if they do not require extensive development expenses. In many cases, however, drugs may be quite inexpensive to produce and may be available as inexpensive antimalarials, especially after patents have expired, as has been the case with some antibiotics.

Folate antagonists, tetracyclines and other antibiotics were developed for their antibacterial properties and were later found to be active against malaria parasites (Clough and Wilson, 2001). Atovaquone was initially identified as an antimalarial, but its development was expedited by the discovery of its activity against *Pneumocystis*. More recently, its potential as an antimalarial (as a component of the combination drug Malarone) has been re-explored, and it was found to have marked antimalarial synergy with proguanil (Canfield et al., 1995). Malarone was subsequently shown to be effective in the treatment and chemoprophylaxis of malaria, and it is now approved for both of these indications (Hogh et al., 2000). Iron chelators, which are used to treat iron overload syndromes, have documented antimalarial efficacy (Loyevsky and Gordeuk, 2001). These examples suggest that it is appropriate to screen new antimicrobial agents and other available compounds as antimalarial drugs. This approach is facilitated by the presence of high-throughput assays for potential antimalarials. As suggested by the recent development of Malarone, the consideration of compounds with activity against other more economically attractive microbial targets may provide a relatively inexpensive means of identifying new antimalarials. In the case of protein farnesyl transferases, development efforts have not yet led to viable anticancer therapies, but nonetheless have expedited the consideration of these targets for antimalarial chemotherapy (Gelb et al., 2003).

Drug resistance reversers

Combining previously effective agents with compounds that reverse parasite resistance to these agents offers another approach to chemotherapy. Many drugs have been shown to reverse the resistance of *P. falciparum* to chloroquine *in vitro*, most notably the antihypertensive verapamil (Martin et al., 1987) and the antidepressant desipramine (Bitonti et al., 1988). In many cases, unacceptably high concentrations of the resistance reversers are needed for their effects, but combinations of two or more of these agents at pharmacological concentrations may provide clinically relevant resistance reversal, as suggested by studies with verapamil, desipramine and trifluoperazine (van Schalkwyk et al., 2001). The commonly used and inexpensive antihistamine chlorpheniramine reversed resistance at safe dosing levels, although the common side-effect of drowsiness might limit acceptance of this therapy (Sowunmi et al., 1997). Efforts to design new reversers of chloroquine resistance are underway (Alibert et al., 2002; Batra et al., 2000). Thus, although chloroquine appears to already have failed as a first-line antimalarial in most of the world, this inexpensive, rapid-acting, well-tolerated antimalarial may be resurrected by combination with effective resistance reversers.

Compounds active against new targets

Arguably the most innovative approach to chemotherapy is

Table 2. *Antimalarial compounds active against old and new targets*

Target location	Pathway/mechanism	Target molecule	Examples		References (new agents)
			Existing therapies	New compounds	
Cytosol	Folate metabolism	Dihydrofolate reductase	Pyrimethamine, proguanil	Chorproguanil	Nzila et al., 2000; Mutabingwa et al., 2001
		Dihydropteroate synthase	Sulfadoxine, dapsone		
		Thymidylate synthase		5-fluoroorotate	Rathod et al., 1992
	Glycolysis	Lactate dehydrogenase		Gossypol derivatives	Razakantoanina et al., 2000
Parasite membrane	Phospholipid synthesis	Choline transporter		G25	Wengelnik et al., 2002
	Membrane transport	Unique channels		Dinucleoside dimers	Gero et al., 2003
Food vacuole	Heme polymerization	Hemozoin	Quinolines	New quinolines	De et al., 1998; Stocks et al., 2002
	Hemoglobin hydrolysis	Plasmepsins		Protease inhibitors	Francis et al., 1994; Haque et al., 1999
		Falcipains		Protease inhibitors	Rosenthal, 2001b; Shenai et al., 2003
	Free radical generation	Unknown	Artemisinins	New peroxides	Vennerstrom et al., 2000; Borstnik et al., 2002
Mitochondrion	Electron transport	Cyt. *c* oxidoreductase	Atovaquone		
Apicoplast	Protein synthesis	Apicoplast ribosome	Antibiotics		
	DNA synthesis	DNA gyrase	Quinolones		
	Transcription	RNA polymerase	Rifampin		
	Type II fatty acid biosynthesis	FabH		Thiolactomycin	Waller et al., 1998
		FabI		Triclosan	Surolia and Surolia, 2001
	Isoprenoid synthesis	DOXP reductoisomerase		Fosmidomycin	Jomaa et al., 1999
	Protein famesylation	Farnesyl transferase		Peptidomimetics	Ohkanda et al., 2001; Chakrabarti et al., 2002

