Origin of Robustness in Generating Drug-Resistant Malaria Parasites

Krittikorn Kümponsin,¹,¹ Charin Modchang,¹,² Adina Heinberg,³ Eric H. Ekland,⁴ Piyaporn Jirawatcharadech,¹ Pornpimol Chobson,¹ Nattida Suwanakitti,⁵ Sastra Chaothong,⁵ Prapon Wilairat,¹ Kirk W. Deitsch,³ Sumalee Kamchonwongpaisan,⁵ David A. Fidock,⁶,⁶ Kirk W. Deitsch,³ Laura A. Kirkman,³,⁷ Yongyuth Yuthavong,⁵ and Thanat Chookajorn*,¹,⁸

¹Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand
²Biophysics Group, Department of Physics, Faculty of Science, Mahidol University, Bangkok, Thailand
³Department of Microbiology and Immunology, Weill Cornell Medical College, New York, NY
⁴Department of Microbiology and Immunology, Columbia University College of Physicians and Surgeons, New York, NY
⁵National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathum Thani, Thailand
⁶Division of Infectious Diseases, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY
⁷Division of Infectious Diseases, Department of Medicine, Weill Cornell Medical College, New York, NY
⁸Center of Excellence in Malaria, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

*Corresponding author: E-mail: thanat.cho@mahidol.edu.
Associate editor: Takashi Gojobori

Abstract

Biological robustness allows mutations to accumulate while maintaining functional phenotypes. Despite its crucial role in evolutionary processes, the mechanistic details of how robustness originates remain elusive. Using an evolutionary trajectory analysis approach, we demonstrate how robustness evolved in malaria parasites under selective pressure from an antimalarial drug inhibiting the folate synthesis pathway. A series of four nonsynonymous amino acid substitutions at the targeted enzyme, dihydrofolate reductase (DHFR), render the parasites highly resistant to the antifolate drug pyrimethamine. Nevertheless, the stepwise gain of these four dhfr mutations results in tradeoffs between pyrimethamine resistance and parasite fitness. Here, we report the epistatic interaction between dhfr mutations and amplification of the gene encoding the first upstream enzyme in the folate pathway, GTP cyclohydrolase 1 (GCH1). gch1 amplification confers low level pyrimethamine resistance and would thus be selected for by pyrimethamine treatment. Interestingly, the gch1 amplification can then be co-opted by the parasites because it reduces the cost of acquiring drug-resistant dhfr mutations downstream in the same metabolic pathway. The compensation of compromised fitness by extra GCH1 is an example of how robustness can evolve in a system and thus expand the accessibility of evolutionary trajectories leading toward highly resistant alleles. The evolution of robustness during the gain of drug-resistant mutations has broad implications for both the development of new drugs and molecular surveillance for resistance to existing drugs.

Key words: drug resistance, evolution, malaria, robustness.

Introduction

Biological robustness plays an important role in the evolutionary process by permitting a given functional process to be maintained while a population of organisms accumulates mutations in the background (Hartman et al. 2001). Diverse genetic repertoires increase the opportunities for a desirable genotype or phenotype to be naturally selected (Kirschner and Gerhart 1998; Masel and Siegal 2009). Improvements in molecular evolution experiments and large-scale genomic sequencing have demonstrated the crucial role of robustness in the evolutionary process (Woods et al. 2011). Despite the conceptual importance of robustness in evolutionary biology, its predictions are often beyond the reach of experimentation, and it has been difficult to find naturally occurring molecular mechanisms that clearly exemplify the origin of robustness.

Heat shock protein 90 (Hsp90) has been shown to function as an “evolutionary capacitor” in several organisms by suppressing phenotypic changes, but the uniqueness of a master switch molecule like HSP90 makes it difficult to assess the general impact that robustness plays in driving evolutionary processes (Quetsch et al. 2002; Sawarkar and Paro 2010; Specchia et al. 2010). The role of robustness can be more easily understood by analyzing a step-by-step process in which a molecule evolves to promote robustness. The step-by-step evolutionary analysis has recently been accomplished by mapping the trajectories of evolutionary pathways and
showing epistasis in protein evolution (Weinreich et al. 2006; Poelwijk et al. 2007; Gong et al. 2013). The trajectory analysis can map evolutionary changes toward an eventual outcome, one mutation at a time. The approach was successfully used to reveal that a series of mutations leading to antibiotic resistance does not occur at random but instead needs to follow a strict mutation order (Weinreich et al. 2006). Even though the trajectory analysis shows stepwise evolutionary processes, it only reflects intralocus epistasis within a single gene and thus does not delineate more complex relationships between multiple loci (Weinreich et al. 2006; Brown et al. 2009; Lozovsky et al. 2009; Costanzo et al. 2011). Trajectory pathways that incorporate the influence of both intra- and interlocus epistasis could reveal in detail the nature of the evolutionary process as well as the origin of robustness.

