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Review Article

New antimalarials targeting both the asexual and gametocyte stages

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CONTENTS

Abstract

Plasmodium falciparum, the most virulent human malaria parasite, has an extended course of sexual development that is required for transmission of the disease. The 48-h asexual cycle is responsible for the clinical symptoms, while 10-12 days are required for the production of transmission-competent mature gametocytes. Mature gametocytes are resistant to all of the current antimalarial compounds, except primaquine, which is not commonly used due to the possibility of serious side effects. Consequently, even after effective treatment to clear asexual parasites, malaria transmission can continue for several weeks, prolonging the spread of the disease in the community. The inclusion of agents acting on gametocytes, as well as other exo-erythrocytic parasite stages, in the drug development pipeline could facilitate the design of combination therapies that reduce morbidity and mortality at both the individual and community levels. In this article, the gametocytocidal potential of compounds currently being developed as antimalarials is reviewed and strategies are proposed for the direct screening of gametocytes and the identification of compounds that target pathways required for sexual maturation.

Introduction

Malaria continues to be a major global health problem, with 3.3 billion people at risk, and 247 million clinical cases and 1 million deaths in 2006 (1). The disease is caused by *Plasmodium* parasites that are transmitted by *Anopheles* mosquitoes. *P. falciparum* and *P. vivax* are responsible for most of the morbidity, while mortality is almost exclusively due to *P. falciparum*. The symptoms associated with malaria are caused by the asexual erythrocytic life cycle stages of the parasites, while transmission requires sexual stage development followed by transfer to a mosquito.

Plasmodium life cycle

Following merozoite invasion of an erythrocyte, the resulting trophozoite undergoes three to five rounds of DNA replication, resulting in the production of a mature schizont containing 8-32 new infectious merozoites. Therefore, the number of infected erythrocytes can increase by 8-32-fold during one asexual cycle, which is 48 h in the tertian malaria parasites *P. falciparum, P. vivax* and *P. ovale* and 72 h in the quartan malaria parasite *P. malariae* (2). This multiplication rate can lead to rapid escalation of the disease, which is characterized by high fever. Fever is induced when the infected erythrocyte ruptures, releasing merozoites. In the case of *P. falciparum*, organ damage, coma and death can result from the adherence of parasite-infected erythrocytes to endothelial cells, which at high levels of parasitemia can occlude the vessel, blocking blood flow. Although responsible for the clinical symptoms, the asexual stages can not be transmitted directly to another person. For transmission, the intraerythrocytic parasite must differentiate into either a single male or female gametocyte and then be taken up in a blood meal by a mosquito. In *P. falciparum* this process takes 10-12 days as the parasite passes through five morphologically distinct stages (I-V) to become a mature stage V gametocyte (3). In the other human malarias gametocyte maturation takes about 2 days.

Gametocyte development

Understandably, the disease-causing asexual stages are the primary target of current malaria chemotherapy. A variety of compounds have been developed as antimalar-

ials and will be reviewed below. Due to distinct differences in asexual and sexual development, most of the approved antimalarials do not affect the viability of mature gametocytes. For example, DNA replication is not required for gametocyte development, and therefore these stages are resistant to drugs that target nucleic acid production, such as sulfadoxine–pyrimethamine (4). There are also marked metabolic differences between asexual and sexual stages (5). This is particularly dramatic in *P. falciparum* after stage II, when the parasite is no longer affected by compounds that block hemoglobin digestion or erythrocyte invasion, such as the 4-aminoquinolines and protease inhibitors (4).