the identification of new targets and subsequent discovery of compounds that act on these targets. Progress towards the characterization of the biology of malaria parasites has been stimulated by the development of technology to disrupt plasmodial genes, although this process remains laborious and inefficient, and the sequencing and annotation of the *P. falciparum* genome. The readily accessible genome sequence facilitates genomic approaches to drug discovery, although the more difficult and risky biochemical and parasitological validation of putative drug targets remains essential and typically limits progress. New targets for antimalarial therapy will be considered based on their locations within the malaria parasite (Table 2).

Cytosolic targets

The cytosol is the location of numerous metabolic pathways, with hundreds of enzymes that are probably essential, and thus potential drug targets. However, many of these pathways are evolutionarily well conserved, such that parasite and host targets are quite similar, and so the identification of compounds that selectively inhibit parasite enzymes may be difficult.

One pathway that has proven to be a valuable target is folate metabolism, as discussed above (Plowe, 2001). Indeed, despite similarities in targets, extensive study has identified antifolates that effectively treat both bacterial and protozoan infections with minimal toxicity. This approach has also benefited from the availability of compounds developed against other diseases, in this case bacterial infections. Unfortunately, resistance to individual DHFR and DHPS inhibitors, including pyrimethamine, proguanil and sulfas, leads to a marked loss in efficacy of even combination regimens (Plowe, 2001). Sulfadoxine/pyrimethamine is inexpensive and it has replaced chloroquine as first-line therapy for malaria in a number of countries in Africa. However, resistance to this agent is already common in many areas, including parts of Africa, and resistance appears to develop quickly, at least in some settings, with widespread use. The new combination of chlorproguanil/dapsone will probably be highly effective in Africa, but not some other areas, due to differences in the *P. falciparum* DHFR and DHPS mutation patterns in different parts of the world, but it remains to be seen how quickly this new drug will select for resistance if it is used alone to treat malaria, as will probably soon be the case. Attempts are now underway to develop improved DHFR inhibitor antimalarials, including biguanides related to proguanil (Kinyanjui et al., 1999). In addition, inhibitors of other folate pathway

enzymes may be effective antimalarials. For example, 5-fluoroorotate exerts antimalarial activity *via* the inhibition of thymidylate synthase (Rathod et al., 1992).

Glycolysis is another cytosolic pathway of interest. Malaria parasites are dependent on this pathway for energy production. *P. falciparum* lactate dehydrogenase has been characterized structurally, and its unique binding site for the NADH cofactor offers opportunities for the design of selective inhibitors (Dunn et al., 1996). Selective inhibitors of *P. falciparum* lactate dehydrogenase have been identified (Deck et al., 1998) and some compounds have demonstrated *in vitro* antimalarial activity (Razakantoanina et al., 2000).

Purine salvage and pyrimidine synthetic pathways also offer potential drug targets. Malaria parasites cannot synthesize purines and rely on salvage of host purines for nucleic acid synthesis. The principal source of purines in *P. falciparum* appears to be hypoxanthine, and hypoxanthine–guanine phosphoribosyltransferase (HGPRT) has been considered as a potential drug target (Keough et al., 1999). Other enzymes in purine salvage may offer useful targets, considering both unique features of the parasite enzymes and the essential role of purine salvage for the parasites, but not humans. In contrast to the case with purines, malaria parasites cannot salvage pyrimidines and are thus reliant on pyrimidine synthesis. Differences between pyrimidine synthetic enzymes of parasites and humans offer potential for exploitation as drug targets. Pyrimidine synthesis also relies on mitochondrial electron transport, linking these targets to those in the mitochondrion (see below).

