

Targeting the hypnozoite reservoir of *Plasmodium vivax*: the hidden obstacle to malaria elimination

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***Plasmodium vivax* is the major species of malaria parasite outside Africa. It is especially problematic in that the infection can relapse in the absence of mosquitoes by activation of dormant hypnozoites in the liver. Medicines that target the erythrocytic stages of *Plasmodium falciparum* are also active against *P. vivax*, except where these have been compromised by resistance. However, the only clinical therapy against relapse of vivax malaria is the 8-aminoquinoline, primaquine. This molecule has the drawback of causing haemolysis in genetically sensitive patients and requires 14 days of treatment. New, safer and more-easily administered drugs are urgently needed, and this is a crucial gap in the broader malaria-elimination agenda. New developments in cell biology are starting to open ways to the next generation of drugs against hypnozoites. This search is urgent, given the time needed to develop a new medication.**

Comparing malarias

Among the five species of *Plasmodium* known to infect humans, *Plasmodium falciparum* and *Plasmodium vivax* are by far the most common. Although infection by *P. vivax* has been called 'benign tertian malaria', it represents a major threat to health in South Asia, Southeast Asia and South America; 2.6 billion people are at risk, with perhaps several hundred million annual infections [1,2]. *Plasmodium vivax* gives more severe cycles of fever, sweats and chills (paroxysms), and higher proinflammatory cytokine levels [3], often leading to severe and even fatal outcomes [4]. Current therapies act against the erythrocytic stages of *Plasmodium* of all species, except where resistance has emerged. However, *P. vivax*, the less common *P. ovale*, and the closely related primate malaria *P. cynomolgi* present an additional problem – relapse. After infection by a biting mosquito, an unknown and probably variable proportion of invading sporozoites develop into dormant forms in hepatocytes, the hypnozoites [5] (Figure 1). Hypnozoites were first identified in tissues in 1982 and are characterised as a persistent uninucleate hepatic stage of around 4 µm diameter observed in relapsing species such as *P. vivax*, but not in *P. falciparum*. They have a distinct pharmacology: inhibition by primaquine at nanomolar concentrations but insensitivity to atovaquone-proguanil (active

against liver stage schizonts) and to chloroquine (a blood schizonticide). The mechanisms that drive dormancy and reactivation are unknown. The dormancy period can be as short as 17 days with the New Guinea Chesson strain, with 60% relapse by Day 28 [6], or can exceed a year in strains from temperate climates. This suggests dormancy is part of the co-evolution of parasite and mosquito with parasite relapse coinciding with seasonal mosquito abundance [7].

With the recent call for the development of a malaria-eradication agenda, therapeutic approaches to the hypnozoite have become part of the front line. The use of artemisinin combination therapies (ACTs) and bednets has reduced the incidence of *P. falciparum* in many countries, with a relatively minor effect on endemic *P. vivax*. The elimination of long-lasting reservoirs of infection represented by the hypnozoite will become an increasingly important target. However, there are few new approaches: although worldwide over 30 agents are in the development pipeline against malaria, only four specifically target hypnozoites [8] (Table 1). New approaches to the discovery of drugs targeting relapse are urgently needed as part of the eradication strategy. This review examines some of the unique obstacles to development of hypnozoitocidal drugs.

'Better than primaquine': the target product profile

Successful treatment of vivax malaria (known as radical cure), treats the episode of fever and parasitaemia, and also prevents relapse. The only clinically validated medication against hypnozoites is primaquine, an 8-aminoquinoline. In clinical trials in adults, primaquine combined with blood schizontocidal therapies showed >95% efficacy using 22.5 or 30 mg per day for 14 days [9,10]. Primaquine-based therapy has three weaknesses. First, it often causes haemolysis and methaemoglobinaemia in patients with a genetic deficiency in glucose-6-phosphate 1-dehydrogenase (G6PD) [11]. Although pre-screening of populations for this deficiency is routine in certain groups (such as the military), widespread pre-screening is problematic, principally because of the hundreds of genotypes with varying degrees of severity. Second, it has a short half-life and the standard dosage regimen requires 14 days to achieve radical cure. A shorter treatment course would improve effectiveness by increasing patient compliance. Third, G6PD deficiency cannot practically be tested in the fetus, and primaquine

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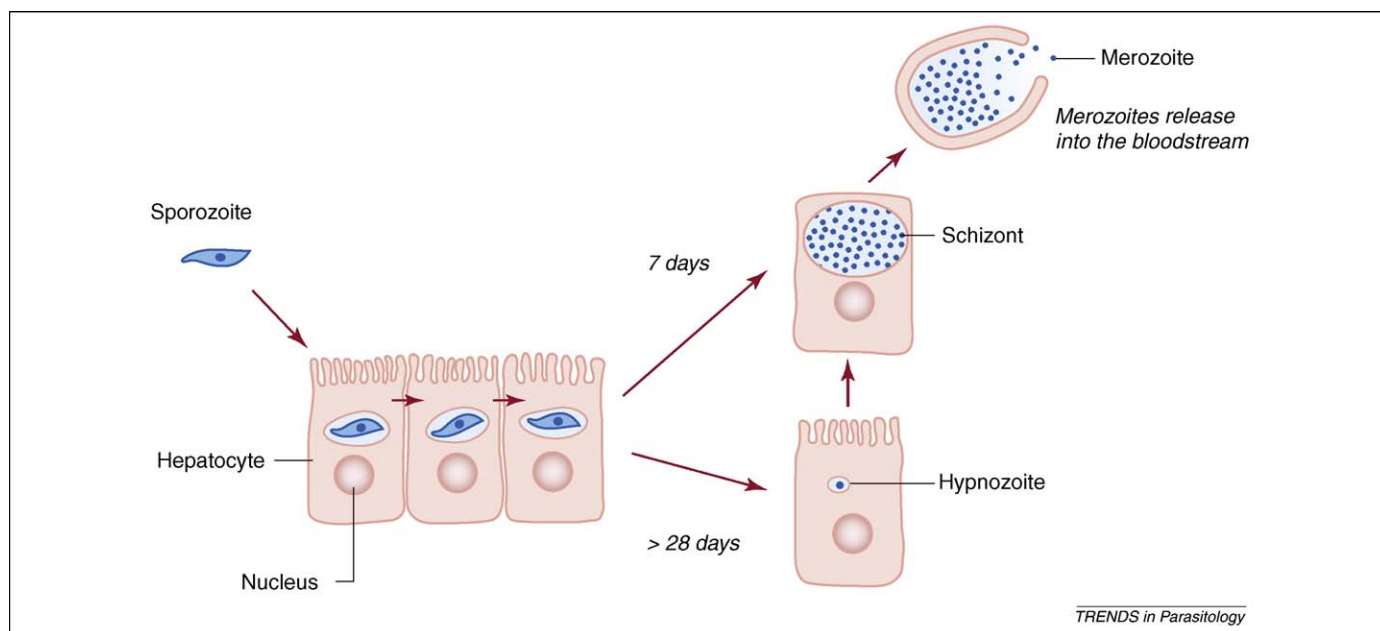