The human parasite *Plasmodium falciparum* causes ~200 million cases of malaria every year and has recently become a model in evolutionary biology due to the large-scale population genetic data available for both the parasite and its human host (Carlton 2007; Manske et al. 2012; Murray et al. 2012). The spread of drug-resistant mutations in *P. falciparum* has been observed multiple times, as new antimalarial drugs have exerted evolutionary pressure on successive resistance loci (Mackinnon and Marsh 2010). Pyrimethamine, a key antimalarial drug, targets *P. falciparum* dihydrofolate reductase (DHFR), which is a part of the bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) in the parasite’s folate metabolic pathway (fig. 1A) (Hyde 2005; Yuthavong et al. 2005). Pyrimethamine acts as a competitive inhibitor of the endogenous substrate dihydrofolate. The level of pyrimethamine resistance is sequentially increased as the drug-sensitive wild-type DHFR enzyme accumulates four specific mutations namely, N51I, C59R, S108N, and I164L (Plowe et al. 1997; Sirawaraporn et al. 1997). Previous studies revealed that the acquisition of these mutations is not random and occurs in a step-wise order, suggesting the influence of additional genetic alterations within dhfr or elsewhere in the genome (Brown et al. 2009; Lozovsky et al. 2009; Chookajorn and Kumpornsin 2011; Costanzo et al. 2011). Genomic and epidemiological studies have also shown that multiple copies of *PFL1155w* (PF3D7_1224000), a gene encoding the first enzyme in the folate pathway, GTP cyclohydrolase I, are commonly found in malaria parasites, with a number of strains from Thailand having more than ten copies of this gene per parasite genome (Kidgell et al. 2006; Nair et al. 2008). This level of gene amplification is surprisingly high considering that most parasites from neighboring Laos have only one copy per parasite (Nair et al. 2008).

GTP cyclohydrolase I is the first enzyme in the folate pathway. It converts GTP into pterin, a core component in this vital metabolic pathway (fig. 1A) (Burg and Brown 1968). Interestingly, the level of amplification of the putative *P. falciparum* GTP cyclohydrolase I (gch1) gene correlates with the presence of the I164L dhfr mutation (Nair et al. 2008). Previous epistasis analyses suggested that the I164L mutation could either be the last mutation [N51I/C59R/S108N → N51I/C59R/S108N/I164L] or the less likely penultimate mutation [C59R/S108N → C59R/S108N/I164L → N51I/C59R/S108N/I164L] in the evolutionary pathway toward becoming the quadruple *dhfr* mutant (fig. 1B, see Results for detail) (Lozovsky et al. 2009). This linkage suggested the possibility of an interlocus epistasis between gch1 copy number polymorphism and *dhfr* mutations, which could be a model for studying how the combination of intralocus and interlocus interactions can affect the trajectory of pyrimethamine resistance evolution.

Here, we utilized evolutionary trajectory analysis to investigate these possibilities. We first confirmed that the protein encoded by *PFL1155w* functions enzymatically as the *P. falciparum* GCH1 using genetic complementation studies. We then demonstrated that amplifying the copy number of gch1 resulted in decreased susceptibility to antifolates. This observation explains why gch1 amplification would arise in a region where pyrimethamine was being used therapeutically. We then analyzed how gch1 amplification influences the trajectory paths leading to the drug-resistant quadruple *dhfr* mutant. We found that *dhfr* mutants with suboptimal catalytic efficiency were fitter on a multicopy gch1 background, presumably because the augmented flux of substrates through the folate synthesis pathway compensated for the impaired DHFR activity. The gain in copy numbers increased the robustness of the system by reducing the fitness costs associated with acquiring mutations in the *dhfr* gene. The findings were confirmed by data from field isolates and transgenic lines. Extra GCH1 allowed evolutionary permutations beyond the conventional trajectories, demonstrating that it may be easier to acquire resistant mutations against novel drugs targeting the folate pathway, or nucleotide metabolism in general, than had previously been appreciated.

**Results**

**Confirmation that PFL1155w Is a GTP Cyclohydrolase**

We first confirmed that PFL1155w is indeed a GTP cyclohydrolyase I by using genetic complementation and enzymatic assays (fig. 2). Our studies showed that recombinant PFL1155W can produce fluorescent neopterin similar to GTP cyclohydrolyase I from other organisms (fig. 2A). This activity was abrogated in a GCH1 mutant in which the key conserved residue at the active site was mutated (H279S). PFL1155W can also complement the loss of bacterial *gch1 (ΔfolE)* in bacteria, confirming its activity as GCH1(fig. 2B).