Gametocytes are also not lysed by sorbitol, suggesting a reduction in uptake pathways such as the plasmodial surface anion channel (PSAC) (6-8). Consequently, *P. falciparum* parasites can continue to be transmitted for over a week following clearance of asexual parasites. The shorter time course of gametocytogenesis in the other species decreases the transmission period. However, both *P. vivax* and *P. ovale* produce dormant liver stages, called hypnozoites, which can be reactivated months to years later and lead to recrudescence. These forms of the parasite are also resistant to most of the commonly used antimalarials. The 8-aminoquinoline primaquine is the only drug recommended for the elimination of hypnozoites, and it also effectively eliminates late-stage gametocytes (2). The mechanism of action of the 8-aminoquinolines remains unknown, but they do generate methemoglobin, which in individuals with genetic deficiencies in glucose-6-phosphate dehydrogenase (G6PD) or NADPH-methemoglobin reductase can lead to hemolytic anemia (9). The potential for this serious side effect, as well as the requirement for a 14-day treatment course, has limited the use of this drug as standard chemotherapy. However, the successful decrease in malaria incidence with the use of transmission-blocking interventions such as insecticide-treated bed nets (10) and indoor residual spraying (11) has prompted a reevaluation of the utility of using primaquine treatment to eliminate mature gametocytes (12-14).

Historically, blocking transmission has been a key element of malaria control efforts. Although not sufficient alone to eliminate malaria mortality in areas of high transmission, blocking the spread of infectious parasites can play an important role in a comprehensive campaign against malaria (15). One approach is to include gametocidal drugs in combination therapy (12-14). Unfortunately, as described above only one drug, primaquine, is available that effectively kills mature gametocytes, but it is not routinely used because it can have significant side effects. Clearly, additional antigametocyte agents are needed. However, this stage of the life cycle is not routinely included in drug screens, because many research labs do not have experience with in vitro gametocyte cultures or transmission-blocking assays. Strategies to identify gametocytocidal reagents will be presented following a brief review of current therapeutic options and compounds in the drug development/discovery pipeline.

Current chemotherapy

Due to the spread of drug-resistant parasites in the last decade, artemisinin combination therapy (ACT) is the current World Health Organization (WHO) recommendation for areas with chloroquine-resistant parasites, which includes most of Africa and Asia. Artemisinin contains an endoperoxide bridge that is required for activity and is thought to mediate ion-dependent alkylation (16). The specific cellular target(s) remains unknown, but it is fast-acting, killing early rings and trophozoites, and thereby preventing the formation of mature forms that can sequester and cause vascular damage (17). In contrast to pyrimethamine and many of the other commonly used antimalarials, artemisinin does have an inhibitory effect on early *P. falciparum* gametocyte stages I-II, but stages III-V are resistant, as are the liver stages and *P. vivax* hypnozoites (18, 19). The remaining *P. falciparum* gametocytes continue to be infectious for the next week, allowing continued transmission in the community (20). Active artemisinin derivatives that are used clinically include artemether, artesunate and dihydroartemisinin (19). The first ACT to be approved was artemether–lumefantrine, produced by Novartis. Additional drug combinations and formulations are in the final stages of clinical testing (www.MMV.org).

Lumefantrine is a 4-aminoquinoline like many other antimalarials, including chloroquine, quinine, mefloquine, amodiaquine and piperaquine (16). This class of compounds concentrates in the food vacuole and inhibits the conversion of toxic heme moieties to inert hemozoin polymers. Damage caused by the free heme is thought to disrupt the development of actively metabolizing trophozoites. Gametocytes are not affected by these compounds, perhaps due to the decreased metabolic activity utilized during sexual differentiation.

Other components used in combination therapies include atovaquone, antifolates, 4-aminoquinolines and antibiotics such as doxycycline and clindamycin. None of these compounds effectively kill gametocytes or *P. vivax* hypnozoites (4, 21). Atovaquone interferes with the binding of ubiquinone to cytochrome bc_1 in the mitochondria, decreasing the generation of pyrimidines which are needed for DNA and RNA synthesis (22). The antifolates also reduce DNA and RNA production by directly inhibiting enzymes required for the synthesis of intermediates in the pathway (16). Sulfadoxine and dapsone inhibit dihydropteroate synthetase (DHPS), while pyrimethamine, proguanil and chlorproguanil inhibit dihydrofolate reductase (DHFR).