Parasite membrane targets

Parasite phospholipid metabolism is one potential target. Intraerythrocytic malaria parasites undergo extensive phospholipid synthesis to produce the membranes necessary to enclose the parasitophorous vacuole, cytosol and multiple subcellular compartments. The most abundant lipid in plasmodial membranes is phosphatidylcholine. Synthesis of phosphatidylcholine requires host choline, and blockage of choline transport has been identified as a promising therapeutic strategy (Vial and Calas, 2001). Extensive medicinal chemistry efforts have identified compounds that exert profound antimalarial effects, probably by inhibition of choline transport. A lead compound, G25, inhibited the development of cultured *P. falciparum* parasites at concentrations that were 1000-fold below those toxic to mammalian cells (Calas et al., 2000; Wengelnik et al., 2002). G25 was also effective *in vivo* against mice infected with rodent malaria parasites and primates infected with *Plasmodium cynomolgi* (a model for *Plasmodium vivax*) and *P. falciparum*. Importantly, the compound was remarkably potent in the primate models, with activity at doses far below 1 mg kg^{-1} day^{-1}, although potency with oral dosing was well below that with parenteral dosing.

Other membrane targets are transport pathways that are unique to malaria parasites. Intraerythrocytic parasites markedly alter erythrocyte transport pathways. Our understanding of parasite transport mechanisms remains incomplete, but it is likely that differences between host and parasite mechanisms offer possibilities for selective antimalarial drugs (Haldar and Akompong, 2001; Kirk, 2001). One possibility is to take advantage of selective transport of cytotoxic compounds into *P. falciparum*-infected erythrocytes. A strategy currently being investigated is the use of dinucleoside phosphate dimers conjugated to antimalarial compounds to improve selective access to parasite targets (Gero et al., 2003).

Food vacuole targets

Malaria parasites contain acidic food vacuoles in which erythrocyte hemoglobin is hydrolyzed. In *P. falciparum* trophozoites, a single large food vacuole is present. The food vacuole appears to be the site of action of a number of existing antimalarials and also offers opportunities for therapies directed against new targets (Banerjee and Goldberg, 2001). In the food vacuole, hemoglobin is degraded into heme, which is polymerized into insoluble hemozoin pigment and globin, which is hydrolyzed to individual amino acids. Antimalarial drugs appear to act by preventing hemozoin formation, producing free radicals in the food vacuole or, in the case of experimental compounds, preventing globin hydrolysis.

The 4-aminoquinoline chloroquine appears to act by blocking the formation of hemozoin from heme molecules once they are liberated from hemoglobin (Sullivan, 2002). Antiparasitic effects are presumably engendered by the toxicity of free heme, possibly by disruption of membranes. It is unclear if other chemically related antimalarials act in a similar fashion. Although chloroquine use is now severely compromised by drug resistance, it is important to note that the >50-year history of successful use of this drug, with only a slow development of resistance, may be due to its non-enzymatic mechanism of action. Many available and experimental antimalarials inhibit specific enzymes, but this approach is likely to routinely suffer from rapid selection of parasites with mutations in target enzymes that mediate drug resistance, as is the case with antifolates. With chloroquine, resistance developed only very slowly. It is now clear that resistance is due primarily to mutations in a putative transporter, PfCRT, and that, although a single mutation mediates resistance *in vitro*, multiple mutations were necessary to select for clinical resistance (Fidock et al., 2000). Although chloroquine-resistant parasites are now common in almost all malarious areas, it seems reasonable to develop other compounds that attack heme polymerization. In this regard, major efforts to synthesize improved quinoline or related compounds as antimalarials are underway (De et al., 1998; Stocks et al., 2001, 2002).

The food vacuole also appears to be the target of artemisinin antimalarials. As noted above, this new class of compounds offers very potent activity without, to date, any reported drug resistance. Artemisinins contain an endoperoxide bridge that is essential for antimalarial activity and that appears to undergo an iron-catalyzed decomposition into free radicals (Meshnick, 2001). The compounds apparently exert antimalarial effects *via*

free-radical damage, possibly by alkylation of plasmodial proteins (Asawamahasakda et al., 1994; Bhisutthibhan et al., 1998), although the specific drug targets are uncertain. As is the case with other compounds active in the food vacuole, a key to the selective antimalarial toxicity of artemisinins may be the specific accumulation of the drug in this parasite organelle. Artemisinin analogs are already proven antimalarials but they are fairly expensive to produce, in part because they are semi-synthetic plant products. Extensive efforts are underway to develop fully synthetic peroxides and related compounds as antimalarials (Borstnik et al., 2002; Vennerstrom et al., 2000).