Figure 1. The liver stages of the life cycle of the species of malaria parasites. The human host is infected by *Plasmodium* sporozoites during the mosquito's bloodmeal. These are taken up by Kupffer cells in the liver and pass through several hepatocytes before establishing a stable infection. In the liver, the sporozoites of all species can replicate as exo-erythrocytic schizonts, forming several thousand merozoites that are eventually released into the plasma after 7–10 days. In the case of the human parasites *Plasmodium vivax* and *P. ovale*, and the primate parasite *P. cynomolgi*, the parasite can produce a dormant form, the hypnozoite. This can remain in the hepatocyte for a period from 17 days to over a year before being reactivated to develop as an exo-erythrocytic schizont, provoking a relapse of the disease.

is contraindicated in pregnancy. Improving on primaquine is not easy: even after 60 years of use its mechanism of action is not understood. Electron micrographs of extra-erythrocytic schizonts from primaquine-treated animals show altered parasite mitochondrial membranes [12], but there are no such studies of the hypnozoite to date. Primaquine efficacy and haemolytic toxicity require metabolism by hepatic cytochromes. A metabolite, 5-hydroxy primaquine, can be oxidised to the quinone imine (Box 1). This can then be attacked by cellular nucleophiles, such as thiols, and causes haemotoxicity *in vivo* [13] in rats by modifying erythrocyte membrane proteins [14]. This changes the rigidity of the erythrocytes, and these are removed by the spleen [15]. The metabolite also alters the cellular redox potential, potentially slowing the reduction of methaemoglobin, further exacerbated by the

lower glutathione concentrations found in G6PD-deficiency. Such metabolites are implicated as the therapeutically active species for other 8-aminoquinolines in other parasites, where redox cycling and mitochondrial membrane modification have been suggested [16]. A second potential anti-hypnozoite drug, RC-12, can similarly form a potential para quinone imine after double dealkylation and oxidation [17] (Box 1), and is effective in primate models of relapsing malaria. It is still not clear if the chemical processes that kill hypnozoites are the same as those causing haemolysis, and separation of these activities, if possible, would have obvious clinical advantages.

Table 1 summarises the current pipeline of drugs against relapse. The search for new analogues of primaquine has involved screening led by the US Army of several hundred molecules in primate models. The most promising

Table 1. The global portfolio of anti-relapse medicines directed against hypnozoites from *P. vivax* and *P. cynomolgi*^a

Active ingredient	Partner	Phase/status	Comments
Primaquine phosphate (Leoprime, Primacip)	Sanofi–Aventis Leo IPCA		8-Aminoquinoline: approved regimen of 22.5–30 primaquine base mg/day for 14 days. Some countries have shorter courses of treatment, because of poor compliance, but these have either unproven or very poor efficacy
Bulaquine (Ablaquin)	CDRI/Nicholas Piramal	Launched in India	Rapidly cleaved prodrug of primaquine; not available outside of India; 25 mg per day for 7 days suggested dose, but little evidence of improved safety
Tafenoquine	GlaxoSmithKline MMV	II	8-Aminoquinoline: (originally from Walter Reed Army Institute of Research) has been in phase III for malaria prophylaxis. Now in a safety study in G6PD deficient subjects for treatment, prior to a phase II/III pivotal study including pediatric use. Potential three-day course of treatment. Next generation 8-aminoquinolines without haemolytic potential are being investigated by a consortium led by WRAIR
Tinidazole	WRAIR	II	5-Nitroimidazole; experimental proof of concept study, repeating earlier Indian study using 2000 mg per day for five days
Inidazolidinone	WRAIR	Preclinical	Compounds show some activity in radical cure in primate models. The key challenge is to identify compounds that are both orally active and have radical cure
CEM-101	Cempra MMV	Preclinical	Macrolide antibiotic shown to be active against parasite, and to concentrate in the liver. Currently undergoing testing in a primate <i>P. cynomolgi</i> model. Other antibiotics, such as clindamycin, have been tested and shown to delay (but not prevent) relapse

^aData from Ref. [8]. Updated portfolio data are available at www.mmv.org.

Box 1. Can next generation 8-aminoquinolines be both safe and effective?