**Tracking the Effect of *P. falciparum* GCH1 on the Course of *dhfr* Evolution**

We then tested how gch1 could affect the trajectory of pyrimethamine-resistant *dhfr* mutations previously mapped based on epistasis analysis and field data, which are paths p13, p15, and p16 (fig. 1B) (Lozovsky et al. 2009). The change from the wild-type *dhfr* allele to the quadruple mutant is designated as the transition from the wild-type 0000 to the quadruple mutant 1111 enzyme, in which the change from 0 to 1 represents the mutation of N51I, C59R, S108N, and I164L, respectively (fig. 1B). The effect of extra amounts of GCH1 was tested for every *dhfr* mutant existing on these evolutionary paths. Because *P. falciparum*...
laboratory-adapted strains and field isolates have diverged in their folate-related genes, after decades of exposure to antifolate drugs, we employed a surrogate bacterial model by replacing endogenous *Escherichia coli* dhfr and ts with bifunctional *Plasmodium falciparum* dhfr-ts and expressing extra *P. falciparum* GCH1 under the control of an inducible promoter. The use of the surrogate system provides the isogenic background for the trajectory analysis without the confounding effect from drug resistance-related polymorphisms that are already widespread in the malaria parasites worldwide. The system was shown to have similar mutational change and drug response as in the parasite under antifolate selection (Chusacultanachai et al. 2002; Lozovsky et al. 2009). The epistasis analysis between gch1 and dhfr mutants was performed, and the results were then compared with experiments performed with transgenic parasites and field data.

We first determined fitness and drug sensitivity of dhfr alleles in p13, p15, and p16. Interestingly, there is a major tradeoff between fitness and drug resistance (fig. 1C and supplementary fig. S1, Supplementary Material online), consistent with a previous report (Brown et al. 2010). Despite high level of resistance to pyrimethamine, mutations leading toward 1111 collectively cripple DHFR function as indicated by the poor fitness (fig. 1C). The tradeoff was confirmed by testing the competitive fitness between 0110 and 1111, with 0110 outcompeting 1111 in the condition of low drug pressure and vice versa under high pyrimethamine pressure (fig. 1D).
Plasmodium falciparum gch1 was then added into the epistasis analysis. The effect of parasite gch1 on dhfr alleles can be divided into two categories namely, cost of mutation and drug resistance. Extra P. falciparum GCH1 significantly reduced the cost of mutation in dhfr mutants as indicated by the rescue of compromised dhfr mutants. For example, in the genetic background 1111, extra GCH1 significantly improves growth when compared with control (fig. 3A). As GCH1 was found to be the rate-limiting enzyme in the folate pathway of model organisms, fitness improvement attributable to the extra GCH1 might come from an increase in folate flux, as previously reported in plants (Hossain et al. 2004). This is supported by the finding that thymidine, a key product of folate metabolism, restored the poor growth of dhfr mutants back to wild-type levels, consistent with rescue of the compromised folate flux (supplementary fig. S2, Supplementary Material online).

Effect of Extra GCH1 on Fitness and Drug Sensitivity
We also assessed the effect of extra GCH1 on pyrimethamine sensitivity. When combined with a sensitive dhfr allele, extra GCH1 increased the IC₅₀ by more than 10-fold (fig. 3B and supplementary table S1, Supplementary Material online).

In contrast, in the context of highly resistant alleles at the end of the trajectory such as 1111, the effect of extra GCH1 on pyrimethamine resistance was negligible. To confirm the validity of the model, these data were compared with those derived from transgenic parasites expressing extra P. falciparum GCH1. We found that the effect of extra GCH1 in the surrogate model correlates with the fold change in drug sensitivity measured for transgenic P. falciparum parasites overexpressing GCH1 (supplementary fig. S3, Supplementary Material online) (Heinberg et al. 2013). The ability of gch1 amplification to confer resistance to pyrimethamine could explain why parasite isolates with wild-type dhfr, including laboratory strains such as 3D7, contain several copies of gch1 (Kidgell et al. 2006). This could also indicate an independent origin of gch1 copy number polymorphism unrelated to dhfr mutations.

We then analyzed every allele by using relative fitness under drug pressure ($f_D$), which takes both relative growth and drug resistance into account (see Materials and Methods for details). These studies revealed the influence of GCH1 on the course of dhfr mutation trajectories that manifests in two phases, namely early and late evolutionary paths (supplementary movie S1, Supplementary Material online). In the early
phase of the evolutionary paths such as 0000 and 0010, *dhfr* alleles were still vulnerable to pyrimethamine. An increased amount of GCH1 led to an improvement in drug resistance levels (fig. 3A and B and supplementary movie S1, Supplementary Material online). The cost of mutation became a major constraint further down the trajectory, that is, 1110 and 1111 where the effect on drug resistance by extra GCH1 was negligible (supplementary movie S1, Supplementary Material online). *gch1* copy number polymorphism was therefore co-opted from its role in drug resistance to that of reducing the cost of mutation (fig. 3A and supplementary movie S1, Supplementary Material online). Extra GCH1 can significantly improve the growth for these highly resistant *dhfr* mutants such as 1110 and 1111 that can withstand strong drug pressure, but would otherwise display poor growth.

