CHAPTER 5

The Medicinal Chemistry of Eradication: Hitting the Lifecycle where it Hurts.

Approaches to Blocking Transmission

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5.1 Introduction

Since the time when malaria and its modes of transmission were first understood, attempts to treat the disease have, perhaps unwittingly, identified two effective modes of intervention. First, to prevent and treat the disease. Second, to prevent the transmission of the parasite from person to person via the mosquito vector. One of the first recorded treatments for malaria, in the 17th century, was quinine, the active ingredient in 'Jesuit's bark'. This was followed by chloroquine in 1934. Whilst quinine is still in use today, widespread resistance to chloroquine has severely restricted its effectiveness. Transmission of the parasite through communities has principally been managed by

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controlling the mosquito vector with bed nets and insecticides such as DDT. Such was the success of these measures that, in 1957, the scientific community felt confident enough to use them as the central theme of a global malaria eradication campaign. Following significant early successes, this campaign was discontinued fourteen years later for many reasons including resistance of the parasite to chloroquine, and of the mosquito to DDT. Future eradication campaigns will need new classes of medicines and insecticides to overcome resistance. Some of these could be specifically targeted towards preventing transmission of the parasite.

5.2 Features of *Plasmodium* Biology Relevant to Drug Design

First, we must appreciate that five species of parasite are known to infect man, *Plasmodium falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi* and at least two others that have done so in the laboratory (*P. cynomolgi* and *P. inui*). Of these it is essential to recognise that the biology of *P. falciparum* (which is in the sub-genus *Laverania*) differs significantly with respect to other malaria species (sub-genus *Plasmodium*).

This raises a number of key points. First, differences in parasite biology mean data gained on other species of parasite need to be reconfirmed on *P. falciparum*, especially those obtained from species which infect rodents. Second, it may be that there is no medicine for targeting all clinical malaria transmission.

Any therapy attacking transmission of the parasite will need components that are designed to stop the appearance of the sexual stages of parasite. These are normally detected in the blood of the patient some 8–10 days after the main wave of disease in a *P. falciparum*-infected patient. They are most likely present in small numbers during the main wave of infection, but their presence is masked by the sheer biomass of the normal asexual parasites. In *P. vivax* the situation is different, and the sexual stages are already present at significant levels when patients first come and seek treatment.

There are two further twists to the problem. For *P. vivax, P. ovale* and the primate infecting strain *P. cynomolgi* there are dormant forms in the liver (known as hypnozoites) which can reactivate, giving the potential of a relapse in the absence of a mosquito bite. Second, there are significant reservoirs of *P. knowlesi* parasites in macaque monkeys and other great apes of Africa.[1] These species can also be transmitted to humans and the medicines need to take this into account.

The lifecycles of all malaria parasites exhibit common properties (See Figure 5.1; Table 1), which help us design new medicines to block transmission.

1. The parasite population undergoes exponential expansion in the asexual blood stages. At the peak of infection an adult patient may host 200 000 parasites per microlitre. This total of 10^{11} parasites is a formidable biomass.
2. Two population bottlenecks occur. The first in the pre-erythrocytic stage in the liver where, following bites from infected mosquitoes, a hundred

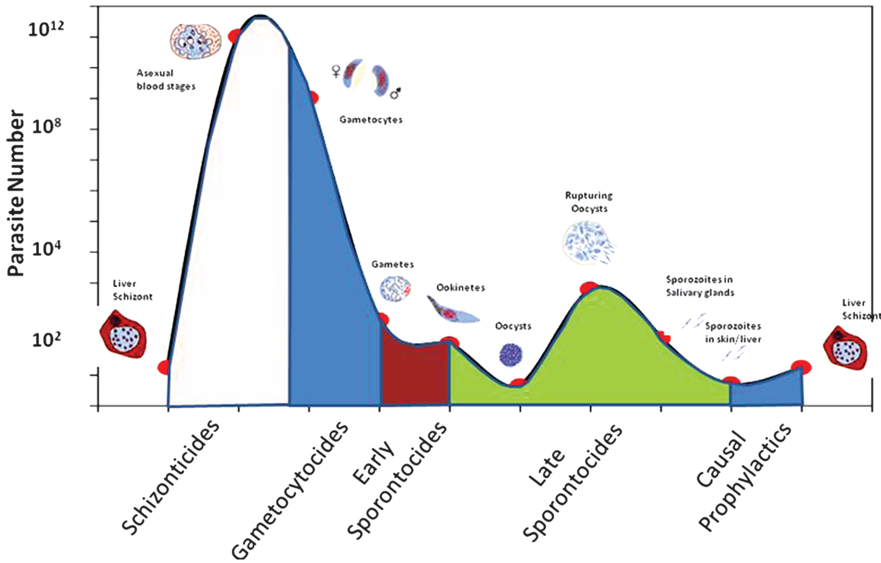

Figure 5.1 The replication cycle of *Plasmodium*. The numbers of parasites at each stage varies considerably. The critical bottlenecks are in the early liver stages in humans, and in the oocysts in the mosquito.

Table 5.1 Key metabolic activities and cellular properties of the different life stages that might influence the design and implementation of drugs targeting each stage.

	Vegetative growth	*DNA replication*	*Energy metabolism*	*Motility*	*Host cell barrier?*
Liver schizont	+++	+++	+++	-	Hepatocyte
Asexual blood stages	++	++	++	+merozoite	RBC
Gametocyte	± (immature)	?	+ (all ages)	-	RBC
Gamete	-	+♂	++♂	++♂	None
Ookinete	±	+ (meiosis)	++ (mitochondria)	++	None
Oocyst	+++	+++	++	-	None/capsule
Sporozoite	-	-	++	++	Salivary gland Kupffer Cell Hepatocyte

parasites can be found in the liver of the infected human host. The second, in the mosquito, where following the very inefficient processes leading to fertilisation, commonly less than five individual parasites survive to form oocysts in the infected mosquito.