The mechanisms of action and toxicity of 8-aminoquinolines are not fully understood. First, the compounds need to be metabolically activated in the liver. The metabolites for primaquine [15] and tafenoquine [20] (Figure 1) generated *in vitro* include reactive quinone imines. Similar species can be predicted for the compound RC12 (Figure 1). These can react with nucleophiles at a variety of specific ring positions and with thiols and glutathione. In addition, infected erythrocytes and hepatocytes contain ferrous iron that facilitates the generation of oxygen radical species. This

presents a double challenge to designing safer next generation compounds. First, the reactive metabolites are short-lived and therefore difficult to confirm in patients. Second, the safety margin is dependent on the relative reactivity of the activated species towards the putative hypnozoite target compared with the erythrocyte targets. Increased selectivity could be a result of altering the physicochemical properties of the compound to make it more likely to accumulate in the liver, and less likely to accumulate in the erythrocyte.

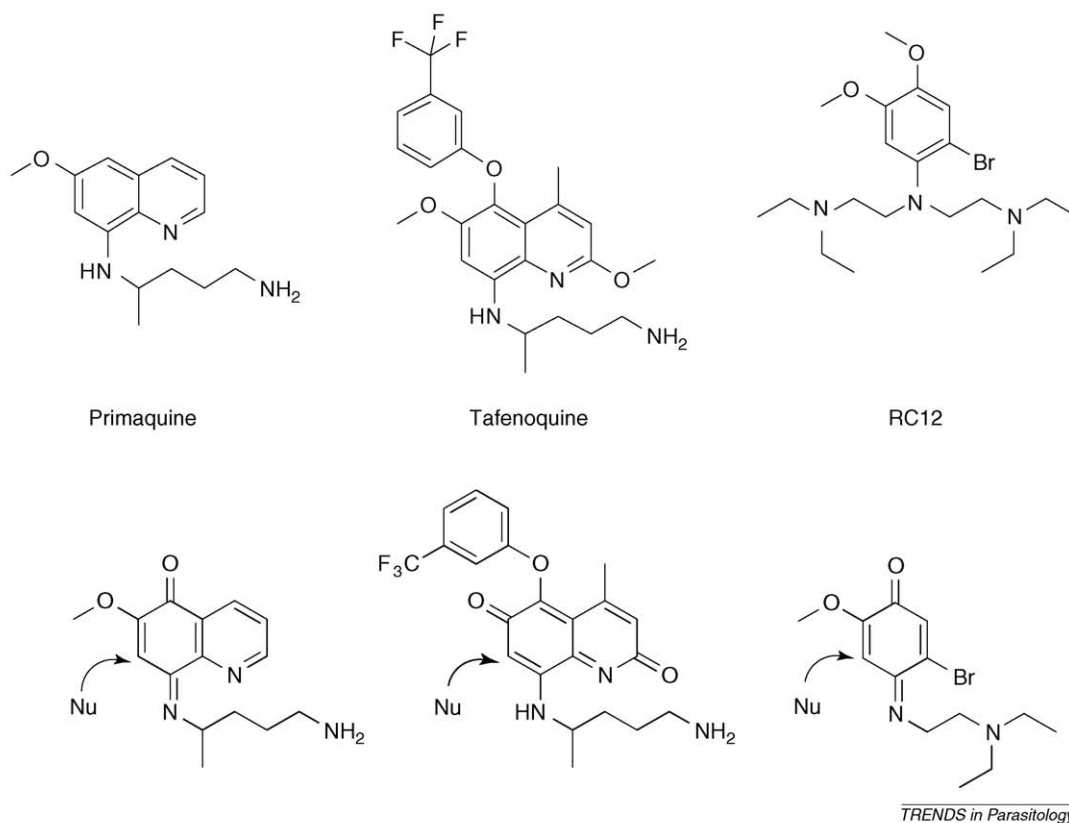


Figure 1. Structures of the anti-hypnozoite therapeutics primaquine, tafenoquine and RC12, showing potential quinone imine intermediates, and potential for attack by free cytosolic or protein thiols (Nu, nucleophile).

compound, tafenoquine (WR238605), has a much longer plasma half-life and has been taken into clinical development with three days of treatment with doses of 200 mg [18]. Tafenoquine can also be metabolised to generate quinone imines [19] and induces haemolysis in G6PD-deficient subjects. Preclinical studies in dogs showed that tafenoquine or the related NPC1161C cause less methaemoglobinemia [20]. Clinical studies are ongoing to determine the dosage safety window in G6PD-deficient patients and in children, with a view to developing tafenoquine as a loose combination with chloroquine for submission to the regulatory authorities in 2014. The final member of this family, bulaquine (CDRI 80/53, *N*-(3-acetyl-4,5-dihydro-2-furanyl)-*N*-(6-methoxy-8-quiniliny)-1-4-pentadiamine), is a rapidly converted pro-drug of primaquine, and protects against *P. cynomolgi* relapse in Rhesus monkeys. In clinical studies, bulaquine had similar efficacy to primaquine. No haemolysis was reported after treatment of three G6PD-deficient patients with 25 mg bulaquine, whereas 30 mg primaquine caused falls in haematocrit in four such

patients. However, the molar dose of bulaquine was lower and, because there was apparently no pharmacokinetic analysis, these results should be interpreted with caution [21].

Recent modifications of primaquine demonstrated that replacing the methoxy group with a bulky tertiary butyl retains the pharmacological activity, but reduces the haemolytic potential of primaquine in rodent models [22]. Another promising approach introduced a second nitrogen into the ring system at the 5-position, producing naphthyridine analogues of primaquine, that again retain blood-stage activity but with no haemolysis [23]. How reliably these models predict haemolysis in humans is not clear, but a validated murine model of G6PD deficiency is a key tool needed for the progression of compounds in humans.

