Commentary

Chemotherapeutic hope on the horizon for *Plasmodium vivax* malaria?

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Plasmodium vivax malaria is less well reported and studied than the more virulent *Plasmodium falciparum* malaria, but it remains a cause of tremendous morbidity (1). Until recently, it was widely believed that this species was not susceptible to the dihydrofolate reductase (DHFR) inhibitors successfully utilized against *P. falciparum* such as pyrimethamine. However, several recent articles have challenged this concept (2–6). An article by Hastings and Sibley (7) in a recent issue of PNAS adds to this work and brings additional perspectives on the future potential role of antifolates for *P. vivax*.

They use an innovative approach involving the complementation of *Saccharomyces cerevisiae* strains that lack endogenous DHFR. Using this approach they confirm other data (4) that a class of triazine DHFR inhibitors, based on WR99210, a metabolite of a potential biguanide prodrug, designed to be active against pyrimethamine-resistant mutants of *P. falciparum*, is also active against *P. vivax*. They go on to propose that pyrimethamine and WR99210 exert opposing selection pressures on *P. vivax* parasites and that they therefore might form a basis for the clinical use of two DHFR inhibitors in combination to reduce the selection of resistance to both compounds.

Hastings and Sibley propose that pyrimethamine and WR99210 exert opposing selection pressures on *P. vivax* parasites.

This commentary covers four main elements: (*i*) a background to *P. vivax* malaria; (*ii*) *P. vivax* resistance to existing DHFR inhibitors; (*iii*) a comparison of Hopkins and Sibley's work with other recent studies; and (*iv*) some future perspectives including an assessment of whether multiple DHFR inhibitors might find clinical use in combination.

P. vivax Malaria

Of the four species of malaria that can infect humans, most experimental attention is directed toward *P. falciparum* (8, 9). It is the most abundant species and causes most of the morbidity associated with the disease: between 300 and 500 million cases annually. In addition, its virulence and ability to sequester in the microvasculature can cause severe anemia and generate severe manifestations of the disease, such as cerebral malaria, which are major causes behind the more than 1 million malaria deaths each year. Most of these deaths occur in sub-Saharan Africa in young children, with pregnant women also forming a particularly vulnerable risk group.

However, a second species of malaria, *P. vivax*, also has a major adverse impact on global health (1), accounting for up to 80 million clinical cases annually. It is responsible for over 50% of malaria outside Africa, notably in Southeast Asia and Central and South America, and has a particularly strong impact on the Indian subcontinent. It also accounts for ≈10% of cases in Eastern and Southern Africa but has only limited prevalence in West Africa because of the presence there of Duffy-negative blood-group variants that limit erythrocyte invasion by *P. vivax* (10).

The dynamics of the symptoms associated with *P. vivax* malaria can be found in ref. 11, which demonstrates that peaks of very high fever correlate with high tumor necrosis factor levels. Such sharply defined clinical paroxysms are repeated every 48 h and may go on for several weeks if the patient is not treated with blood schizonticides. The situation is complicated further by the fact that *P. vivax* parasites can remain dormant in the liver stage as hypnozoites. Thus, even if the blood stage of *P. vivax* infection is cleared, reactivation of these liver forms can result in relapses within a few months. This results in recurrent malaria that can impact an individual's health severely, especially if not treated with primaquine, the only drug that is effective against *P. vivax* hypnozoites and is widely available.

An idea of the potential impact of *P. vivax* malaria, should adequate chemotherapy and other control measures cease to be available, is provided by historical reports of what is presumed to have been *P. vivax* malaria, common in Western Europe in the early 19th century (see ref. 1 and references therein). One memorable quote comes from the British physician John Macculloch, who in 1827 described the typical inhabitant of the malarious parts of Europe as "the ghost of a man, a sufferer from his cradle to his grave; aged even in childhood and laying down in misery that life which was but one disease." The average life expectancy in such areas could be as little as 20–25 years of age (1), and its impact on communities is vividly illustrated in a painting by Maurice Sand in the mid 19th century entitled "The Ghost of the Swamp."

P. vivax Resistance to Existing DHFR Inhibitors

For the last 50 years or so, the impact of *P. vivax* malaria, although significant, has been limited because of the effectiveness of the 4-aminoquinoline, chloroquine, in treating the blood stage of the disease and the ability of primaquine, an 8-aminoquinoline, to clear hypnozoites from the liver. However, the impact of drug resistance on *P. falciparum* chemotherapy that now is well documented (9) and is limiting the effectiveness of standard drugs such as chloroquine and antifolates such as sulfadoxine–pyrimethamine is beginning to be observed also for *P. vivax*. The urgent need to undertake appropriate biological research, medicinal chemistry, and clinical research to discover and develop new drugs for *P. falciparum* (9) is now also becoming a priority for *P. vivax*.

When seeking to develop new drugs to treat malaria, the optimal goal is to have drugs that are efficacious against both *P. falciparum* and *P. vivax*. This is because in areas where both species exist, especially in areas where health systems are underresourced, it is often not possible to distinguish between the two species in an initial diagnosis. Furthermore, mixed infections can occur in areas of high trans-

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mission, and for these infections drugs are needed that clear both species from the circulation simultaneously. Chloroquine, being active against the blood stages of both malaria species, was an ideal drug in this regard, but evidence of mounting chloroquine resistance in both *P. falciparum* (9) and *P. vivax* (1) means that new drugs will have to be discovered and developed against both species.