3. The parasites are intracellular for most of their lifecycle. Pre-erythrocytic schizonts are resident in liver cells for several days. Blood-stage schizonts reside in anucleate red blood cells. The mosquito stages of parasite

development are extracellular, but the oocyst is enveloped by a capsule that is highly permeable to amino acids;[2] nucleotides[3] and drugs.[4] Conversely, all gametic and invasive stages are extracellular.

4. Mature gametocytes, salivary gland sporozoites and hypnozoites are all terminally differentiated cells that require specific external cues to progress through the cell cycle. As such they may share certain regulatory and metabolic similarities. It is therefore possible that drugs which kill the mature stage V gametocytes will have an impact on the hypnozoites. Indeed this is true for the 8-aminoquinoline family including primaquine and tafenoquine.

Prevention of disease requires that we reduce the large asexual blood-stage population, in which we might anticipate rapid selection of resistant mutants. However, if we want to reduce transmission we might profitably attack the small populations of parasites responsible for the transit of the parasite between human and mosquito. Here we might hypothesise that the selection of resistant genotypes will be substantially delayed (in inverse proportion to the population size).

5.3 Status of Current Biological Assays and Future Needs

For a thorough review of the early work on assays used we direct the reader to the excellent two-volume thesis of Peters.[5]

5.3.1 Pre-erythrocytic (Liver-stage) Assays

In vivo: Although *in vivo* assays have very low throughput, they have been the mainstay of drug development for compounds addressing the liver stages. These could be for causal prophylaxis (preventing the parasite leaving the liver prevents malaria blood stage infections) or for radical cure (wiping out the dormant reservoirs of *P. vivax, P. ovale* or *P. cynomolgi* in the liver). Until recently, the impact of drugs was primarily determined by the delay or cessation of blood patent parasitemia. The major development in recent years is using PCR to detect the appearance of parasites in the blood. This can be used in human studies since detection precedes clinical symptoms by a safe margin.[6] The sensitivity of PCR-based methodologies has *inter alia* permitted quantisation of pre-erythrocytic parasites directly in liver biopsies from model species, notably the rodent malarial parasites. The recent transgenic parasites (commonly in the rodent malaria species) expressing reporter molecules such as green fluorescent protein (GFP) or luciferase at selected points in the lifecycle permits their widespread application to time-series observations of parasites *in situ*,[7] and might provide a route to *in vivo* observations of the hypnozoite stages of *P. vivax* and *P. ovale* (or the model *P. cynomolgi*); or gametocyte sequestration in *P. falciparum*.

In vitro: The successful culture of rodent and then human parasite species in hepatoma cells,[8,9] and subsequently primary hepatocytes[10,11] has raised the importance of such techniques in drug discovery, especially now the assays can be conducted in 96-well formats using automated microscopic analysis,[12] thereby permitting the screening of large libraries of compounds.[13] Questions still remain as to the metabolic relevance of the hepatocyte monoculture and, to a lesser extent, the absence of 3D organisation of the host cells. Attempts to address this by the use of prefabricated 3D matrices are at an early stage.

Attempts have been made to investigate the hypnozoites of *P. vivax in vitro*,[14] but these are still experimental. The problem is how to prevent the host cell from totally outgrowing the static parasite population in culture. Recognising that the hypnozoite is an arrested parasite, identification of markers to distinguish hypnozoites from young primary schizonts is problematic. In the literature there are clear descriptions of strains of *P. vivax* (Nickolaiev strain) that exclusively produce relapsing forms.[15] Might it be feasible to re-discover these parasites and adapt them to culture?

5.3.2 Asexual Blood-stage (Schizonticide) Assays

Since the majority of drug discovery over the last seventy years has focused on the asexual blood stages responsible for the symptoms of malaria, there is a wide range of assays available.

In vivo: The main development in the field has been that of transgenic reporter parasites. However, they have yet to receive widespread application in understanding the impact of drugs on the dynamics of infection *in vivo*.

In vitro: The revolution in drug screening was brought about by the culture of the asexual blood stages of the most pathogenic of human parasites: *P. falciparum*.[16] The subsequent use of radio-labelling methods ([^{3}H] –hypoxanthine uptake), and more recently of non-radioactive (SYBR-green) approaches to measure parasite replication will remain a mainstay of antimalarial discovery. However, despite these successes there is still no routine procedure to support the growth of the blood-stages of *P. vivax* and the other human pathogens, and this is an area which requires more attention.

5.3.3 Mature Gametocyte (Gametocytocide) Assays

Development of gametocytocidal assays have been heavily influenced by the atypical biology of sexual development in *P. falciparum*.

In vivo: The specific search for gametocytocidal compounds was pioneered by Terzian,[17] but has received scarce attention since. Studies on the rodent malaria parasites in the 1980s, at the Department of Entomology at the Walter Reed Army Institute of Research, however, remain notable.[18] Studies on *P. falciparum* gametocytes are complicated by their biology. *In vivo* immature gametocytes are sequestered in the deep tissue for the first six to eight days and so are not seen in the bloodstream. The mature crescent-shaped gametocytes

appear nine to twelve days after the waves of asexual parasites. If the infection is treated with a pure schizonticide (classically this was chloroquine), all the blood stages and the immature gametocytes are killed up to stage III.[19,20] Thus, there is a short period where no parasites can be detected in the blood. This is followed by the emergence in the peripheral circulation of the late-stage gametocytes (which are not affected by 4-aminoquinolines such as chloroquine). Some have interpreted such data[21] to suggest that drugs with such selective killing activity induce gametocytogenesis, but have not recognised they may simply be observing the inevitable consequence of stage-specific lethality. Methods to distinguish gametocyte-selection from induction require careful design.[22] Concepts on the use of drug combinations to avoid enhanced transmission[23] require a more secure understanding of gametocyte biology. Studies on all of the other species apart from *P. falciparum* are free of this confounder.

