

# Virulence and drug resistance in malaria parasites

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**Virulence and drug resistance are traits that pathogens can acquire independently, albeit these traits can influence each other. A recent publication has reported on the co-evolution of virulence and pyrimethamine resistance in malaria parasites. Here, we discuss this finding in the context of the folate biosynthesis pathway and explain how mutational changes in this pathway can affect both parasite replication rates and the development of drug resistance.**

## The dogma: drug resistance incurs a biological cost

Resistance to first- and second-line antimalarial drugs, such as the quinoline derivative chloroquine and the folate antagonist pyrimethamine, has repeatedly set back efforts to control malaria, an infectious disease that causes an estimated 515 million clinical cases and 1.0 million deaths annually [1]. Although efforts to develop novel antimalarial drugs are ongoing, the drug-discovery process is costly and time consuming. Some studies have raised hopes that drugs might be reintroduced in the clinics after a period of suspended use. This belief is based primarily on previous observations that, while drug resistance mechanisms confer a selective advantage under drug pressure, they can bestow upon the parasite a biological disadvantage under drug-free conditions. For example, in several countries where chloroquine (CQ) use has been suspended because of widespread resistance, sensitive *P. falciparum* strains have re-emerged and are expanding [2–5]. *In vitro* studies confirm these epidemiological data by showing that genetic traits associated with CQ resistance can incur a fitness cost to *P. falciparum* in the absence of drug pressure [6]. Similar data exist for pyrimethamine and the rodent malaria parasites *P. berghei* and *P. chabaudi*. For example, pyrimethamine resistant *P. berghei* strains seem to have a transmission disadvantage under drug-free conditions as compared to pyrimethamine sensitive strains from the same genetic background [7]. Similarly, a pyrimethamine-sensitive *P. chabaudi* strain outgrew a pyrimethamine-resistant isogenic strain in mice in two out of three experiments [8]. These data are explained by resistant parasites carrying mutational changes in key factors that, while allowing the parasite to evade the deleterious effects of the drug, compromise their natural physiological function(s).

## The challenge: virulent parasites are less susceptible to pyrimethamine

Common to all these studies is that they follow the same experimental design. First, isogenic parasites with

different drug responses were obtained and then analyzed for their fitness. In a recent publication, Schneider *et al.* [9] investigated the reverse scenario and made an unexpected observation.

Schneider *et al.* took a virulent and an avirulent *P. chabaudi* strain from the same genetic background [10] and infected mice with them. The virulent strain multiplied faster and developed a patent blood-stage infection much earlier than the avirulent strain. Moreover, it grew to a much higher parasitemia. As to the host, the mice infected with the virulent strain had a lower body weight and a lower red blood cell count than those infected with the avirulent strain. Thus, the virulent strain was far better accommodated to blood-stage development in the mouse. It was fitter and more virulent than the avirulent strain from which it was derived.

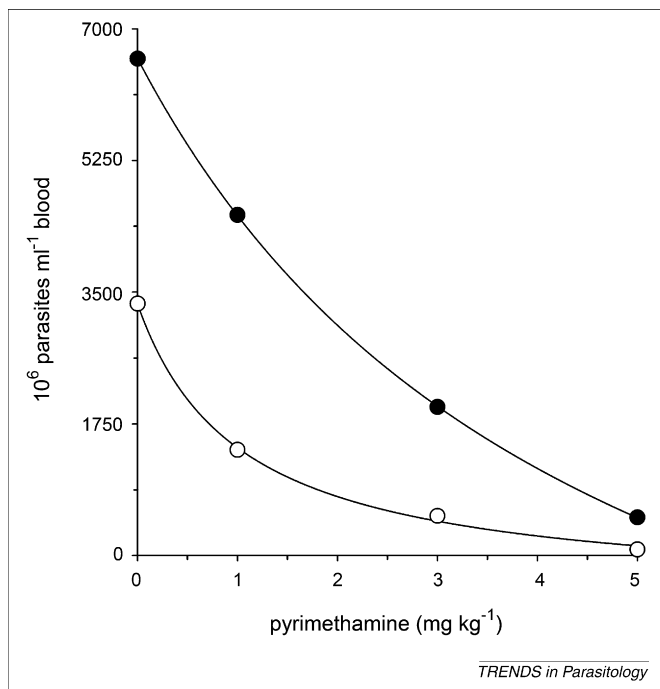
Now Schneider *et al.* treated the infections with different concentrations of pyrimethamine. Again, the virulent strain multiplied within the host faster than the avirulent strain. Figure 1 shows a replot of some data presented by Schneider *et al.* [9]. For both strains, the parasitemia drops continuously with increasing pyrimethamine concentration, but does so at a very different rate. For the avirulents, the drug has half of its inhibitory effect at a pyrimethamine concentration of 1.0 mg kg<sup>-1</sup> (amount of drug per kg body weight), whereas for the virulent parasites this rises to 4.6 mg kg<sup>-1</sup>. Overall, the drug is 3–4 times less effective in the virulent strain than it is in the avirulent strain. This is drug resistance in its classic definition.

It is important to note that the parasites were not exposed to the drug during the period that they developed virulence. So this is not the type of drug resistance that microorganisms acquire when they are exposed to sub-optimal drug concentration for a lengthy period. Here, pyrimethamine resistance has developed independently of drug exposure, simply as part of the parasite's new metabolic state that has brought about virulence.