**Gain of Robustness in the Evolutionary Processes Toward Pyrimethamine Resistance**

A more complete picture emerges once the effect of *P. falciparum* GCH1 was analyzed in the context of the entire evolutionary trajectories based on the data from each step of the mutations from wild-type (0000) to the quadruple mutant (1111) (Weinreich et al. 2006; Lozovsky et al. 2009; Brown et al. 2010). The effect of GCH1 on the trajectories is presented in a color scale map indicating the probability for each trajectory path to reach 1111 (fig. 4). The probability heat maps are presented with the genetic contributions from either *dhfr* or *gch1* on the cost of mutation and drug resistance as x- and y-axes, respectively. The large warm area (red color) means that the evolutionary trajectory toward 1111 can be completed over broad parameters, which increases the chances of becoming a highly resistant genotype. To represent the effect of drug pressure on the trajectories, the increase in drug pressure was gradually applied as shown by compiled movie clips (supplementary movies S2 and S3, Supplementary Material online). When *dhfr* mutations are the sole factor in the evolutionary process, the probabilities for completing the trajectories can only occur at high drug pressure as indicated by the small warm area under the high drug pressure region (fig. 4, left panel; supplementary movie S2, Supplementary Material online). When the biphasic influence of GCH1 on both drug resistance and growth was applied on the trajectories, extra GCH1 improved the accessibility for every trajectory to complete the evolutionary process toward achieving 1111 (fig. 4 and supplementary movies S2 and S3, Supplementary Material online). In fact, the robustness of the system was improved as demonstrated by the increase in pathway accessibility under broader parametric criteria (expansion of the warm area). Field data analysis also indicated the influence of GCH1 (fig. 5). When the cost of mutation was too high, the gain of *gch1* copies became apparent among the field isolates.

**Effect of GCH1 on Unconventional Evolutionary Pathways**

In order to confirm the general role of GCH1 in antifolate resistance evolution, we tested whether it allowed unfavorable mutations outside the 0000 → 1111 pathway. The *dhfr* mutant library was screened for unconventional mutants (Japrung et al. 2007). Additional alleles, such as E21D/Y35F/C50R and E30G/C50R, also had poor fitness compared with that of the wild-type allele, but the expression of extra GCH1 helped compensate for this reduced fitness (fig. 6A). In addition, we explored whether overexpressing GCH1 conferred resistance to other anti-DHFR compounds in a different evolutionary trajectory. For these experiments, we used the *P. falciparum* FCR3 strain that has the unconventional S108T *dhfr* allele and a single copy of *P. falciparum gch1* (Peterson et al. 1988; Kidgell et al. 2006). Overexpressing GCH1 in this strain rendered the parasites 6-fold more resistant to cycloguanil and 7-fold more resistant to pyrimethamine, demonstrating a similar effect of increased GCH1 on pyrimethamine as well as other anti-DHFR compounds as previously shown. The IC50 values for chloroquine, an antimalarial that does not interfere with folate metabolism, were unaffected in these lines (fig. 6B). GCH1 was fused to GFP to confirm its localization and was found to be dispersed

---

**Fig. 3.** Extra GCH1 affects the cost of *dhfr* mutation and pyrimethamine resistance. (A) Extra GCH1 improves the fitness in a highly resistant mutant with poor fitness such as 1111. Extra GCH1 alone does not intrinsically boost growth as seen in 0110. Extra GCH1 even caused lower relative fitness in the condition without drug (Relative Fitness<sub>0</sub>) in wild-type 0000, probably due to the adverse effect of depleting GTP. (B) Effect of extra P. falciparum GCH1 on pyrimethamine sensitivity. GCH1 increased IC<sub>50</sub> values especially when combined with pyrimethamine-sensitive *dhfr* alleles (*P* value < 0.05 and **P** value < 0.001). For the complete picture, see supplementary movie S1, Supplementary Material online.

---

**Downloaded from https://academic.oup.com/mbe/article-abstract/31/7/1649/2925814 by guest on 14 March 2019**
throughout the cytoplasm (fig. 6C). This pattern concurs with what has been observed in plants, in which folate synthesis is initiated by GCH1 in the cytoplasm, with subsequent steps being carried out in the plastid, the mitochondria, or the cytoplasm (Hossain et al. 2004). The GCH1-GFP expressing parasite lines also demonstrated increased resistance to cycloguanil and pyrimethamine (supplementary fig. S5, Supplementary Material online).