***In vitro*:** Methods to grow and monitor (by microscopy) mature gametocytes of *P. falciparum in vitro,*[24] in 96-well format[25] and by flow cytometry[26] have advanced our understanding of the metabolic organisation and drug sensitivities of these stages.[27] However, we need to carefully evaluate these assays in the light of *Plasmodium falciparum* as explained above. The biology of the first three stages of gametocyte development is sensitive to drugs which kill the blood-stage asexual parasites, and so there is less need for drugs specifically targeting these stages. Where assays are really needed are the metabolically down-regulated stage IV and V gametocytes. Currently, the problem is that most markers such as Pfs16 are for early gametocytes. The search for later markers is ongoing. Current assays therefore focus on whether the infected cell is dead or can progress to gametes. Alternatives would be to look at the ability of stage IV and V gametocytes to develop into metabolically active cells.

Molecular assays: Whilst we currently understand little of the molecular pathways regulating gametocytogenesis, markers of sexual development are being described e.g. Pfs16;[28] telomerase,[29] and proteosomes.[30] These are a first step to describing molecular targets.

5.3.4 Mosquito-stage Assays (Gametogenesis; Ookinete and Oocyst Formation)

***In vivo*:** The ability to transmit many species of malarial parasite through the mosquito in the laboratory provides a powerful (but expensive) *in vivo* assay for transmission-blocking agents – widely exploited in the vaccine field as the 'gold' standard membrane feeding assay. Clearly the most amenable (safe) systems are the rodent parasites when transmitted by laboratory vectors such as *A. stephensi*. The success of transmission of the parasites from a drug-treated/infected host may be measured at many stages in development, including the ookinete, oocyst and the sporozoite. Clearly monitoring gametocyte-to-sporozoite development embraces the widest biology. All endpoints can now be measured conveniently using genetically tagged reporter parasites.[31,32] Data have in the past been commonly represented as the average reduction in

parasite number/intensity, or more rarely as a reduction in the prevalence of infected mosquitoes. Notwithstanding that these two parameters are mathematically related,[33] it is easier for the non-specialist to understand the impact of the intervention using the latter format.

Use of infected rodent hosts in standard membrane feeding assays benefits from the inclusion of host–drug PK/PD, but may be less appropriate for compounds targeted at *P. falciparum*. It is therefore convenient that *P. falciparum* gametocytes can be cultured throughout their development and then offered in membrane feeders to mosquitoes – a highly relevant assay.

Antivector drugs (smart insecticides) to disturb parasite–mosquito interactions delivered through the human host are a theoretical, but currently sidelined possibility, as demonstrated by studies using insect-immune peptides and their analogues SHIVA,[34] magainin[35] and cercropin.[36] *In vivo* studies on vivax, ovale, malariae and knowlesi malaria, such as those on *P. falciparum,* remain feasible using non-human primate hosts, and therefore have an important position in the development pipeline.

***In vitro*:** The culture of the entire mosquito phase of parasite development is now technically possible, but currently impractical for drug screening.[37,38] Nevertheless, useful assays have been developed to screen for compounds that block early gametic and sporogonic development.[39] Exflagellation of mature cultured drug-treated male gametocytes of *P. falciparum* is a glycolysis-powered event.[40] It represents perhaps the most relevant assay to monitor drugs that kill mature gametocytes. It could also be applied to all species of parasite.

The importance of finding new compounds has led to testing known compounds in these assays, with some success. Compounds already demonstrated to possess particular activity in this assay include atovaquone and thiostrepton. On published evidence this assay could readily be adapted to *P. vivax*.[41,42] Assays on cultured ookinetes of *P. falciparum* are still not at the stage where they can be used for routine testing.[43,44]

Molecular assays: Molecular pathways, targets and inhibitors that may be appropriate to monitor in the search for inhibitors of microgametogenesis include: Cysteine proteases,[45] PfPK7,[46] Pfnek-2,[47] Pfnek-4,[48] cGMP-dep protein kinase,[49] pbmap-2,[50] pbKch1,[51] cdpk3,[52,53] PMSF protease inhibitors,[54] PLA2,[55] antimicrobial peptides,[56] protein synthesis,[57] *Azadirachta indica*,[58] and NADPH-dependant dual oxidases.[59]

Compounds known to categorically impact transmission *via* either the gametocytes or mosquito stages are listed in Figure 5.2.

5.4 Clinical Aspects of Transmission-blocking Approaches

The differences between the timing of gametocyte appearance for *P. falciparum* and the other species of malaria mean they must be considered separately.

In all malaria species a small fraction of asexual blood stages are committed at each round of cell division in the red blood cell to become sexual stages.