The apicoplast, which is thought to be derived from an ancient endosymbiotic cyanobacteria, is the target of antibiotics with antimalarial activity (23). Genes derived from this prokaryotic endosymbiont are still found in *P. falciparum* and the corresponding proteins are sensitive to antibacterial agents such as ciprofloxacin, rifampicin, clindamycin, azithromycin and doxycycline (24). The apicoplast is required for asexual reproduction, but its role in gametocyte development is largely unknown (25). Field

studies have indicated that clindamycin and tetracycline, an analogue of doxycycline, reduce asexual parasitemia but do not decrease gametocyte carriage (12). In addition to the apicoplast housekeeping functions targeted by the antibacterial agents listed, the apicoplast is also involved in a number of pathways that are not present in the human host and thus represent potential drug targets.

Drug discovery

Current drug discovery efforts can be divided into several categories: 1) further optimization of existing antimalarials; 2) identification of metabolic pathways that are distinct from the human host; and 3) high-throughput screening of libraries of small molecules, known drugs and natural products. Evaluating derivatives of effective drugs for enhanced activity is an established approach. However, as is the case with most of the current antimalarials, when the targeted pathway is not essential for sexual differentiation, it is unlikely that analogues of the drugs will effectively kill gametocytes. Consequently, novel quinolines structurally related to chloroquine or amodiaquine and additional compounds that interfere with DNA or RNA synthesis are unlikely to effectively block transmission by killing mature gametocytes. However, since it is not yet possible to accurately predict all the properties of novel compounds, the development of an efficient screen against gametocytes would allow an initial estimate of the transmission potential of new candidates. This information could then be factored into further drug design decisions.

A number of derivatives are currently being developed as drugs through the Medicines for Malaria Venture (www.MMV.org), including *N*-tertiary butylisoquine, a derivative of chloroquine, inhibitors of DHFR and dihydroorotate dehydrogenase (DHODH) (26), 4(1*H*)-pyridone, which targets cytochrome bc_1 like atovaquone (27), analogues of azithromycin (28, 29) and antagonists of the PSAC (8). Other possible targets that are likely to affect asexual but not mature gametocytes are the shikimate pathway involved in the production of *para*-aminobenozate (PABA), an intermediate in folate biosynthesis (30), and key enzymes in polyamine metabolism, such as the unique bifunctional ornithine decarboxylase *S*-adenylmethionine decarboxylase (ODC-AdoMetDC), which is inhibited by α-difluoromethylornithine (DFMO) (31). Also being considered as drug targets are polyamine and nucleoside membrane transporters (32-34) and enzymes involved in purine metabolism, such as hypoxanthine-guanine phosphoribosyltransferase (HGPRT), adenylosuccinate synthetase (AdSS) and *S*-adenosyl-L-homocysteine hydrolase (35-38).

Compounds with gametocytocidal potential

Primaquine and synthetic artemisinin derivatives

Several compounds in the MMV portfolio could potentially reduce gametocyte carriage. One of the most promising is Pyramax®, an ACT comprised of pyronaridine–artesunate that is in phase III clinical trials. Pyronaridine, a 9-anilino-azaacridine synthesized by the Chinese Antimalarial Research Group, was found to be even more effective against mature gametocytes in vitro than primaquine (39); the IC_{50} values for pyronaridine and primaquine are 20 nM and 2.1 μM, respectively. Other MMV-targeted compounds are tafenoquine, an 8-aminoquinoline that kills *P. vivax* hypnozoites, and novel imidazolidinediones that inhibit liver stages. In analogy with primaquine, both types of compounds could also have an effect on gametocytes.

The success of artemisinin has led to the development of a number of synthetic peroxide derivatives, including trioxanes and ozonides (www.MMV.org). Their effect on gametocyte formation has not been reported, but synthetic hybrid compounds called trioxaquines, which combine the trioxane motif from artemisinin with an aminoquinoline moiety from chloroquine, have been reported to kill stage IV and V gametocytes in culture (40). Further study of the mechanisms of action of these effective gametocytocidal compounds should identify pathways that are essential for gametocytes. These critical pathways could then be targeted for future drug development efforts.