**Discussion**

**Gain of Robustness in Drug Resistance Evolution**

Our analysis has uncovered a role for GCH1 in promoting robustness that arose during the acquisition of pyrimethamine resistance in *P. falciparum*. The process relies on the co-option of an existing copy number polymorphism to compensate for the gain of deleterious mutations at another gene in the same metabolic pathway. The trajectory analysis approach, which represents step-by-step evolutionary changes, is a useful tool to demonstrate two roles of *gch1* copy number polymorphism, namely drug resistance and reduction of the cost of *dhfr* mutations.

The evolutionary paths toward antifolate resistance either via *gch1* amplification or stepwise *dhfr* mutations are not mutually exclusive. Nevertheless, the increase in the level of pyrimethamine resistance via extra GCH1 alone is not comparable to that observed in *dhfr* mutants especially in those with more than two mutations. Some field isolates with triple and quadruple *dhfr* mutants tend to have more copies of *gch1* than in the *dhfr* alleles early in the stepwise evolutionary pathway. These findings could imply that the major role of *gch1* amplification in the evolution of antifolate resistance might be to facilitate the fixation of unfavorable *dhfr* mutants rather than playing a role in enhancing the level of drug resistance. Still, it is not possible to exclude the significance of *gch1* amplification on the gain in pyrimethamine resistance during the early part of the *dhfr* evolutionary process since...
certain *Plasmodium falciparum* strains with the wild-type *dhfr* allele were found to contain multiple copies of *gch1*.

The gain of a permissive mutation that accommodated subsequent drug-resistant mutations with poor fitness in the same gene was observed in oseltamivir resistance in influenza virus (Bloom et al. 2010). Our analysis suggests that the trajectory analysis can go beyond a one-gene system. We expect that high-throughput whole-genome approaches will open the door for more multidimensional epistasis studies between multiple genetic variations. Nevertheless, increases in variables or trajectories will exponentially confound the outcome. Even an epistasis analysis within the folate pathway could become complicated by several orders of magnitude by including other known polymorphic genes or by introducing trajectory reversion. High-throughput sequencing and automated culture systems have been shown to be promising solutions in dissecting epistasis in drug resistance with multiple variants (Toprak et al. 2011).

**Prevention and Surveillance of Malaria Drug Resistance**

Complacency on the emergence of malaria drug resistance could cost the lives of millions, especially among young African children, as indicated by the recent history of chloroquine and sulfadoxine–pyrimethamine resistance (White 2012). The emergence of artemisinin-resistant parasites has gradually expanded from a few isolated cases to a common trend among *P. falciparum* malaria patients at the Thailand–Cambodia and Thailand–Myanmar borders (Carrara et al. 2013). Malaria parasites in Southeast Asia have proven to be highly adept at developing resistance. The gain of *gch1* copies might explain why the costly I164L mutation can be highly adept at developing resistance. The gain of a permissive mutation that accommodated subsequent drug-resistant mutations with poor fitness in the same gene was observed in oseltamivir resistance in influenza virus (Bloom et al. 2010). Our analysis suggests that the trajectory analysis can go beyond a one-gene system. We expect that high-throughput whole-genome approaches will open the door for more multidimensional epistasis studies between multiple genetic variations. Nevertheless, increases in variables or trajectories will exponentially confound the outcome. Even an epistasis analysis within the folate pathway could become complicated by several orders of magnitude by including other known polymorphic genes or by introducing trajectory reversion. High-throughput sequencing and automated culture systems have been shown to be promising solutions in dissecting epistasis in drug resistance with multiple variants (Toprak et al. 2011).

**Materials and Methods**

**Fitness Analysis in Surrogate Model**

Wild-type 0000 and mutant (0010, 0110, 1010, 1110, and 1111) alleles of *P. falciparum* *dhfr-ts (PF3D7_0417200)* were tested individually by cloning into the pET17b plasmid and transforming into *E. coli* BL21 (DE3) *Athere* *AfolA* generated through the red recombinase method (Datsenko and Wanner 2000). Growth analysis was performed with overnight preculture in LB broth (Bio Basic) supplemented with 300 μM thymidine (Sigma) and 100 μg ml⁻¹ ampicillin (Bio Basic). A starting culture with OD₆₀₀ at 0.005 was grown in the same media without thymidine supplement at 37°C.
Bacterial growth was determined using a spectrophotometer at optical density at 600 nm (OD600). Each experiment was completed independently in at least triplicate. *Plasmodium falciparum* GCH1 and *P. falciparum* GCH1 (H279S) were cloned into pBAD33. The H279S mutation was introduced using QuikChange II Site-Directed Mutagenesis (Agilent Technologies). Growth analyses with *P. falciparum* GCH1, *P. falciparum* GCH1 (H279S), and pBAD33 vector control were performed as previously stated with the addition of 34 μg ml⁻¹ chloramphenicol (Sigma).