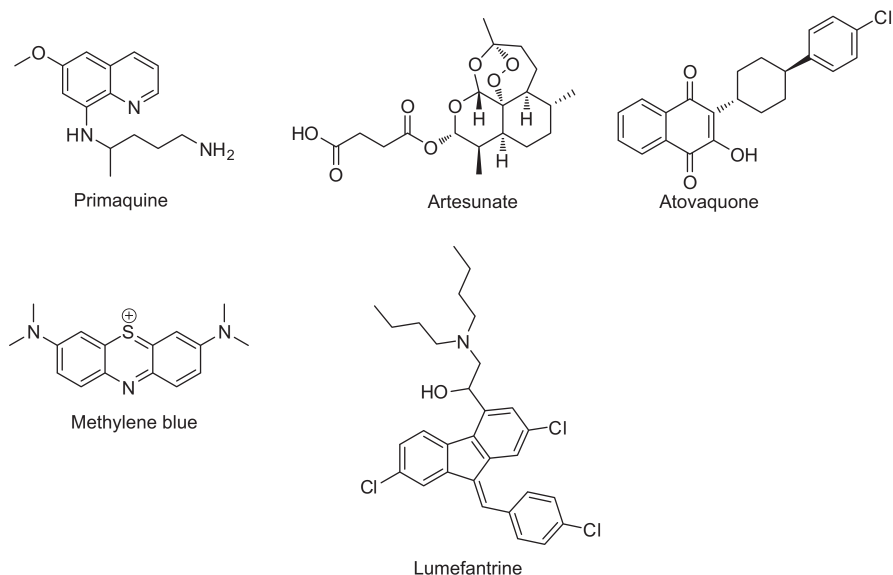

Figure 5.2 Drugs known to impact transmission in *P. berghei* or *P. falciparum*.[60,61]

Where the gametocyte and asexual blood stage maturation are of similar duration, the infected human must be considered to be infectious to the mosquito from the moment the patient has schizonts present in the blood. The waves of synchronous rupture of schizonts, which mark the clinical course of disease, have been shown to temporarily suppress this infectivity.[62] The short half-life of the mature gametocyte emphasises the notion that co-administration of a schizonticide and a (mature) gametocytocide is a very desirable strategy, but is this stratagem applicable to *P. falciparum? P. falciparum* gametocyte maturation is significantly slower than that of the asexual blood stage, onset of theoretical infectivity is delayed by 8–12 days and the population persists for 22 days (half life of 4–7 days). Periods of high gametocytaemia and infectivity follow the waves of asexual burden for extended periods of infection.[63] Does this temporal 'dislocation' influence the strategy of drug delivery? Do gametocytocidal compounds need to be delivered 8–12 days after a *P. falciparum*-infected patient has visited the clinic, and then must it persist in the bloodstream for a further 22 days? We suggest that the delivery of an effective schizonticide (blood-stage killer) combined with a fast-acting compound targeted to kill the late-stage gametocytes should also be effective here. The former will kill all input to the gametocyte population and potentially the standing population of immature gametocytes (stages I-III); and the latter will kill all the standing population of mature (stages IV-V) gametocytes. There will be no ensuing infectious population. The key conclusion from this hypothesis is that the half-life of a gametocytocidal drug needs to be sufficient to ensure that the gametocyte population is killed, though it need not be inordinately long.

5.4.1 Development of Transmission-blocking Drugs

One of the key questions raised by the concept of transmission blocking is to clearly explain the benefit of such a medicine to the patient. A gametocytocidal or sporozonticidal (killing the mosquito stages) molecule will benefit the recipient because it lowers the probability of being reinfected. Mosquitoes do not travel far and often feed on many people within the same room. Treating infectious patients to prevent transmission is not an altruism; it is sound public health policy.

One important question is how to test the efficacy of a transmission-blocking medicine clinically. Here the best model is another transmission-blocking agent, namely insecticide-treated nets (ITNs), and use randomised cluster approaches. What endpoint should we use? Whilst in the laboratory the screens have correctly measured the reduction in number of parasites (exflagellating microgametocytes; ookinetes; oocysts; sporozoites, or the prevalence of infected mosquitoes), we venture to suggest that this is not the endpoint that should be used in field trials other than early proof-of-concept studies. Here the key parameter to be measured is the reduction in the number of new infections in man following the introduction of the intervention. Clearly the issue of identifying new infections is key, but if impractical, the reduction in the total number of cases may be an indirect but relevant endpoint as has been used in bed net trials.[64]

5.5 Medicinal Chemistry Perspectives on Transmission Blocking

5.5.1 Liver-stage Parasites

Plasmodium vivax

The substantial worldwide burden of vivax malaria and its relapsing dormant liver-stage (hypnozoite) coupled with inadequate first-line therapy has raised the drug discovery priority for this indication. Consequently, it is important to have new drugs which kill liver-stage vivax hypnozoites.[65] Primaquine, an 8-aminoquinoline is the only marketed vivax malaria anti-relapse agent, though is contraindicated in patients with a glucose-6-phosphate dehydrogenase (G6PD) deficient phenotype and in expectant mothers, where the G6PD classification of the foetus is unknown.

From a medicinal chemistry perspective, a range of potential issues need to be considered for successful prosecution of a drug discovery project and these fall broadly into two categories. First, those dependent on the limitations of available assays and thus the nature of the test cascade. Second, what combination of parameters are necessary to be clinically relevant. These areas can be summarised for a *P. vivax* liver-stage drug as follows.

1. Test cascade: A *P. vivax in vitro* hepatocyte assay exhibiting both liver schizonts and hypnozoites is still not available. There are many reasons for this and these have been discussed at length elsewhere.[68] Furthermore, although an

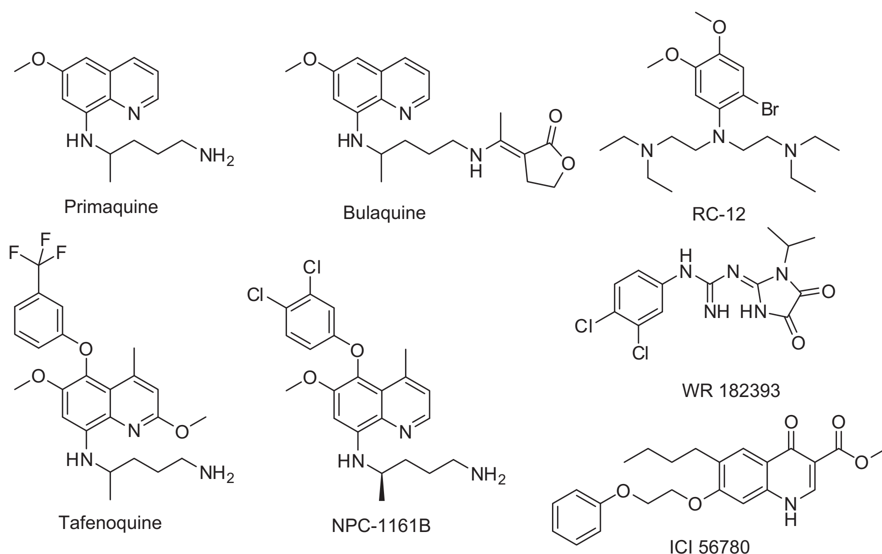

Figure 5.3 Structures of compounds known to block relapse in either *P. vivax, P. ovale* or *P. cynomolgi.*[66,67]