Searching for new chemotypes – the cell assay as the gatekeeper

It is important to understand that, historically, the search for effective hypnozoiticide has been limited

almost exclusively to 8-aminoquinolines. The original 8-aminoquinoline, pamaquine, was first synthesised by Bayer in the 1920s and further developments have largely been restricted to 8-aminoquinoline analogues. A systematic search for new chemical families with activity against hypnozoites is long overdue. In the absence of a clear understanding of the molecular mechanisms the whole parasite is the best place to start such a search. Screening large collections of compounds against whole parasites is now a matter of course for *P. falciparum* [24], where 'high content' image analysis systems are used. Millions of compounds have been tested, with 0.1–0.25% able to inhibit parasites at sub-micromolar concentrations, and with no toxicity against human cell lines. This provides a 'treasure trove' of new starting points for medicinal chemistry programs. To target *P. vivax* relapse, cellular assays for three key steps of liver-stage biology are needed: hepatocyte infection, hypnozoite formation and reactivation to hepatic schizonts. The new technologies of image-based high-content screening can distinguish these activities. Assays already exist for liver stages of *Plasmodium* species that do not form hypnozoites: *P. falciparum*, *P. yoelii* and *P. berghei* [25]. The additional challenge of distinguishing the hypnozoite from the hepatic schizonts is not trivial (Figure 2), and

will require parasites that express fluorescent labels in the intra-hepatic stages. Screening millions of compounds against the hypnozoite is a far horizon, but being able to screen a highly selected few hundred is the first step.

There are several challenges to overcome before cellular screening for anti-relapse reagents becomes a reality. First, securing a supply of viable sporozoites: because sporozoites are produced inside infected mosquitoes there are many logistical challenges. The major challenge is to be able to maintain *P. vivax* in continuous *in vitro* culture so as to provide a clonal supply of parasites on which the mosquitoes can feed. If this problem can be solved then production of viable frozen stocks of sporozoites should be feasible, as has been shown by work towards attenuated sporozoite vaccines [26]. Second, stable and infectable hepatocyte lines are needed, because primary human liver cells are often of variable quality and lose their differentiation in culture. Human cell lines such as HepG2-A16 (Ref. [27]) or the more recent HC-04 have been used [28], but infection rates are low. Cell lines must remain differentiated in long-term culture and without overgrowing. Third, hypnozoite detection is difficult because of their relatively small size, and also because of the low infection rate – typically <0.1% for *P. vivax*. Cell lines could be