Competitive fitness was studied using 0110 and 1111. Overnight precultures of 0110 and 1111 were prepared as described above and mixed at the ratio of 1:1 in new LB broth without thymidine supplement. Pyrimethamine was added to the final concentrations of 0, 10, 100, 500, and 750 μM. The cultures were reinnoculated everyday by adding 1% of each culture to the new LB broth supplied with respective concentrations of pyrimethamine. After 300 generations, serially diluted cultures were grown on thymidine-supplemented LB agar without pyrimethamine to select colonies for direct sequencing.
Functional Study of *P. falciparum* GCH1

Genetic complementation of *P. falciparum* GCH1 was performed in *E. coli* K12 MG1655 ΔfolE (a gift from Professor Andrew Hanson, University of Florida, Gainesville, FL, USA) (Klaus et al. 2005). *Plasmodium falciparum* GCH1 and GCH1(H279S) in pBAD33 were transformed by heat shock with 300 μM thymidine supplement. Assays employed four different media conditions, as follows: 1) LB broth or agar without arabinose and thymidine, 2) LB broth or agar with 300 μM thymidine without arabinose, 3) LB broth or agar with 0.02% (w/v) arabinose without thymidine, and 4) LB broth or agar with 0.02% (w/v) arabinose and 300 μM thymidine. Growth was monitored using OD600 measurements.

Enzymatic assays of *P. falciparum* GCH1 were performed with recombinant protein. *Plasmodium falciparum* GCH1 was cloned into pET45b(+) and expressed in *E. coli* BL21(DE3)RIL. Protein production was induced with 0.4 mM IPTG at 16°C with constant shaking at 220 rpm for 18 h. Protein was purified by Ni2+-Sepharose (GE Healthcare) using the manufacturer’s protocol. The purified protein was dialyzed against 50 mM Tris–HCl pH 7.8, 100 mM KCl, 20% glycerol, 250 μM GTP, and 2.5 μM *P. falciparum* GCH1. The reaction was incubated in the dark at 37°C for 90 min and stopped by 67 mM HCl. The product of reaction, the nonfluorescent 7,8-dihydronicotinamide triphosphate, was oxidized to the fluorescent neopterin product with 0.067% iodine (dissolved in 2% KI). Then, 0.12% ascorbic acid and 55.6 mM NaOH were added. The product was measured using a spectrofluorometer (RF-5301PC, Shimadzu) with neopterin (Sigma) as the standard.

Drug Sensitivity Analysis

*Escherichia coli* BL21 (DE3) ΔthyA ΔfolA harboring pET17b with *P. falciparum* dhfr-ts and pBAD33 with *P. falciparum* gch1 were propagated as previously described. Cells were cultured under various concentrations of pyrimethamine [5-(4-chlorophenyl)-6-ethyl-2,4-pyrimidinediamine] (Sigma) prepared in dimethyl sulfoxide (Fisher). The sigmoidal dose response plot of OD600 at the time of stationary phase as a function of pyrimethamine concentrations was used to determine IC50 value (Graphpad Prism Software). Each experiment was completed independently in at least triplicate.

To generate parasite lines that differ only in gch1 copy number and expression levels, *D. salmositica* parasites (genotype: 0000) were stably transfected with a plasmid encoding either GCH1 or Renilla luciferase as a control. Using the regulatable transgene expression system described previously (Epp et al. 2008), we cultured parasites in the presence of 5 or 20 μg/ml blasticidin to modulate copy number and expression of gch1 or Renilla luciferase in cultured parasites. To determine pyrimethamine IC50 values, SYBR Green-based drug assays were performed as described previously (Smilkstein et al. 2004).

Fitness Analysis

We modified a previously reported fitness model (Schulz zur Wiesch et al. 2010) to estimate the roles of dhfr mutations and to incorporate the effect of *P. falciparum* gch1. In this model, the relative fitness values of the dhfr alleles in the absence (fND) and presence (fD) of drug pressure were given by

\[
 f_{ND} = 1 - C_M + e_{GM},
\]

\[
 f_D = [1 - a(1 - e_R)(1 - e_{CD})]/(1 - C_M + e_{GM}).
\]

Model parameters are summarized in supplementary table S2, Supplementary Material online. All relative fitness values were expressed relative to the fitness of wild-type dhfr (0000) without drug pressure. The wild-type dhfr allele has the highest fitness value in the absence of drug pressure (fND) and has a reduced fitness in the presence of drug, whereas other dhfr alleles generally gain more advantage in the presence of drug.

The amount of fitness loss of wild-type dhfr was assumed to be dependent on drug activity (a). Unless stated otherwise, a was set at 0.99 to maximize the drug effect. The fitness gain of mutant dhfr alleles during drug treatment (fD) can be described by the efficiency of the resistance mutation (eR) in reducing the drug activity. The efficiency of resistance for each dhfr allele was set to be proportional to its half maximal inhibitory concentration (IC50).