Protease inhibitors

Cysteine protease inhibitors are also MMV candidates that could have transmission-blocking affects. Cysteine proteases are involved at multiple steps in sporogonic development in the mosquito (41-44). Other classes of proteases have also been shown to play critical roles in asexual growth, including schizont rupture, red blood cell (RBC) invasion and proteasome activity. Libraries of protease inhibitor derivatives have been generated and are being screened for effects at specific points in the asexual cycle using parasite lines that express fluorescent protein at particular stages of asexual development (45). Gametocytes have not yet been included in these screens, but recently serine protease inhibitors have been shown to decrease exflagellation (46) and the ability of the rodent malaria parasite *P. berghei* to be transmitted to mosquitoes (47).

Apicoplast pathways

The recognition that *Plasmodium* contains a relict plastid, the apicoplast, has provided several new drug targets. In addition to the presence of nonmammalian housekeeping pathways described above, the apicoplast also plays a key role in fatty acid and isoprenoid production (23). The role of these pathways in gametocytes has not been reported, but throughout sexual differentiation the parasite continues to grow in size, requiring expansion of both plasma and parasitophorous membranes. Once taken up by the mosquito, male gametocytes also need to produce eight individual microgametes, suggesting an important role for membrane synthesis.

The fatty acid biosynthesis type II pathway used by *Plasmodium* includes several enzymes without close orthologues in humans. Acetyl-CoA carboxylase, ketoacyl-ACP synthase and enoyl-ACP reductase are among those that are being actively investigated as drug targets (48-50). The antibacterial agents thiolactomycin and triclosan target this pathway and show micromolar IC_{50} values against *P. falciparum,* and serve as the initial leads for further drug development (www.MMV.org). In contrast to most eukaryotes, *Plasmodium* only utilizes the nonmevalonate pathway for isoprenoid production and consequently is sensitive to fosmidomycin, which inhibits 1-deoxy-D-xyulose-5-phosphate (DOXP) reductoisomerase (51). Fosmidomycin monotherapy resulted in a rapid decrease of parasitemia, but there was a high recrudescence rate (52). The addition of clindamycin, which targets translation in the apicoplast, improved parasite clearance when treatment was administered for 4-5 days (53).

Additional compounds and enzyme targets in this pathway are also being evaluated (54). Inhibitors of protein prenylation, which is required for the posttranslational modification of proteins involved in signal transduction, such as the oncogene ras, have been developed as anticancer therapies. Analogues of these drugs have high potency against *Plasmodium* both in vitro and in mouse studies (55, 56). Their effects on gametocytes have not yet been reported.

Antioxidant systems

Antioxidant pathways are also being investigated as potential drug targets (54). The sensitivity of the parasites to oxygen is clearly demonstrated by the low-oxygen conditions required to grow *P. falciparum* in culture. The parasite lacks catalase and glutathione peroxidases, but has both glutathione and thioredoxin systems to regulate the redox environment. In fact, thioredoxin reductase has been shown to be essential for the survival of asexual parasites, demonstrating the importance of this pathway and its potential as a drug target (57). Inhibitors of glutathione and thioredoxin reductases, such as 1,4-naphthoquinones and phenols, have been shown to have antimalarial activity. These are also being linked to 4-aminoquinolines to generate dual drugs that concentrate in the food vacuole and increase the release of unconjugated heme by decreasing the formation of hemozoin and inhibiting the antioxidant effect of both the glutathione and thioredoxin systems (58). The roles of these inhibitors have not been directly tested on gametocytes. Additionally, the roles of the enzymes in gametocytes are also not known, because in *Plasmodium* standard gene knockout or disruption approaches require asexual growth to generate a transformed parasite line. The transformed line can then be tested for the ability to undergo sexual differentiation or sporogonic development. Systems are just now being developed to generate conditional, stage-specific deletions of specific genes (59, 60).