in vivo model utilising a *P. vivax* surrogate, *P. cynomolgi,* to infect rhesus monkeys is available with a reproducible relapse frequency,[69] a direct, simple validated *P. vivax* translational relapse model is not available.

Several models with validation underway include the following *in vitro* assays: *P. cynomolgi*-infected primary rhesus hepatocytes[70] and *P. vivax* infected HepG2 cells.[71] A validated *P. cynomolgi* assay will have the advantage that it should, assuming acceptable pharmacokinetics, translate well to the *in vivo* standard model (same host and parasite), though is not the human relevant parasite.

Current test cascades are pragmatic and can be influenced by hypotheses regarding the higher likelihood of finding anti-hypnozoiticidal agents from amongst liver schizonticides, rather than from non-liver acting molecules. In such scenarios, high-throughput rodent liver-stage malaria assays are performed and used as a filter for the lower throughput, more demanding vivax or cynomolgi malaria liver-stage assays. Alternatively, screening against vivax or cynomolgi liver-stage malaria can be performed with advanced compounds optimised for erythrocyte activity.

Such an approach is a profiling, rather than an optimisation strategy, and can be applied to any exo-erythrocytic stage, as shown in Figure 5.4. Should any activity be observed, follow-up according to the more detailed test cascades is possible.

The ideal test cascade for liver-stage vivax would have a validated and high-throughput vivax hypnozoite assay as the primary assay, in a metabolically functional human cell line. This is followed by *in vitro* metabolism, physical

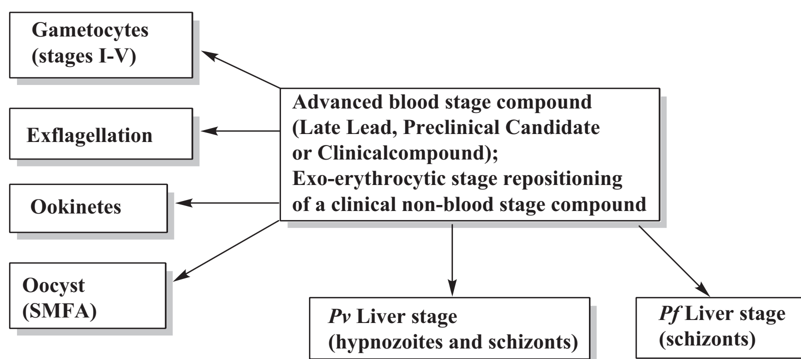

Figure 5.4 Profiling to determine extra-erythrocytic activity of advanced compounds. *Pv* = *Plasmodium vivax; Pf* = *Plasmodium falciparum*; SMFA = standard membrane feeding assay.

property measurements, and *in vivo* pharmacokinetics. Ultimately, the impact on relapse in the *in vivo P. cynomolgi* rhesus efficacy model, including chloroquine to eliminate blood-stage parasites, is the pre-clinical *in vivo* model. Given the issues of haemolysis with 8-aminoquinolines in G6PD deficient patients, a further requirement for a new molecule would be safety in an *in vivo* G6PD deficiency model.[72] One such idealised test cascade for a vivax liver drug discovery project is shown in Figure 5.5. It is to be noted that currently some of the specific parasitological assays do not exist, whilst other non-pharmacological assays are part of the standard drug discovery battery.

2. Parameters required for clinical utility: The lifetime of a hypnozoite is highly variable, from 26 days to a year. For a single exposure radical cure, exposure over a time period equal to three- to four-times (or less) the human half-life, after which the drug is likely to be below efficacious levels, must be sufficient to completely sterilize the liver. If not, multiple doses will be necessary. Clinically, we are searching for something as safe for everyone as primaquine is in G6PD normal patients, but with a single administration sufficient to kill all hypnozoites.

Plasmodium falciparum

Plasmodium falciparum does not form hypnozoites. A compound such as atovaquone, which kills the liver schizonts can be used as a causal prophylactic. Other examples of compounds with this activity are shown in Figure 5.6.

In those instances where, for pragmatic reasons, the parasite used in the assay is different from that relevant for human disease there is always a question relating to species differences. For full confidence, it is always important to demonstrate activity in the most physiologically relevant setting: the human parasite, in the human cell in the right tissue context.