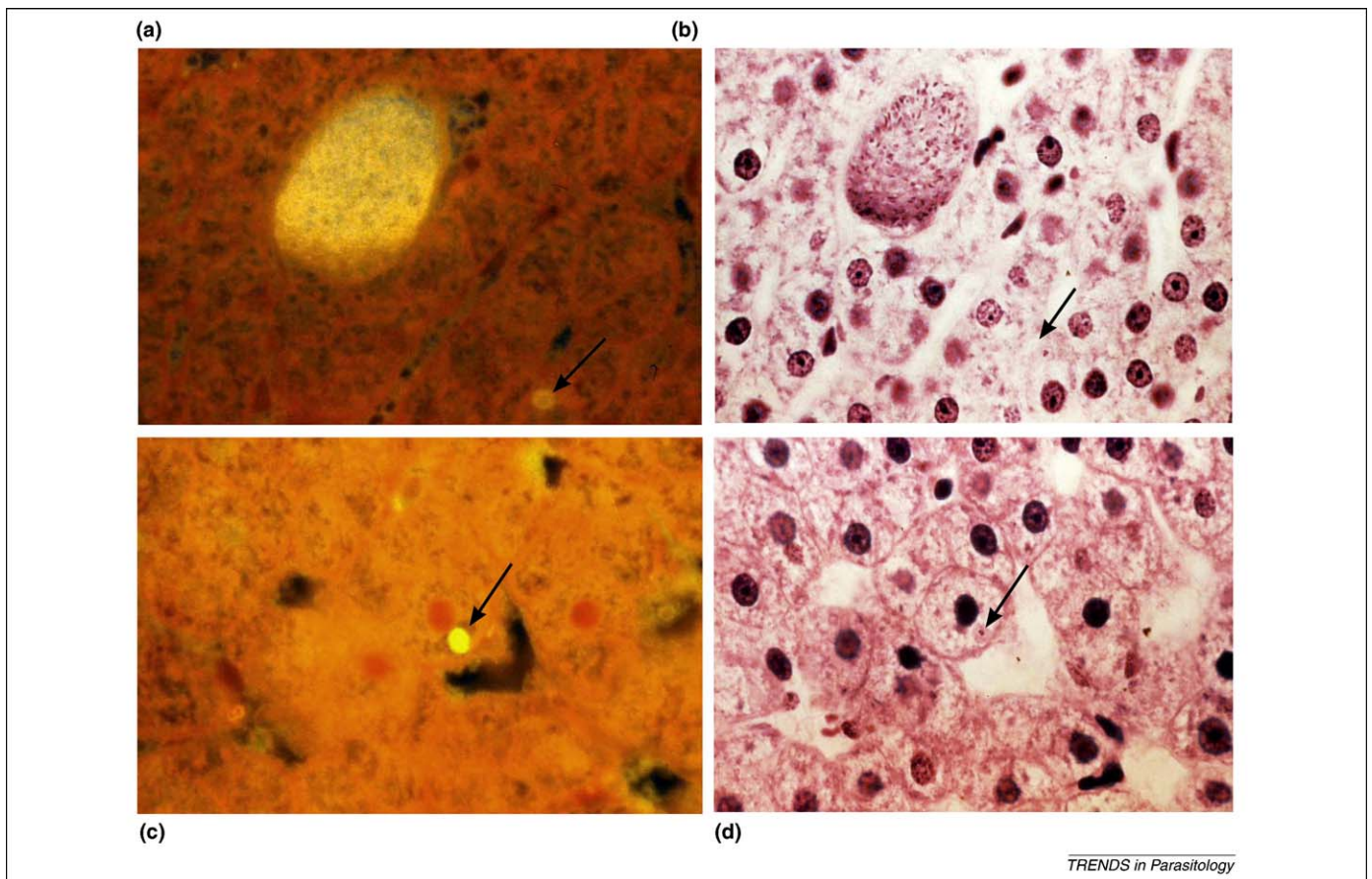


Figure 2. Hypnozoites: spotting the needle in the haystack. Comparison of the liver-stage infections of *P. cynomolgi bastianelli* [51]. The tissues were taken five days after sporozoite infection and clearly show the hepatic schizonts (size ~18 μ m) in the upper panels (a,b) compared with the much smaller hypnozoites (size ~4 μ m) in the lower panels (c,d) (marked by arrows). Note that there is some lightly stained material in the upper panels that corresponds with a weaker staining, and hypnozoite morphology. The images used specific immunofluorescence (a,c) using rabbit polyclonal anti sporozoite serum, and the same sections with subsequent counterstaining by Giemsa colophonium (b,d). Currently there are no biomarkers, and the hypnozoite is detected on the basis of parasite surface proteins and its distinctive morphology. Because infection rates are less than 0.1%, stable fluorescent-labeled parasites will be a key to developing a robust assay. Photographs reproduced by permission from Ref. [52] (also available from the collection of the late Dr Herman Zaiman, available from the Division of Infectious Disease & International Medicine, University of South Florida).

selected for higher infection rates: markers such as CD81 (Ref. [29]) and SR-BI (Ref. [30,31]) have been shown to be important for *P. falciparum* infection. It will be important to find their functional equivalents in *P. vivax* infection. In addition, because assay validation requires demonstration of primaquine inhibition of hypnozoites, and this requires metabolism of primaquine, it is crucial that hepatocyte lines similarly process the drug. Cell culture of hypnozoites would facilitate the identification of hypnozoite biomarkers. Currently, morphology, parasite staining and pharmacology are the only reliable characteristics, and it is important to distinguish between dormant parasites and those that are dying or apoptotic [32]. Such cultures will give key information on the biology of the reactivation process: the cellular triggers remain unknown, but stress signals in the patient and also mosquito factors have been proposed [7].

The role of liver architecture in hypnozoite formation and activation is not understood. Precision-cut hepatic slice cultures retain some architecture, and are used as model systems for hepatitis C [33], but are difficult to maintain for long periods. Alternatives, such as three-dimensional cultures [34] or differentiated embryonic stem cells [35], should be considered. Other cells could play a role in hypnozoite formation: in mice, sporozoites pass from the bloodstream to Kupffer cells before entering hepatocytes. These cells could also be targets: 8-aminoquinolines are active against the intracellular forms of *Leishmania* within macrophages.

An alternative cellular system is the infection of hepatocytes from Rhesus monkeys (*Maccaca mulatta*) with *P. cynomolgi bastianelli* [36]. This simian system provides a more reliable supply of both parasites and primary cells. The current frequencies of infection are higher than for *P. vivax* (0.1–1.0%) (C. Kocken, personal communication). This model was originally used in the screening that led to the discovery of the drug primaquine, and also in defining the activity of proguanil and cycloguanil against liver stage schizonts. Building on these original activities, 2-guanidine imidazolidinediones have been shown to be active against both the schizonts and hypnozoites *in vivo* [37].

Prioritising molecules for testing in anti-relapse assays

Once an assay is available, the first compounds to test are those already in clinical or preclinical development against the erythrocyte stages of *P. falciparum* malaria [8]. Second, compounds can be selected by 'orthologue' approaches: in cases were orthologous targets are present in both human and *Plasmodium*, and the human has already been a target in drug discovery, the approach involves testing a range of compounds including those structurally related to molecules active against the human target [38]. For this, no *a priori* assumption is needed about the relevance of the target in hypnozoite metabolism. A third approach is to use a selection of the thousands of compounds identified by high throughput screens of *P. falciparum* blood stages and known to be active at sub-micromolar concentrations [24]. These could be enriched by pre-screening against molecular targets before testing in a hypnozoite assay. They could also be enriched by selecting for compounds that concen-

trate in the liver. Although we have no information either on potential drug targets, nucleotide synthesis, energy supply and protein synthesis are all good candidates, as well as related cellular signalling cascades. The availability of the *P. vivax* genome sequence [39] will allow molecular targets to be prioritised on the basis of the likelihood that a small molecule inhibitor might be found [40].

In vivo models of mice, monkeys and men

A modern approach to the discovery and development of drugs would start from a simple cell assay, confirm the activity of compounds *in vivo* in an animal model, and then proceed via clinical proof of concept to full human clinical trials (see Box 2). The mainstay of preclinical assessment of anti-relapse chemotherapy for *P. vivax* in the past 60 years has been the infection of rhesus monkeys with *P. cynomolgi* [41]. Here, the objective should be to determine the plasma and hepatic concentrations required to prevent relapse, assess if such an exposure is safe, and to evaluate (from allometric scaling) whether the exposure is achievable in humans. In most historical studies pharmacokinetic data are available, making interpretation virtually impossible. Because these studies can cost as much as \$250000, validated cellular assays are clearly needed. Progressing directly to primate models is risky, although interesting data have been obtained for the lincomycin analogue antibiotic mirincamycin [42] that might control replication [43]. The nitroimidazole tinidazole has also been shown to have an effect in rhesus models, and is currently being tested in patients [44] (R.S. Miller, personal communication). For drugs that have already been tested in humans for other diseases, pharmacokinetic modelling and a validated cell model are all that is required for rapidly moving into clinical studies. Mice have been suggested as an alternative pharmacological system. Severe combined immunodeficient (SCID) mice with human erythrocytes are used to study the erythrocyte and liver stages of *P. falciparum* [45].