Globin hydrolysis appears to be mediated by a number of classes of proteases, including food vacuole aspartic (plasmepsins; Banerjee et al., 2002), cysteine (falcipains; Shenai et al., 2000; Sijwali et al., 2001) and metalloproteases (falcilysin; Eggleson et al., 1999), and at least one cytosolic metalloaminopeptidase (Gavigan et al., 2001). These enzymes all offer potential targets for chemotherapy (Rosenthal, 2001b). In the case of the plasmepsins and falcipains, the repertoire of proteases that mediate hemoglobin hydrolysis is now known to be more complicated than originally envisioned, as biochemical studies and the availability of the full *P. falciparum* genome sequence have identified additional members of these families. Four plasmepsins are believed to participate in hemoglobin hydrolysis in the food vacuole (Banerjee et al., 2002). It has been argued that plasmepsin II and perhaps additional plasmepsins mediate initial cleavages of hemoglobin, allowing additional processing by other proteases, but the specific roles of these or any other proteases in the process remain uncertain. Plasmepsin inhibitors have demonstrated antimalarial effects (Francis et al., 1994; Haque et al., 1999; Jiang et al., 2001; Moon et al., 1998; Nezami et al., 2002; Noteberg et al., 2003; Silva et al., 1996) but these have not clearly correlated with inhibition of hemoglobin hydrolysis. Cysteine protease inhibitors appear to act by inhibiting falcipain-2 and falcipain-3, the principal cysteine protease mediators of hemoglobin hydrolysis (Shenai et al., 2000; Sijwali et al., 2001). Falcipain inhibitors have been shown to prevent hemoglobin hydrolysis, block parasite development and cure murine malaria (Batra et al., 2003; Rosenthal, 2001b; Rosenthal et al., 1991, 1993, 1996, 2002; Shenai et al., 2003; Singh and Rosenthal, 2001). Importantly, the antimalarial activity of cysteine protease inhibitors is accompanied by a block in parasite hydrolysis of hemoglobin, with the accumulation of intact hemoglobin in the food vacuole. This morphological abnormality confirms the specific action of these compounds on falcipain targets. Of interest, cysteine and aspartic protease inhibitors exert synergistic antimalarial effects *in vitro* and *in vivo* (Semenov et al., 1998). These results suggest that an optimal protease inhibitor antimalarial might include inhibitors of both classes of proteases.

Mitochondrial targets

One new antimalarial has a mitochondrial target. Atovaquone acts against ubiquinol–cytochrome *c* oxidoreductase (complex III), inhibits electron transport and collapses mitochondrial membrane potential, which is required for a number of parasite biochemical processes (Vaidya, 2001). The drug has potent antimalarial activity but suffers from rapid selection of resistant parasites with mutations in the target enzyme, and so is inappropriate as monotherapy. Atovaquone proved to be surprisingly effective in combination with the antifolate proguanil, and this combination is now marketed as Malarone, an effective, but very expensive, drug for both chemoprophylaxis (Hogh et al., 2000) and therapy (Radloff et al., 1996) of falciparum malaria. The combination probably benefits from synergistic action of atovaquone and proguanil rather than the antifolate activity of the metabolite cycloguanil, and for this reason it is more effective than would have been predicted in areas with high levels of antifolate resistance (Vaidya, 2001).

Apicoplast targets

The apicoplast has recently been identified as a chloroplast-like organelle of apicomplexan parasites (Kohler et al., 1997). The apicoplast apparently resulted from endosymbiosis, leading to an organelle that maintains certain specific functions, probably including fatty acid, heme and amino acid metabolism (Roos et al., 2002). Like the mitochondrion, the apicoplast has a separate, prokaryote-like genome, and this fact probably explains the antimalarial effects of a number of antibacterial compounds that otherwise do not attack eukaryotes. However, most apicoplast proteins are encoded in the nucleus and then transported to the apicoplast by a specific mechanism involving two amino-terminal targeting sequences (Foth et al., 2003; Waller et al., 1998).