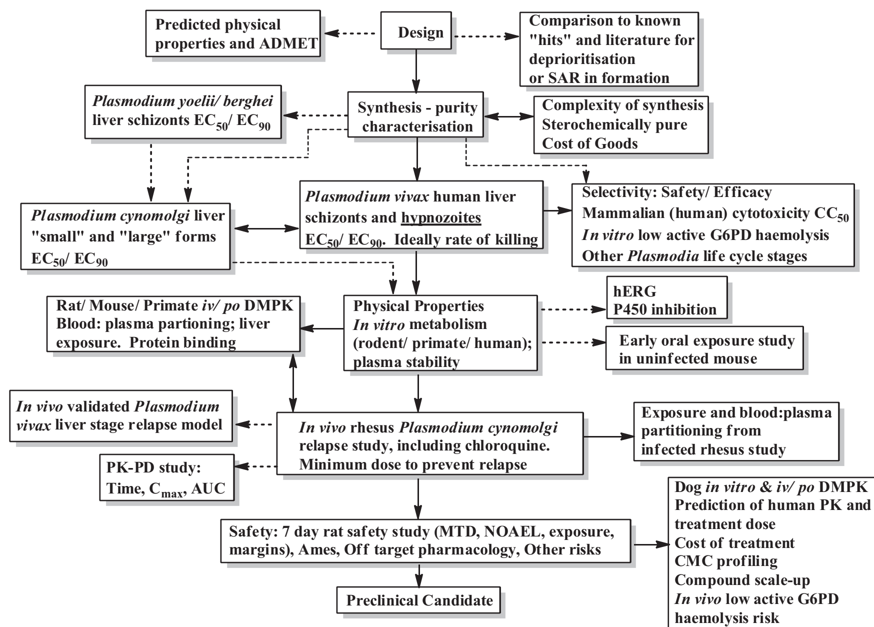

Figure 5.5 Idealised test cascade for *Plasmodium vivax* liver stage. ADMET = Absorption, Distribution, Metabolism, Excretion, Toxicity; SAR = Structure Activity Relationship; EC_{50}/EC_{90} = concentration of compound necessary to inhibit growth of the parasite by 50%/90% respectively; CC_{50} = concentration of compound resulting in 50% inhibition of an endpoint linked with cytotoxicity; DMPK = Drug Metabolism and Pharmacokinetics; hERG = human ether-a-go-go related gene (an ion channel implicated in possible cardiovascular risk); P450 = Cytochrome enzymes (those liver enzymes effecting metabolism of a compound); *iv/po* = *intra venous/per oral* (dosing routes directly into the vein or by mouth; *in vitro/in vivo* within glass/within living organisms); PK = Pharmacokinetics; PK-PD = Pharmacokinetic-Pharmacodynamic relationship; Tmax, Cmax, AUC = Time associated with maximal concentration, maximum concentration, area under the curve; MTD = maximum tolerated dose; NOAEL = No adverse effect level; Ames = a bacterial genotoxicity assay; CMC = chemistry, manufacturing and control; G6PD = glucose-6-phosphate dehydrogenase.

Consequently, primary cultures give more physiological readouts.[76] It is critical to understand whether activity is driven by parent molecule or a metabolite and to be aware to what extent turnover is an issue in the primary assay. This can be explored in several ways; comparing with metabolically inert cell lines, monitoring metabolic stability in the *in vitro* assay conditions or pre-treatment with pan-P450 inhibitors.[77] Usually, it is better for activity to be driven by the parent compound and to optimize the structure to reduce metabolism and thus extend half-life and duration of action.

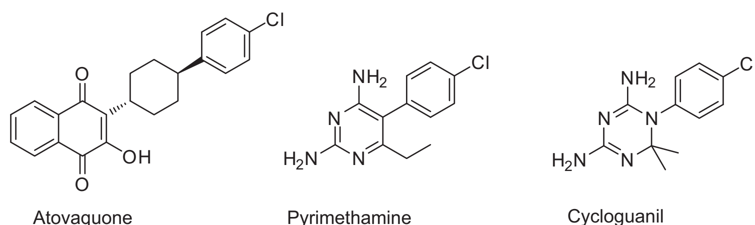

Figure 5.6 Compounds affecting pre-erythrocytic parasites.[73–75]

Liver targeting may also be an advantage and comparisons of liver *versus* blood exposure can be instructive. Strategies to selectively increase higher free concentrations in the liver usually involve exploiting active transport mechanisms and designing in characteristics that make the compounds better substrates for active uptake, such as has been demonstrated with the statins. One such transporter is OATP, the oxyanion transporter protein that is particularly beneficial for anionic species.[78] To the authors' knowledge, no substantial examples of such active transport strategies have been adopted in the malaria community, although many compounds are highly concentrated in the liver such as primaquine.

5.5.2 Gametocyte-stage Parasites

Disrupting transmission to the mosquito vector to render the mosquito non-infective to humans can be achieved by either eliminating the sexual gametocyte pool in the patient (gametocytocidal) or by eliminating the ookinete, oocyst and sporozoite load within the mosquito (sporozonticidal).[79–81]

Whilst the existing and developing host gametocyte assays measure various endpoints and stages, it is clear that the preferred phenotype would be rapid killing of all five stages. Clearly, killing of stage IV and V gametocytes, especially the latter, is of most value given that they are the infective parasite species. Furthermore, although in falciparum malaria there is an 8-day peak delay between asexual and sexual parasite load, at the point when a patient experiences symptoms, committed gametocytes will be present in the blood. For vivax malaria the gametocyte differentiation and production is coincident with the emergence of the blood-stage infection.[82] Consequently, having a drug combination treatment that kills stage V gametocytes is critical for full gametocytocidal efficacy. It is important to note that, particularly for vivax malaria, there is a window prior to symptoms, in which a patient may be infective.

1. Test cascade: The medicinal chemistry strategy and test-cascade adopted for such a transmission-blocking programme could depend on whether gametocytocidal activity was the chief optimisation parameter or not. Most pragmatically, advanced falciparum asexual acting compounds can be profiled *in vitro* against falciparum gametocyte stages I–V and for the differential stage activity to be assessed. The most relevant subsequent transmission-blocking

assay would be to incubate the compound in *P. falciparum* culture, for a duration and at a concentration relevant to the predicted *in vivo* duration and trough level following three daily doses in humans. Membrane feeding with the female *Anopheles gambiae* vector followed by dissections and oocyst counting then gives an idea of transmission-blocking potential.