Determining clinical activity against hypnozoites is more complicated for several reasons. First, there is a need to distinguish relapse from reinfection. In *P. falciparum* therapeutic failures the parasite can reappear from expansion of residual blood parasites (recrudescence) or reinfection by a new mosquito. These possibilities cannot always be resolved by parasite genotyping – in *P. vivax* infection, genotyping cannot distinguish reinfection from reactivation of the hypnozoite (relapse). This is because, parasitaemias caused by relapse can be either clonal to the original parasitaemia or distinct genotypes [46–48], and our understanding of this biology and epidemiology is limited. To be sure that new parasitaemias are from reactivation of hypnozoites, subjects need to be moved to a place free from risk of reinfection. This restricts long-term anti-relapse studies to special populations such as repatriated soldiers and travellers. Second, distinguishing relapse delay from relapse prevention is important. When analogues of Pamaquine were tested in the fast relapsing tropical strains of *P. vivax* in North America in the 1940s and 1950s, most therapeutic failures occurred after 28 days, even though untreated controls relapsed earlier. For the slower-relapsing temperate zone strains, the follow-up period can be as long as 12 months.

Box 2. The challenges of discovering new chemical classes

The 8-aminoquinolines, first identified over 80 years ago, are the only class of compounds shown to be clinically effective against hypnozoites. If new classes of anti-relapse therapy are to be found, further

development will be needed at all stages in the drug discovery cascade (Figure 1).

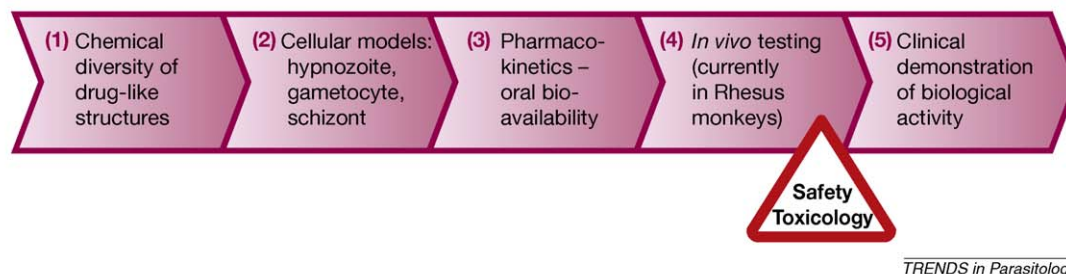


Figure 1. (1) Chemical diversity. Over four million compounds have been tested in assays for *Plasmodium falciparum*. For *P. vivax* hypnozoites, such throughputs will not be possible in the next 10 years. A smaller collection of a few thousand compounds could be made based on chemical diversity, known activity against the blood stages, or drug classes already in development that are activated by or concentrated in the liver. (2) Cellular models. The two available cell systems are *P. vivax*-infected primary human cells and *P. cynomolgi*-infected primate cells. Hypnozoites and hepatic schizonts can be distinguished by using antibody staining, and in the near future stable fluorescently labelled parasites. The challenge will be to maintain culture systems for sufficient time and to find ways to overcome the extremely low infection rates (<0.1%). Here the three-dimensional architecture of the hepatocyte could be an important factor. The big logistical problem is in supplying sporozoites. Current assays are labour intensive, but there is no reason why there should not be a future 'Moore's law' increase in throughput – perhaps doubling assay capacity every 18 months. These models are validated pharmacologically (response to 8-aminoquinolines, but not to chloroquine or antifoates) and microscopically (small 4 µm particles that do not develop over 10 days). The validity of these tests is determined by how well they predict responses in primates or humans. (3) Pharmacokinetics: before moving to disease models it is important to assess the bioavailability and exposure of the compounds. (4) Animal models. Rodent strains of malaria do not form hypnozoites, and studies *in vivo* are currently limited to primates. If these models are to be useful, then the plasma exposure needs to be measured and a dose-response study will need to be carried out. Full pharmacological characterisation of a single compound costs about \$250000. Primate models of relapse are close to the disease, but ultimate validation requires that several classes of compound active in these models are also shown to be clinically active. (5) Experimental medicine. With reliable cellular and pharmacokinetic data, the next step is pilot clinical studies. This is extremely cost-effective with medicines approved for human use. Clinical models are being generated that distinguish between relapse and reinfection. This means challenging volunteers, or working with patients who do not return to the infectious region. These studies can be relatively small because the 'gold standard' treatment primaquine is extremely effective.

Third, parasitaemic subjects would need a rapid-action blood schizontocidal co-therapy to rule out recrudescence as a source of the parasitaemia, adding another drug to the protocol. This co-therapy would need to be excreted rapidly so that it does not interfere with subsequent relapses.

There are additional hurdles caused by the partner drugs. Early studies suggest that primaquine requires concomitant administration of quinine or chloroquine to prevent relapse of Chesson *P. vivax* [49,50]. Neither quinine nor chloroquine exerts any overt effect upon relapse when given without primaquine or other 8-aminoquinolines, and so primaquine could require a companion drug to be fully effective against hypnozoites. It is also important to understand drug interactions and the pharmacokinetics of combinations in patients. This is illustrated by the example of the 9-aminoacridine atabrine and pamaquine. Atabrine was the only synthetic blood schizontocide available in 1942 and was rushed into wartime production, but caused increases in plasma concentrations of pamaquine up to tenfold, and extended the plasma half-life of primaquine by tenfold, resulting in toxicity. Late-stage trials will be required to study the paired blood schizontocide and hypnozoitocide therapies, with radical cure as the therapeutic endpoint and a close watch on safety. There is no consensus as to how to conduct such trials, but clearly the goal would be to prove superiority over primaquine and/or chloroquine in either safety or efficacy, or both.