In conditions without drug pressure, the fitness of resistant mutants was usually found to be lower than that of the wild-type dhfr. The loss of fitness due to mutations is described by the cost of mutation parameter (Cm). In our model, the cost of mutation for each dhfr allele is proportional to the growth difference in time, relative to wild-type dhfr allele, for which its optical density at 600 nm (OD600) reaches the value of 0.5. The growth analysis by OD600 was calculated by fitting the OD600 data with the following Gompertz function:

\[
 OD_{600} = a \exp[-\exp(-b(t-c))].
\]

where t is time in hours, and a, b, and c are constants to be determined by nonlinear regression curve fitting. The equation was solved for t at OD600 equal to 0.5.

The effect of extra *P. falciparum* GCH1 on the relative fitness of dhfr alleles can be divided into two categories. First, extra *P. falciparum* GCH1 can alter the cost of mutation. This effect was represented in the model by the efficiency of GCH1 in mutation cost parameter (eGM). The parameter eGM for a specific dhfr allele is proportional to its relative change in cost of mutation when extra *P. falciparum* GCH1 was added. Second, extra *P. falciparum* GCH1 can change the drug resistance levels of dhfr alleles. In the model, this effect was quantified by the efficiency of GCH1 in the drug resistance parameter (eCD). This parameter for a given dhfr allele can be calculated from the change in IC50 value after adding extra *P. falciparum* GCH1. Definitions of parameters are summarized in supplementary table S2, Supplementary Material online. To calculate probabilities of evolutionary trajectories with a broad range of parameter values, these
four parameters $C_M, e_R, e_{GD}$, and $e_{GM}$ were scaled with the following relations:

$$C_M = C_{M, \text{scaling}} \hat{C}_M,$$
$$e_R = e_{R, \text{scaling}} \hat{e}_R,$$
$$e_{GD} = e_{GD, \text{scaling}} \hat{e}_{GD},$$
$$e_{GM} = e_{GM, \text{scaling}} \hat{e}_{GM},$$

where $C_{M, \text{scaling}}, e_{R, \text{scaling}}, e_{GD, \text{scaling}},$ and $e_{GM, \text{scaling}}$ are scaling factors for $C_M, e_R, e_{GD},$ and $e_{GM}$, respectively, and the parameters $\hat{C}_M, \hat{e}_R, \hat{e}_{GD}$, and $\hat{e}_{GM}$ represent the normalized $C_M, e_R, e_{GD},$ and $e_{GM}$ respectively. The values of these normalized parameters were between 0 and 1 and were calculated from growth analyses and IC50 values (supplementary table S1, Supplementary Material online). Unless stated otherwise, $C_{M, \text{scaling}} = 0.8$.

Calculation of Evolutionary Trajectories

We used previously established methodology to determine the evolutionary trajectories that are accessible for dhfr evolution to attain pyrimethamine resistance (Lozovsky et al. 2009; Brown et al. 2010). Our evolutionary model assumes that selection pressure is strong relative to mutation pressure, and that the time between mutations arising is much longer than the time to lose new mutations, which allows the fixation of at most one mutation during each interval of time. At each interval, all mutations that will result in an increase in fitness ($f_D$) are considered. The probabilities of moving from the low fitness wild type (0000) to an allele of a higher fitness, 1111 in the presence of drug, via pathway 13, 15, or 16 are given, as in Weinreich et al. (2006) and Brown et al. (2010), by

$$P_{13} = P_{0000 \rightarrow 0010} \cdot P_{0010 \rightarrow 1010} \cdot P_{1010 \rightarrow 1110} \cdot P_{1110 \rightarrow 1111},$$
$$P_{15} = P_{0000 \rightarrow 0010} \cdot P_{0010 \rightarrow 0110} \cdot P_{0110 \rightarrow 1110} \cdot P_{1110 \rightarrow 1111},$$
$$P_{16} = P_{0000 \rightarrow 0010} \cdot P_{0010 \rightarrow 0110} \cdot P_{0110 \rightarrow 1111} \cdot P_{1111 \rightarrow 1111},$$

respectively (Weinreich et al. 2006; Brown et al. 2010). In our model, the probability of fixation ($P_{i \rightarrow j}$) equals to $f_{Dj} - f_{Di}$ if $f_{Dj} > f_{Di}$ and equals to zero if $f_{Dj} < f_{Di}$, where $f_{Dj}$ and $f_{Di}$ are the fitness value of alleles $i$ and $j$ in the presence of drug pressure, respectively. The biphasic probability was calculated in the model by setting $e_{GM, \text{scaling}}$ in the early evolutionary trajectory and $e_{GD, \text{scaling}}$ in the late trajectory to zero. The values of $e_{GM, \text{scaling}}$ and $e_{GD, \text{scaling}}$ represent the co-option of GCH1’s role from drug resistance improvement during the early trajectory to mutational cost reduction in the late trajectory. On the other hand, the monophasic probability was calculated using the same $e_{GM, \text{scaling}}$ and $e_{GD, \text{scaling}}$ value for all mutants in the whole trajectory, thereby guaranteeing the equivalent role of GCH1 in drug resistance improvement and mutational cost reduction for all mutants along the mutational trajectory.