Historically for *P. falciparum*, the back-up drug if chloroquine is inactive has been the antifolate sulfadoxine–pyrimethamine. Until recently it was assumed that antifolates, particularly components such as pyrimethamine, which act by inhibiting DHFR, were inactive against *P. vivax* malaria. This assumption was based on early clinical studies that failed to show efficacy of antifolates against *P. vivax* malaria (12). The implications of this for future drug discovery was that these data suggested the active sites of the *P. falciparum* and *P. vivax* enzymes might differ significantly, making it difficult to obtain inhibitors active against the enzymes of both species.

It now transpires that these studies took place in regions previously exposed to antifolates for the treatment of *P. falciparum* malaria, and resistant strains of *P. vivax* had already had a chance to develop. The successful use of proguanil, an early antifolate, after its initial deployment in Malaysia in 1947 lends credence to this hypothesis (13, 14). This interpretation has been supported further by recent analyses documenting point mutations in the *P. vivax* DHFR gene that are analogous to those found in DHFR genes from pyrimethamine-resistant *P. falciparum* strains (2–6). The existence of such mutants correlates geographically with antifolate use against *P. falciparum* (2). The most significant of these mutations seems to be a double mutation (S58R and S117N) corresponding to the mutations C59R and S108N observed in *P. falciparum* DHFR (2, 5).

These data radically alter our perception of DHFR as a drug target for *P. vivax*, because it suggests that inhibitors can be identified that are efficacious against the two most important malaria species, making them more valuable components of antimalarial drugs.

Comparison of Hastings and Sibley's Work (7) with Other Recent Studies

The formal demonstration that the mutations observed in *P. vivax* DHFR are the basis for the lack of efficacy of antifolates against *P. vivax* in the clinic has proved more difficult for *P. vivax* than *P. falciparum*. For example, it is not possible yet to culture *P. vivax* parasites to easily determine their drug susceptibility. Also, there are no small animal models of the infection. Hastings and Sibley took the elegant approach of expressing different variants of the *P. vivax* DHFR gene in a yeast strain that lacks its own DHFR (7). In essence, this establishes a yeast surrogate for assessing inhibition of *P. vivax* DHFR and its impact on cell growth. By using this model, the effect of a large number of different mutations on inhibitor activity could be assessed.

Table 1. Comparison of relative resistance of mutant *P. vivax* DHFR enzymes to different drugs by different investigators

Allele and drug evaluated	Hopkins and Sibley (7), relative resistance*	Leartsakulpanich *et al*. (4), relative resistance†	Tahar *et al*. (3), relative resistance‡
Pyrimethamine			
Wild type	1	1	1
S58R	2	12	
S117N	87	4,192	
S58R/S117N	460	325	15
Chlorcycloguanil			
Wild type	1		
S58R	5		
S117N	14		
S58R/S117N	350		
WR 99210			
Wild type	1	1	
S58R	7	2	
S117N	0.1	9	
S58R/S117N	1	1	

*Hopkins and Sibley (7) data determined by IC_{50} values determined against recombinant yeast.
†Leartsakulpanich *et al*. (4) data determined by K_i values determined against functional recombinant enzyme, purified from bacteria, that required no refolding.
‡Tahar *et al*. (3) data determined by K_i values determined against functional recombinant enzyme, purified from bacteria, that required extraction from inclusion bodies and refolding.

Other recent studies (3, 4) have attempted to address the issue of *P. vivax* DHFR mutant susceptibility to antifolates through the bacterial gene expression, purification, and evaluation of functional mutant *P. vivax* DHFRs, which also has included an estimation of K_i values with different inhibitors. Different mutant enzymes were assessed in the different studies, but there were some in common. Hastings and Sibley (7) evaluated 10 mutants against wild type, with a strong focus on double and triple mutants. Leartsakulpanich *et al.* (4) assessed five single mutant forms and the double mutant (S58R/S117N) against wild type, whereas Tahar *et al.* (3) just assessed the double mutant against wild type. Table 1 attempts to collate and compare some of these data by focusing on the relative resistance observed for the mutants, as measured by the different investigators using their different methodologies.

For simplicity of comparison, Table 1 has been limited to the wild-type strain, the S58R/S117N double mutant, and the single mutants that compose the double mutant. Hastings and Sibley in particular look at many more mutants in their analysis. The drugs under comparison in Table 1 are limited to (*i*) pyrimethamine, a 2,4-diaminopyrimidine that is the most widely used antimalarial DHFR inhibitor and is a key component of the drug sulfadoxine–pyrimethamine, (*ii*) chlorcycloguanil, a triazine DHFR inhibitor and the active metabolite of chlorproguanil, which is a key component of a fixed dose combination of chlorproguanil-dapsone (or Lapdap), which has undergone phase-three studies in a partnership involving GlaxoSmithKline and WHO/TDR and is about to be submitted for regulatory approval (15), and (*iii*) WR99210, a phenoxypropoxydiaminotriazine, a member of a family of DHFR inhibitors that are generated as metabolites from the corresponding biguanide, some of which are being considered for development (16).