Concluding remarks

The hypnozoitocide is clearly the largest scientific challenge facing the malaria-elimination agenda. Although preclinical studies are being carried out on *P. vivax* vaccines [51] it is clear that drugs remain a major factor in disease control; the

challenge will be to provide new drugs that will enable this role to continue and to increase. Primaquine remains the mainstay of anti-hypnozoite therapy but suffers the combined drawbacks of inconvenient dosage (leading to low compliance) and the need for clinical and laboratory screening for safe use. A safer and more easily administered drug is required for application across vast endemic ranges where medical supervision could be lacking or absent. In addition, in the absence of contemporary evidence of efficacy, it is possible that resistance to primaquine already occurs widely. In brief, the only weapon that is available against relapse is not safe or practical in the real world, and it is uncertain whether it works at all as prescribed. This represents a gaping deficiency in our aspiration to eliminate malaria. Nonetheless, the long relative neglect of vivax malaria relapse appears to be acknowledged, and work is beginning to address such gaps. The commitment to fund exploration of the basic biology of this parasite by interdisciplinary collaboration among malariologists, clinicians, chemists and engineers must be made to build the depth of understanding that will yield new drugs active against the hypnozoites.

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References

- Price, R.N. *et al.* (2007) Vivax malaria: neglected and not benign. *Am. J. Trop. Med. Hyg.* 77 (Suppl 6), 79–87
- Hay, S.I. *et al.* (2004) The global distribution and population at risk of malaria: past, present and future. *Lancet Infect. Dis.* 4, 327–336