Probabilities of evolutionary trajectories were estimated using simulations in Matlab software. The possible evolutionary paths were explored by randomly choosing single-step mutations. Each new mutant allele was accepted if its fitness value was larger than that of the current allele; otherwise, the new mutant allele was rejected. Evolution on each simulated landscape was continued until the allele with maximum fitness was reached. To estimate the trajectory probability, we simulated 1 million rounds of evolution (see Lozovsky et al. 2009 for complete details of the model).

Published dhfr genotype and gch1 copy data from malaria field isolates were analyzed in the context of the cost of mutation (Nair et al. 2008). The cost of mutation associated with different dhfr alleles was compared with the copy number of gch1.

Effect of GCH1 on Antimalarial Drug Sensitivity in Malaria Parasite

Plasmodium falciparum FCR3 parasites, kindly provided by Dr Elizabeth Winzeler (University of California, San Diego), contain one copy of P. falciparum gch1 (Kidgell et al. 2006). Asexual blood-stage parasites were maintained in human red blood cells diluted to a 4% hematocrit in RPMI 1640 medium (Gibco) supplemented with 5% human serum, 5 mg/ml Albumax (Gibco), 2.4 mg/ml Na2HCO3 (Gibco), 25 mM HEPES, 50 µg/ml hypoxanthine (Sigma–Aldrich), and 10 ng/ml gentamycin (Gibco). Parasites were incubated at 37°C in 6-well culture plates in a humidified chamber (Billups-Rothenberg), gassed with 5%CO2/5%O2/90%N2.

FCR3 gch1 was PCR amplified from genomic DNA and cloned into the expression vectors pDC2-BSattP-PbeF1a-GCH1 and pDC2-BSattP-GCHPr-GCH-GFP, which expresses GCH1 fused to GFP under the control of the endogenous promoter. FCR3 parasites transfected with episomal copies of the construct were selected for using 2 µg/ml blastidicin (Invitrogen). Control lines were transfected with the “empty” vector pDC2-BSattP-GCHPr, which expresses the selectable marker, but has no coding region downstream of the gch1 promoter.

Expression of the PfGCH1-GFP fusion protein was confirmed by fluorescence microscopy using an Eclipse Ti inverted microscope (Nikon Instruments) with the NIS-Elements software (Nikon). Cells were stained in 300 µl of phenol red-free RPMI medium (Gibco) containing 1 µg/ml Hoechst 33342 (Sigma–Aldrich). Cells were allowed to adhere for 15 min at 37°C on poly-d-lysine-coated glass-bottom culture dishes (MatTek). Before imaging, the medium was exchanged with fresh phenol red-free RPMI containing 5 mg/ml Albumax. Transfected parasites were tested for altered susceptibility to pyrimethamine, cycloguanil, and chloroquine as previously described (Ekland et al. 2011).

Ethics statement

Plasmodium falciparum reference laboratory strains used in this research were obtained from the malaria research and reference reagent resource center (MR4). Anonymized red blood cell and serum for P. falciparum culture were kindly provided by the national blood service center.
Acknowledgments

This work is dedicated to the memory of the late Dr. Sastra Chaotheing. The authors thank our colleagues especially D.M. Weinreich, E.R. Lozovsky, and P. Jaru-Ampornpan for discussions and comments on the article. They are grateful for the comments and suggestions from anonymous reviewers. Illustrations were prepared by T. Kochakarn and P. Ponsuwanna. This work was supported by CPMO-National Science and Technology Development Agency to T.C., C.M., S.K., and Y.Y.; Grand Challenges Canada to T.C., C.M., and K.K.; the Faculty of Science, Mahidol University to T.C. and K.K.; the National Institutes of Health AI099327 to K.W.D., AI50234 to D.A.F., and AI76635 to L.A.K.; and the Commission of Higher Education-Thailand Research Fund-Mahidol University RMU5380054 to T.C. This work was also supported by The Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program Grant No. PHD/0044/2554 to K.K. and T.C. S.K. was supported in part by the International Research Scholar grant, the Howard Hughes Medical Institute. The Department of Microbiology and Immunology at Weill Medical College of Cornell University acknowledges the support of the William Randolph Hearst Foundation. L.A.K. is a William Randolph Hearst Foundation Clinical Scholar in Microbiology and Infectious Diseases.

Supplementary Material

Supplementary tables S1 and S2, figures S1–S5, and movies S1–S3 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

References