Naturally the clinical endpoint (measuring oocyst counts in the female *Anopheles gambiae* vector) is the same, whether performed in a non-clinical or clinical setting, providing a useful translational link. However, in mosquitoes ingesting a blood meal through the skin of an infected human in which gametocyte load has been impacted by the drug, the metabolites and the immune response is the most relevant measure of response. Some compounds are only active via their metabolites, such as primaquine.

A medicinal chemistry strategy to optimise potency against gametocytes clearly requires a primary assay to examine killing of the required stages *in vitro*. *In vivo* assays looking at impact on gametocytes are feasible though will commonly involve both rodent malaria parasites and rodent liver drug metabolism. Gametocyte carriage in the *P. falciparum* severe combined immunodeficient (SCID) mouse model can be assessed,[83,84] though again rodent drug metabolism is in operation and the immunological component absent. The more complex *in vivo* transmission-blocking assays involve mosquito feeding of *Anopheles stephensi* or *Anopheles gambiae* (for the *Plasmodium falciparum* SCID mouse) on an infected mouse previously treated with the oral drug. Oocysts are then counted in the mosquito vector as a measurement of the transmission blocking potential. As described earlier, this assay can be substituted by the membrane feeding study with human parasites and human blood, with the metabolic caveats already mentioned.[85]

An idealised test cascade for this approach is shown in Figure 5.7.

2. Parameters required for clinical utility: Understanding the full gametocyte stage profile of a compound is key. For a compound acting on multiple lifecycle stages then it can be suggested that a similar mechanism of action is responsible for killing in the different phases. Since resistance generation is related to parasite numbers, any transmission-blocking agent with asexual-stage activity could potentially be rendered ineffective over time due to asexual resistance induction. From this specific perspective a pure gametocytocidal-only agent would be preferable. In practice, primaquine, which has only weak blood-stage efficacy, kills mature gametocytes and has shown impact on gametocytes in clinical trials. Consequently, it illustrates the target product profile and is the compound to compare new molecules against to ensure superiority.

It is critical that the half-life in humans of a gametocytocidal drug is appropriate relative to the rate of killing of gametocytes. In other words, that three once-daily doses of the agent results in exposure that will eliminate gametocytes below an infective concentration.[86] Rapid action would be preferred to truncate the transmission window.

Since gametocyte cultures are non-proliferating there is no opportunity for resistance generation through errors occurring via clonal expansion. Resistant

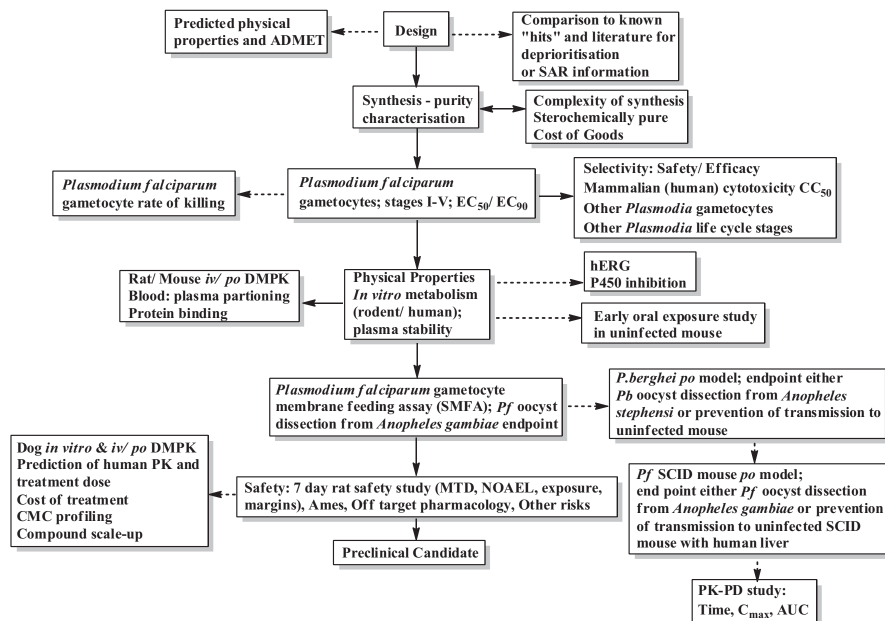

Figure 5.7 Idealised test cascade for gametocyte stages. ADMET = Absorption, Distribution, Metabolism, Excretion, Toxicity; SAR = Structure Activity Relationship; EC_{50}/EC_{90} = concentration of compound necessary to inhibit growth of the parasite by 50%/90% respectively; CC_{50} = concentration of compound resulting in 50% inhibition of an endpoint linked with cytotoxicity; DMPK = Drug Metabolism and Pharmacokinetics; hERG = human ether-a-go-go related gene (an ion channel implicated in possible cardiovascular risk); P450 = Cytochrome enzymes (those liver enzymes effecting metabolism of a compound); *iv/po = intra venous/per oral* (dosing routes directly into the vein or by mouth; *in vitro/in vivo* within glass/within living organisms); PK = Pharmacokinetics; PK-PD = Pharmacokinetic-Pharmacodynamic relationship; Tmax, Cmax, AUC = Time associated with maximal concentration, maximum concentration, area under the curve; MTD = maximum tolerated dose; NOAEL = No adverse effect level; Ames = a bacterial genotoxicity assay; CMC = chemistry, manufacturing and control.

gametocytes presumably thus occur as an end product of the asexual stage or via direct chemo-mutagenic events once committed. The ideal combination would thus include compounds with orthogonal gametocytocidal mechanisms to eliminate the transmission of the resistant phenotype.

5.5.3 Vector-stage Parasites

A transmission-blocking target product profile is also achievable *via* killing the parasites in the mosquito (sporozonticidal), in addition to or independent of host-stage parasiticidal action. Due to parasite numbers, the ookinete and

oocyst are extremely attractive choke-point targets for intervention. The idealised test cascade is covered in Figure 5.8.