- 3 Hemmer, C.J. *et al.* (2006) Stronger host response per parasitized erythrocyte in *Plasmodium vivax* or *ovale*, than in *Plasmodium falciparum* malaria. *Trop. Med. Int. Health* 11, 817–823
- 4 Tijtra, E. *et al.* (2008) Multi-drug resistant *Plasmodium vivax* associated with severe malaria in children: a prospective study in Papua New Guinea. *PLoS Med.* 5, e128
- 5 Cogswell, F.B. (1992) The hypnozoite and relapse in primate malaria. *Clin. Microbiol. Rev.* 5, 26–35
- 6 Dao, N.V. *et al.* (2007) *Vivax* malaria : preliminary observations following a shorter course of treatment with artesunate plus primaquine. *Trans. R. Soc. Trop. Med. Hyg.* 101, 534–539
- 7 Huldén, L. *et al.* (2008) Natural relapses in *vivax* malaria induced by *Anopheles* mosquitoes. *Malar. J.* 7, 64
- 8 Oliaro, P. and Wells, T.N.C. (2009) The global portfolio of new antimalarial medicines under development. *Clin. Pharmacol. Ther.* 85, 584–595
- 9 Baird, J.K. and Rieckmann, K.H. (2003) Can primaquine therapy for *vivax* malaria be improved? *Trends Parasitol.* 19, 115–120
- 10 Goller, J.L. *et al.* (2007) Regional difference in the response of *Plasmodium vivax* malaria to primaquine as anti-relapse therapy. *Am. J. Trop. Med. Hyg.* 76, 203–207
- 11 Beutler, E. *et al.* (2007) Glucose-6-phosphate dehydrogenase deficiency and antimalarial drug development. *Am. J. Trop. Med. Hyg.* 77, 779–789
- 12 Boulard, Y. *et al.* (1983) The chemotherapy of rodent malaria XXXIV. *Ann. Trop. Med. Parasitol.* 77, 555–568
- 13 Bowman, Z.S. *et al.* (2004) Primaquine-induced hemolytic anaemia: susceptibility of normal versus glutathione-depleted rat erythrocytes to 5-hydroxyprimaquine. *J. Pharmacol. Exp. Ther.* 309, 79–85
- 14 Bowman, Z.S. *et al.* (2005) Primaquine-induced hemolytic anaemia: role of membrane lipid peroxidation and cytoskeletal protein alterations in the hemotoxicity of 5-hydroxy primaquine. *J. Pharmacol. Exp. Ther.* 314, 838–845
- 15 Bowman, Z.S. *et al.* (2005) Primaquine-induced hemolytic anaemia; role of splenic macrophages in the fate of 5-hydroxyprimaquine treated rat erythrocytes. *J. Pharmacol. Exp. Ther.* 315, 980–986
- 16 Tekwani, B.L. and Walker, L.A. (2006) 8-Aminoquinolines: future role as antiprotozoal drugs. *Curr. Opin. Infect. Dis.* 19, 623–631
- 17 Schmidt, L.H. *et al.* (1985) Antimalarial activities and subacute toxicology of RC-12 a 4-amino substituted pyrocatachol. *Antimicrob. Agents Chemother.* 28, 612–625
- 18 Elmes, N.J. *et al.* (2008) The efficacy and tolerability of three different regimens of tafenoquine versus primaquine for post-exposure prophylaxis of *Plasmodium vivax* malaria in the Southwest Pacific. *Trans. R. Soc. Trop. Med. Hyg.* 102, 1095–1101
- 19 Idowu, O.R. *et al.* (1995) Metabolism of a candidate 8-aminoquinoline antimalarial agent WR238605 by rat liver microsomes. *Drug Metab. Dispos.* 23, 1–17
- 20 Nanayakkara, N.P. *et al.* (2008) Antiparasitic activities and toxicities of individual enantiomers of the 8-aminoquinoline 8-[(4-amino-1-methylbutyl)aminol]-6-methoxy-4-methyl-5-[3,4-dichlorophenoxy]quinoline sulphate. *Antimicrob. Agents Chemother.* 52, 2130–2137
- 21 Krudsdod, S. *et al.* (2006) Safety and tolerability of elubaquine (Bulaquine, CDRI 80/53) for the treatment of *Plasmodium vivax* malaria in Thailand. *Korean J. Parasitol.* 44, 221–228
- 22 Jain, M. *et al.* (2004) Discovery of a bulky 2-*tert*-butyl group containing primaquine analogue that exhibits potent blood-schizonticidal antimalarial activity and complete elimination of methemoglobin toxicity. *J. Med. Chem.* 47, 285–287
- 23 Zhu, S. *et al.* (2007) Synthesis and evaluation of naphthyridine compounds as antimalarial agents. *Bioorg. Med. Chem. Lett.* 17, 6101–6106
- 24 Plouffe, D. *et al.* (2008) *In silico* activity profiling reveals the mechanism of action of antimalarials discovered in a high-throughput screen. *Proc. Natl Acad. Sci. U. S. A.* 105, 9059–9064
- 25 Gego, A. *et al.* (2006) New approach for high-throughput screening of drug activity on *Plasmodium* liver stages. *Antimicrob. Agents Chemother.* 50, 1586–1589
- 26 Luke, T.C. and Hoffman, S.L. (2003) Rationale and plans for developing a non-replicating, metabolically active, radiation-attenuated *Plasmodium falciparum* sporozoite vaccine. *J. Exp. Biol.* 206, 3803–3808
- 27 Hollingdale, M.R. *et al.* (1986) *In vitro* culture of exoerythrocytic parasites of the North Korean strain of *Plasmodium vivax* in hepatoma cells. *Am. J. Trop. Med. Hyg.* 35, 275–276
- 28 Sattabongkot, J. *et al.* (2006) Establishment of a human hepatocyte line that supports *in vitro* development of the malaria parasites *Plasmodium falciparum* and *P. vivax*. *Am. J. Trop. Med. Hyg.* 74, 708–715
- 29 Silvie, O. *et al.* (2006) Expression of human CD81 differently affects host cell susceptibility to malaria sporozoites depending on the *Plasmodium* species. *Cell Microbiol.* 8, 1134–1146
- 30 Yalaoui, S. *et al.* (2008) Scavenger receptor BI boosts hepatocyte permissiveness to *Plasmodium* infection. *Cell Host Microbe.* 4, 283–292
- 31 Rodrigues, C.D. *et al.* (2008) Host scavenger receptor SR-BI plays a dual role in the establishment of malaria parasite liver infection. *Cell Host Microbe* 4, 271–282
- 32 Undomsangpet, R. *et al.* (2008) Cultivation of *Plasmodium vivax*. *Trends Parasitol.* 24, 85–88
- 33 Chang, M.L. *et al.* (2009) A liver slice culture based *ex vivo* assay to predict the outcome of antiviral therapy for chronic hepatitis C. *J. Viral Hepat.* 16, 359–366
- 34 Pamploni, F. *et al.* (2007) The third dimension bridges the gap between cell culture and live tissue. *Nat. Rev. Mol. Cell Biol.* 8, 839–845
- 35 Preynat-Seauve, O. *et al.* (2009) Development of human nervous tissue upon differentiation of embryonic stem cells in three dimensional culture. *Stem Cells* 27, 509–520
- 36 Fisk, T.L. *et al.* (1989) *In vitro* activity of antimalarial compounds on the exoerythrocytic stages of *Plasmodium cynomolgi* and *P. knowlesi*. *Am. J. Trop. Med. Hyg.* 40, 235–239
- 37 Guan, J. *et al.* (2007) Malarial causal prophylactic activity of imidazolidinedione derivatives. *J. Med. Chem.* 50, 6226–6231
- 38 Grundner, C. *et al.* (2007) Structural basis for selective inhibition of *Mycobacterium tuberculosis* protein tyrosine phosphatase PtpB. *Structure* 15, 499–509
- 39 Carlton, J.M. *et al.* (2008) Comparative genomics of the neglected malaria parasite *Plasmodium vivax*. *Nature* 455, 757–763
- 40 Agüero, F. *et al.* (2008) Genomic-scale prioritization of drug targets: the TDR Targets database. *Nat. Rev. Drug Discov.* 7, 900–907
- 41 Schmidt, L.H. (1983) Relationships between chemical structures of 8-aminoquinolines and their capacities for radical cure of infections with *Plasmodium cynomolgi* in Rhesus monkeys. *Antimicrob. Agents Chemother.* 24, 615–652
- 42 Schmidt, L.H. (1985) Enhancement of the curative activity of primaquine by concomitant administration of mirincamycin. *Antimicrob. Agents Chemother.* 27, 151–157
- 43 Dahl, E.L. and Rosenthal, P.J. (2008) Apicoplast translation, transcription and genome replication: targets for antimalarial antibiotics. *Trends Parasitol.* 24, 279–284
- 44 Sarma, P.S.A. (1988) Tinidazole a new drug in the treatment of *Plasmodium vivax* malaria. *Current Therapeutic Research* 43, 954–956
- 45 Morosan, A. *et al.* (2006) Liver stage development of *Plasmodium falciparum* in a humanised mouse model. *J. Infect. Dis.* 193, 996–1004
- 46 Imwong, M. *et al.* (2007) Relapses of *Plasmodium vivax* infection usually result from activation of heterologous hypnozoites. *J. Infect. Dis.* 195, 927–933
- 47 Chen, N. *et al.* (2007) Relapses of *Plasmodium vivax* infection result from clonal hypnozoites activated at predetermined intervals. *J. Infect. Dis.* 195, 934–941
- 48 Collins, W.E. (2007) Further understanding the nature of relapse of *Plasmodium vivax* infection. *J. Infect. Dis.* 195, 919–920
- 49 Alving, A.S. *et al.* (1955) Potentiation of the curative action of primaquine in *vivax* malaria by quinine and chloroquine. *J. Lab. Clin. Med.* 46, 301–306
- 50 Edgcomb, J.H. *et al.* (1950) Primaquine SN13272: a new curative agent in *vivax* malaria a preliminary report. *J. Natl. Malar. Soc.* 9, 285–292
- 51 Galkinski, M.R. and Barnwell, J.W. (2008) *Plasmodium vivax*: who cares? *Malar. J.* 7 (Suppl 1), S9
- 52 Krotowski, W.A. *et al.* (1982) Observations on early and late post-sporozoite tissue stages in primate malaria. *Am. J. Trop. Med. Hyg.* 31, 211–225