Signal transduction

Genes involved in signaling pathways such as protein kinases are also important drug targets in many systems and are actively being investigated in *Plasmodium*. Analysis of the genome data identified 75-99 protein kinases, depending on the criteria used, including 20 members of a family that is unique to apicomplexa, named FIKK for the conserved amino acid motif common to the family members (61). There are also several other kinases that do not belong to any previously established group. Even the *Plasmodium* kinases that can be categorized usually diverge significantly from the corresponding genes found in higher eukaryotes, providing potential for drug development. No kinase inhibitor has been reported to kill mature gametocytes, but four kinases have been found to play critical roles once mature gametocytes are taken up in a blood meal by a mosquito (62-67). These include CDPK4 and CDPK3, calcium-dependent protein kinases with a domain architecture that is only also found in plants and some protists, MAP-2, an atypical mitogen-activated protein kinase, and Nek4, a NimA (never in mitosis/*Aspergillus*)-related protein kinase. Analysis of the kinases expressed in the early stages of gametocytogenesis may provide additional targets to inhibit intraerythrocytic sexual development.

Drug screens

The critical need for alternatives to artemisinin, which is the only antimalarial remaining for which widespread resistance has not been reported, has led to the development of high-throughput assays to screen compound libraries. To date, these have all been developed for asexual parasites and use fluorescent DNA stains (68-70) to measure parasite replication. Since gametocytes do not replicate, these approaches can not be used. Standard immunofluorescent techniques also can not be used as screens because gametocytes develop within the erythrocyte and no sexual stage-specific antigens have been found to be accessible on the erythrocyte surface. A possible alternative is to use parasites transformed with a fluorescent protein under the control of a gametocyte-specific reporter. Several *P. falciparum* lines have recently been produced that express GFP (green fluorescent protein) at distinct stages through gametocytogenesis and could be used to evaluate the efficacy and sensitivity of this method (71). As described in the previous section, a similar approach has been used with asexual parasites to screen protease inhibitors for stage-specific effects using transformed lines that express GFP at distinct stages.

In vitro conditions are available to allow screening of *P. falciparum* from the induction of gametocytogenesis, through the 10-12 days of maturation, up to 6 h after mosquito uptake is simulated by decreasing the temperature below 30 °C and adding xanthurenic acid or increasing the pH above 8. To evaluate later time points in the life cycle of *P. falciparum,* the parasites must be taken up by a mosquito from a membrane feeder. This

assay can be used to test specific compounds, but significant technical advances are needed to use this even as a medium-throughput screen. In contrast, in vitro conditions are available to produce motile ookinetes using the rodent malaria *P. berghei. P. berghei* is also commonly used for initial in vivo drug analysis, which would allow assessment of transmission-blocking activity in the initial evaluation. However, in *P. berghei* and other rodent malarias gametocytogenesis and the asexual cycle have similar time courses. In the case of *P. berghei* both take 24 h, and consequently it is difficult to separate the effects of a compound on asexual parasites and gametocytes.

Compound libraries

The *P. falciparum* parasite screens described above have been used to evaluate libraries of small molecules, known drugs, analogues of enzyme inhibitors and extracts of marine organisms, resulting in a number of hits which are being further evaluated. A recent marine microorganism screen led to the identification of salinosporamide A, produced by *Salinispora tropica,* a marine actinomycete (70). This compound has been evaluated in mice in preclinical trials as a chemotherapeutic agent against human lymphoma and has been shown to inhibit the 20S subunit of the proteasome. The compound had an IC_{50} of 11.4 nM against *P. falciparum* in an in vitro assay and a single s.c. dose of 130 μg/kg to *P. yoelii*-infected mice successfully suppressed an increase in parasitemia. Other proteasome inhibitors that are in preclinical development as anticancer drugs have also been shown to effectively kill asexual parasites at nanomolar levels (72, 73). The effect of these compounds on gametocytes or other life cycle stages has not been reported.

Summary

The unique characteristics of *P. falciparum* gametocytes and other exoerythrocytic parasite stages protect them from many of the commonly used antimalarials and related derivatives. The incorporation of these life cycle stages into drug development strategies is likely to enhance malaria control efforts by reducing transmission.

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