As noted above, a number of antibacterial compounds are effective, albeit slow-acting, antimalarials (Clough and Wilson, 2001). These compounds probably act by targeting apicoplast and/or mitochondrial processes that are similar to those in bacteria (Ralph et al., 2001). Tetracyclines, clindamycin, macrolides and chloramphenicol inhibit different steps of prokaryote-like protein synthesis. Quinolone antibiotics inhibit DNA gyrase, and rifampin inhibits RNA polymerase, again with specificity to prokaryote-like activity. It is not clear why all of these compounds appear to exert only slow antimalarial activity. It seems most likely, based in part on studies with the related protozoan *Toxoplasma*, that apicoplast toxicity primarily impacts on the life cycle after that which is initially incubated with these drugs (Fichera and Roos, 1997). In some cases, mitochondrial toxicity may also play a role. Despite the slow action of antibacterial compounds as antimalarials, some, including tetracyclines and clindamycin, are already well-validated as effective antimalarial agents, and additional study of related drugs is warranted.

Apicoplast biology includes a number of biochemical pathways that are present in bacteria, plants and apicomplexan parasites but are absent in the human host and thus provide obvious opportunities for chemotherapy (Ralph et al., 2001;

Roos et al., 2002). The type II fatty acid biosynthesis pathway is absent in humans, but genes encoding homologs of bacterial enzymes from this pathway are present in the *P. falciparum* genome and contain putative apicoplast coding signals (Waller et al., 1998). One type II fatty acid biosynthesis subunit, β-ketoacyl-acyl-carrier protein synthase (FabH), is the target of the antibiotic thiolactomycin, and this antibiotic was active against cultured malaria parasites (Waller et al., 1998). Another subunit, enoyl-acyl-carrier protein reductase (FabI), is also encoded by *P. falciparum* and is the target of the antibacterial triclosan (Surolia and Surolia, 2001). Triclosan demonstrated activity against cultured *P. falciparum* parasites and against murine malaria, inhibited the target enzyme and blocked parasite fatty acid synthesis, validating this target.

Isopentenyl diphosphate, the precursor for isoprenoids, is synthesized in plants and animals *via* the mevalonate pathway, but an alternative pathway, known as the 1-deoxy-D-xylulose 5-phosphate (DOXP) or non-mevalonate pathway, is present in bacteria and chloroplasts. Genes encoding two enzymes in this pathway, DOXP reductoisomerase and DOXP synthase, are encoded by *P. falciparum* and contain putative apicoplast targeting signals (Jomaa et al., 1999). The antibiotic fosmidomycin inhibited the activity of recombinant DOXP reductoisomerase, inhibited the growth of cultured *P. falciparum* parasites and cured murine malaria (Jomaa et al., 1999). Fosmidomycin was previously developed as an antibacterial, so it could quite quickly be brought to human trials for malaria. In an initial trial of safety and efficacy for uncomplicated malaria, fosmidomycin was well tolerated and demonstrated 100% initial cure rates (Lell et al., 2003). However, the use of fosmidomycin as monotherapy will be limited by the apparent need for frequent and prolonged dosing and the common occurrence of recrudescence after therapy. The inclusion of this compound or other inhibitors of apicoplast processes in combination antimalarial regimens may be appropriate.

The DOXP pathway provides precursors for protein farnesylation. Inhibitors of protein farnesyltransferases have been studied as potential cancer therapies. Plasmodial farnesyl transferase activity has unique biochemical features (Chakrabarti et al., 2002), and inhibitors of this process have *in vitro* antimalarial activity (Chakrabarti et al., 2002; Ohkanda et al., 2001).

Conclusion

It is very concerning that the resistance of malaria parasites to available drugs continues to grow, increasingly limiting our ability to control this serious disease. However, it is reassuring that many new approaches to antimalarial drug discovery are now under evaluation, as reviewed here. Recent increases in the pace of progress in this area suggest that, if support for antimalarial drug discovery is adequate, new approaches should lead to the development of valuable new strategies for antimalarial therapy in the near future.

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