## The explanation: different avenues to pyrimethamine resistance

The molecular basis of resistance to pyrimethamine is well understood in malaria parasites. It is generally brought about by mutations in the drug's target, the bifunctional enzyme dihydrofolate reductase-thymidylate synthase (DHFR-TS) [11]. For *P. falciparum*, pyrimethamine resistance in Africa has arisen multiple times, with double or triple mutations, at several of the positions 50, 51, 59, 108, and 164 of PfDHFR-TS being very common [12]. Schneider *et al.* checked whether the pyrimethamine resistance associated with virulence was due to any such mutations [9]. It was not. They genotyped the *dhfr-ts* genes in these

## Update



**Figure 1.** Effect of virulence on pyrimethamine susceptibility in *Plasmodium chabaudi*. Mice were infected with a virulent (closed circles) and an avirulent progenitor strain, followed by treatment with different concentrations of pyrimethamine. The parasitemia determined in the different mice was analyzed as a function of the corresponding pyrimethamine concentration. Re-analyzed from Schneider *et al.* [9].

parasites, both before and after serial passaging in mice, and found none of the mutations known to confer resistance to pyrimethamine within this gene. Thus, the development of virulence does not involve mutations of the target enzyme, DHFR-TS. What, then, is the molecular basis of pyrimethamine resistance that develops with virulence?

To address this question, one has to consider the result in light of the biochemical pathway in which DHFR-TS partakes. DHFR-TS is a key enzyme in the folate biosynthesis pathway (reducing folate to dihydrofolate and concerting deoxyuridine 5' phosphate (dUMP) to deoxythymidine 5'-phosphate (dTTP)). Folates are essential metabolites, and the folate-dependent generation of DNA precursors in the form of deoxythymidine 5'-phosphate is essential for the replication of malaria parasites. Thus, parasites that display an increased multiplication rate are expected to have an increased demand for the products of the folate biosynthesis pathway, in particular pyrimidines. There are several possibilities to satisfy this need without mutating the *dhfr-ts* gene itself.

First, the *dhfr-ts* gene may be amplified or overexpressed. Amplification and overexpression of the *dhfr-ts* gene would result in more DHFR-TS enzyme and, as shown in other systems, in increased synthesis of pyrimidines, which, in turn, would allow the cells to multiply faster [13]. Amplification of the *dhfr-ts* gene does occur in *Plasmodium* and, importantly, it results in reduced susceptibility to pyrimethamine [14] since higher drug concentrations are required to inhibit the DHFR-TS in cells that overexpress it.

A second possibility would involve overexpression of genes upstream in the folate biosynthesis pathway to

produce more substrates of the DHFR-TS. As shown in other systems, the enzymatic activity of DHFR, but also expression of the *dhfr* gene, can be stimulated by the substrate dihydrofolate and inhibited by the product tetrahydrofolate [13]. Such feedback mechanisms are quite common, and it is likely that the *Plasmodium* DHFR-TS is regulated accordingly. Consistent with this model, a recent study has found amplification of the GTP cyclohydrolase gene, the first enzyme in the folate biosynthesis pathway, to be associated with pyrimethamine resistance in *P. falciparum* [15]. Apparently, the GTP cyclohydrolase is a rate-limiting factor in the folate biosynthesis pathway in *Plasmodium*. If this bottleneck is overcome by gene amplification, then the parasite seems to possess a higher intracellular dihydrofolate pool and possibly an augmented DHFR-TS enzymatic activity, which would out-compete the intracellular pyrimethamine concentration, leading to reduced susceptibility. Such parasites would further produce more of the precursor molecules essential for DNA replication and, thus, would multiply faster.

A third model would invoke altered transport processes. The role of transport systems in inducing drug resistance is very well documented, both for antimalarials, such as CQ and quinine, and for anticancer drugs [16–20]. But pyrimethamine resistance is unlikely to be due to a transport system for pyrimethamine itself, since pyrimethamine's site of action is in the cytoplasm, which the lipophilic drug can reach with no difficulty.

A very interesting mode of resistance to antifolates has been reported in human leukemia cells that have been selected for resistance to the anti-cancer drug methotrexate [21]. In these cells, resistance is brought about by augmentation of a folate carrier that brings folate into wild-type cells, but to far higher levels in mutant drug-resistant cells [21]. Methotrexate's blockade of dihydrofolate reductase is circumvented in these cells by the elevated intracellular folate pool out-competing the intracellular drug concentration. The same final result was found in hamster ovary cells that had suffered, in this case, a loss of folic acid exporter function, resulting in markedly augmented folate accumulation, which again out-competed the intracellular methotrexate concentration [22]. Genes that encode folate transporters (members of the folate-biopterin transporter family) have been identified in the genome of malaria parasites, including *P. chabaudi* and *P. falciparum* [23], and could thus be possible sites for the source of pyrimethamine resistance in the virulent parasites.

### The outlook

Which of the different models, if any, underpin the virulence-associated pyrimethamine resistance in *Plasmodium* remains to be determined. Whatever the cause, it is a chilling thought that parasites may evolve in Nature that multiply faster, are more virulent and, thus, more deadly, and, at the same time, are less susceptible to drug treatment because of their increased state of fitness. However, there is hope that the co-dependent evolution of virulence and pyrimethamine resistance remains a singular event due to the nature of the folate biosynthesis pathway and the crucial role this pathway plays in parasite replication.

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