There are several main conclusions from this comparative analysis that are reinforced by the paper of Hastings and Sibley (7). First, the mutations from wild-type DHFR seem to confer resistance to pyrimethamine on *P. vivax*. This result suggests that the lack of efficacy of current antifolates in the field is due primarily to resistance rather than an innate lack of efficacy against the wild-type *P. vivax* DHFR enzyme. The data for pyrimethamine compare quite well between Hastings and Sibley and Leartsakulpanich *et al.*, despite the widely different means by which the results were generated. A major discrepancy occurs in the data related to the S117N mutant. However, this enzyme had a very low k_{cat}/K_m value in the work of Leartsakulpanich *et al.*, which may indicate that in their study it is not reflecting the properties of the native mutant enzyme.

Second, WR99210 retains its efficacy against the mutant pyrimethamine-resistant *P. vivax* enzymes. Once again, the

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correlation between the work of Hastings and Sibley and Leartsakulpanich *et al.* compares well with the exception of the S117N single mutant. It therefore seems that biguanides capable of metabolism to generate analogs of WR99210 could be developed as components of antifolate drugs for treatment of both drug-resistant *P. falciparum* and drug-resistant *P. vivax*.

Interestingly, Hastings and Sibley also looked at the effect of *P. vivax* DHFR mutations on the efficacy of chlorcycloguanil. This compound lacked significant efficacy against the *P. vivax* pyrimethamine-resistant double mutants, which contrasts with the results obtained when chlorcycloguanil was evaluated against pyrimethamine-resistant mutants of *P. falciparum*. Against *P. falciparum*, it retains good efficacy against all but the most highly pyrimethamine-resistant tetravalent mutants (15). Based on these data, chlorproguanil-dapsone may have a more limited impact against *P. vivax* than against *P. falciparum* malaria. Clinical trials are needed to evaluate this hypothesis.

Future Outlook

Taking the results of all these papers together, there are strong grounds for hope that improved DHFR inhibitors could be useful agents against both *P. falciparum* and *P. vivax* malaria. Work around biguanide prodrugs of WR99210 and other triazine series offers the possibility of a short-term success in identifying a compound suitable for development. Discussions are under way currently between Jacobus Pharmaceutical Company and the Medicines for Malaria Venture (www.mmv.org) to select and develop one such molecule. In addition, the recent crystallization and solution of the crystal structures of both *P. falciparum* and *P. vivax* DHFR crystal structures (Y. Yuthavong, personal communication) undoubtedly will provide improved opportunities for rational drug design of other new inhibitors.

It is worth commenting on the proposal (7) that because of evidence indicating that pyrimethamine and WR99210 exert opposing selection on *P. vivax* DHFR, the combination of pyrimethamine and the corresponding biguanide in antimalarial treatment could be desirable to limit the generation of further drug resistance. Similar proposals have gained acceptance in other fields of chemotherapy such as HIV/AIDS, where cocktails of protease inhibitors are used. However, the approach would probably stand a better chance of success in malaria if two inhibitors exerting different selection pressures were being introduced into clinical use at around the same time, ensuring that there was limited preexisting selection pressure against either inhibitor. Resistance to pyrimethamine is so widespread already, especially in the case of *P. falciparum*, that its future use in combination with the biguanide that generates WR99210 seems to be an unlikely scenario.

This conclusion is reinforced further by the fact that even if the clinical development of such a biguanide analog proceeds smoothly, it will take 5–6 years. *P. vivax* resistance to chloroquine and sulfadoxine-pyrimethamine is likely to worsen during this period. In the short term, other drugs, probably in combination with artemisinin derivatives (9), will gain extended use to treat blood-stage *P. vivax* malaria.

The concept of looking for new 2,4-diaminopyrimidine DHFR inhibitors, analogous to pyrimethamine, that are both active against pyrimethamine-resistant strains and can complement the triazine analogs of WR99210 as part of a combination treatment may be more worthy of investigation. However, such a treatment would have to compete with a range of other potential combination approaches (9).

Finally, the ability of parasites to generate resistance to new drugs should not be underestimated. It is particularly notable that WR99210-resistant phenotypes have been generated in bacteria (*Escherichia coli*) carrying the *P. falciparum* DHFR gene after they were mutated at random and selected for resistance (17). This bacterial model has picked up known mutations in the past correctly and might be a good prediction of how the malarial parasite will respond to the new biguanides.

The work of drug discovery and development is long-term and needs to be sustained over many years to guarantee success. Many new opportunities for antimalarial drug discovery and development are being generated by genomics and other technical developments (9). However, it is likely that inhibition of the folate pathway, a tried and tested drug target, will remain a major cornerstone of malaria research and development activities for many years to come. Equally important for the future of *P. vivax* treatments, however, is the urgent need to identify drugs that can improve on primaquine and cure patients of the liver hypnozoite forms that lead to relapses.

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