1. Test cascade: The medicinal chemistry strategy and test cascade adopted for such a transmission-blocking programme would depend on whether vector-stage activity was seen as a selective property or as an additional value to a blood-stage acting compound. Most pragmatically, compounds acting against asexual stages can be profiled in the dedicated falciparum or rodent vector-stage assays. These include gametocyte exflagellation, ookinete and oocyst

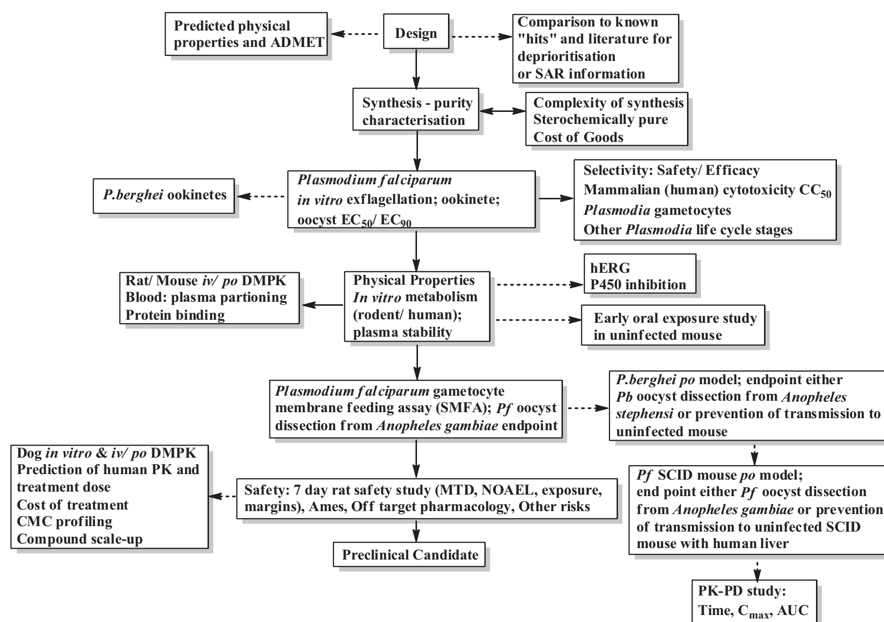

Figure 5.8 Idealised test cascade for mosquito stages. ADMET = Absorption, Distribution, Metabolism, Excretion, Toxicity; SAR = Structure Activity Relationship; EC_{50}/EC_{90} = concentration of compound necessary to inhibit growth of the parasite by 50%/90% respectively; CC_{50} = concentration of compound resulting in 50% inhibition of an endpoint linked with cytotoxicity; DMPK = Drug Metabolism and Pharmacokinetics; hERG = human ether-a-go-go related gene (an ion channel implicated in possible cardiovascular risk); P450 = Cytochrome enzymes (those liver enzymes effecting metabolism of a compound); *iv*/*po* = *intra venous*/*per oral* (dosing routes directly into the vein or by mouth; *in vitro*/*in vivo* within glass/within living organisms); PK = Pharmacokinetics; PK-PD = Pharmacokinetic-Pharmacodynamic relationship; Tmax, Cmax, AUC = Time associated with maximal concentration, maximum concentration, area under the curve; MTD = maximum tolerated dose; NOAEL = No adverse effect level; Ames = a bacterial genotoxicity assay; CMC = chemistry, manufacturing and control.

inhibition (Figure 5.4). As with gametocytes, the most relevant transmission-blocking assay involves counting oocysts in the vector following a membrane feed of an infective culture and drug. There is clearly a trade-off between throughput and relevance of the assay.

Alternatively, if the objective is a mosquito-stage-only compound, then the approach would be to screen compounds in the mosquito-based parasite assays. The results can be confirmed through periodic testing of optimised compounds in the membrane-feeding assay. *In vivo* rodent studies could be performed to determine the dose and duration of transmission blocking.

Pharmacokinetics and metabolism in the vector could be considered a potential issue for such a strategy, but the pragmatic way forward is simply to only pursue series that present correlations between a primary phenotypic assay and the oocyst count because, by definition their properties must have been sufficiently good in the vector to elicit the desired response.

2. Parameters required for clinical utility: For a compound to be clinically useful on mosquito-stage parasites, the drug must be present in blood, at concentrations commensurate with transmission blocking, for as long as infective gametocytes are in circulation. Given the data showing sub-microscopic gametocyte concentrations out to beyond 50 days,[87,88] it is clear that the major challenge with this approach is ensuring sufficient compound cover (i.e. long half-life). Therefore, it is likely to be viable only for a vector-stage-acting compound to be dosed as part of a combination that also kills gametocytes, and thus inherently reduces circulating mature gametocytes.

From a translational perspective, a concentration response curve in the standard membrane feeding assay with falciparum gametocytes can be obtained (i.e. defining the concentration at which oocyst counts are inhibited). This minimum effective sporozonticidal concentration can then be used, along with the desired duration of cover, to model the dose necessary in man to achieve blocking of transmission. The caveats of this are the lack of metabolism, immunological factors and the feeding *via* a non-skin model.

5.6 Conclusions

Antimalarial drug discovery, historically, has focused on treating the asexual blood stages of the infection. With increased understanding of the parasite lifecycle and an availability of assays to measure infectivity, researchers are finally starting to have the tools that will allow a new era of antimalarial drug discovery: focused for the first time on exoerythrocytic stages. In particular, hitting the lifecycle where it hurts – at the key choke points – remains a compelling strategy. Only by addressing transmission between the infected human and the vector (and *vice versa*) can the moral imperative of eradication ever be realised.

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