PERSPECTIVES

The evolutionary landscape of antifolate resistance in *Plasmodium falciparum*

MARNA S. COSTANZO and DANIEL L. HARTL*

Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138, USA

[Costanzo M. S. and Hartl D. L. 2011 The evolutionary landscape of antifolate resistance in *Plasmodium falciparum. J. Genet.* **90,** 187–190]

Resistance to antifolates in Plasmodium falciparum is well described and has been observed in clinical settings for decades. At the molecular level, point mutations in the dhfr gene that lead to resistance have been identified, and the crystal structure of the wildtype and mutant dihydrofolate reductase enzymes have been solved in complex with native substrate and drugs. However, we are only beginning to understand the complexities of the evolutionary pressures that lead to the evolution of drug resistance in this system. Microbial systems that allow heterologous expression of malarial proteins provide a tractable way to investigate patterns of evolution that can inform our eventual understanding of the more complex factors that influence the evolution of drug resistance in clinical settings. In this paper we will review work in Escherichia coli and Saccharomyces cerevisiae expression systems that explore the fitness landscape of mutations implicated in drug resistance and show that (i) a limited number of evolutionary pathways to resistance are followed with high probability; (ii) fitness costs associated with the maintenance of high levels of resistance are modest; and (iii) different antifolates may exert opposing selective forces.

Introduction

Resistance to antifolate drugs targeted at disrupting the folate pathway in *Plasmodium* is well described and has been observed in clinical settings for decades. Noted as early as 1969 in murine malaria, pyrimethamine resistance was observed to occur 'in a single step' (Diggens *et al.* 1970). Once resistance arises, pyrimethamine is no longer able to effectively bind *P. falciparum* dihydrofolate reductase (PfDHFR) in order to disrupt the folate pathway and interfere with DNA and amino acid synthesis (Sibley *et al.* 2001; Gregson and Plowe 2005). At the molecular level, the 'single step' to resistance came to be associated with particular point mutations in the *dhfr* gene that codes for dihydrofolate reductase (Cowman *et al.* 1988; Peterson *et al.* 1988). The most commonly observed amino acid changes in PfDHFR are A16V, N51I, C59R, S108N/T, and I164L. Homology modelling of wildtype and mutant PfDHFR enzymes show that these mutations occur mostly near the active site and may cause steric interference with the docking of competitively inhibiting antifolates (Rastelli *et al.* 2000).

While the molecular basis for antifolate resistance is well understood, we are only beginning to understand the complexities of the evolutionary pressures that lead to the evolution of drug resistance in this system. Microbial systems that allow heterologous expression of malarial proteins provide a tractable way to investigate patterns of evolution that can inform our eventual understanding of the more complex factors that influence the evolution of drug resistance in clinical settings. Such systems have been developed in *S. cerevisiae* (Wooden *et al.* 1997) and in *E. coli* (Sirawaraporn *et al.* 1990; Chusacultanachai *et al.* 2002) in which endogenous DHFR is knocked out by mutation or inhibited, respectively, and replaced by plasmids carrying various alleles of the parasite *pfdhfr* gene.

Using targeted mutagenesis to create the mutant amino acids most regularly associated with drug resistance, all possible combinations of these mutations are constructed. These studies focus on mutations already known to be associated with drug resistance in the field, and thus explore a well defined but limited portion of the adaptive landscape. Studies of the wider landscape of drug-resistance mutations generated by selection in laboratory experiments would also be

*For correspondence. E-mail: dhartl@oeb.harvard.edu.

Keywords. malaria; drug resistance; dihydrofolate reductase; pyrimethamine; chlorcycloguanil; antifolates.

of great interest, but have not as yet been applied to malaria DHFR.

Drug resistance values are determined using growth rate assays where growth rate in the presence or the absence of drug functions as a proxy for fitness. Using the 'fitness' (resistance) values obtained in this manner, it is possible to completely reconstruct the underlying adaptive landscape for the specific mutations in response to a particular drug. Evolutionary pathways (also called trajectories) are then determined by computer simulations that probe the landscape to discover the most probable progression of evolution. Brown *et al.* (2010) provide a detailed description of this approach.

In this paper, we will review the results of work in *E. coli* and *S. cerevisiae* expression systems showing that a limited number of evolutionary pathways to resistance are followed the vast majority of the time. These experiments also indicate that fitness costs associated with the maintenance of high levels of resistance are modest, and that different antifolates may exert opposing selective forces.

Highway to resistance: accessibility of fitness peaks on the adaptive landscape

Studies of beta-lactamases in bacteria have shown that only a limited number of pathways to antibiotic resistance are accessible to evolution, largely due to the existence of epistatic interactions among mutant sites within the gene coding for the enzyme (Weinreich *et al.* 2006; Salverda *et al.* 2011). In a study using an *E. coli* expression system, Lozovsky and colleagues used the measurement of the half maximal inhibitory concentration (IC50) to simulate the evolution of pyrimethamine resistance from the wildtype to the most drug resistant (quadruple) mutant of *P. falciparum* DHFR (Lozovsky *et al.* 2009). Their results show that one pathway accounts for more than half of the pathways that lead to pyrimethamine resistance. Moreover, the top three most likely pathways accounted for almost 90% of all realizations of the evolution of drug resistance. Similarly, a study conducted by Brown *et al.* (2010) assayed all combinations of five sites in a yeast system and found that the top three most likely pathways leading from the wildtype to the most highly resistant quadruple mutant account for approximately 85% of realizations of pyrimethamine resistance. Evaluating the pathways to chlorcycloguanil resistance in the same yeast system suggests that the trend of a few pathways dominating the evolutionary landscape is not merely a function of the type of antifolate being assayed, as the top five pathways account for over 80% of total pathway realizations for resistance to chlorcycloguanil (Costanzo *et al.* 2011).

The finding that very few mutational trajectories account for most of the probability of realization is thus robust to variability in experimental design. Expression in a prokaryotic versus eukaryotic system did not reveal substantially different numbers of pathways traversed. Neither did the addition of a fifth site in the pyrimethamine study with yeast materially change the degree of accessibility of the landscape or lead to a wider variety of trajectories being followed than four sites did in the pyrimethamine study with bacteria. The findings in these experimental systems correlate well with field data where a few mutations dominate and are repeatedly observed in the same combinations, despite having independent origins (Mita *et al.* 2008).

The findings for PfHDFR contrast with those for bacterial beta lactamase. Whereas only a handful of mutations recovered from natural populations or found in laboratory experiments appear to contribute significantly to the resistance of PfDHFR to pyrimethamine or chlorcycloguanyl (Peterson *et al*. 1988; Chusacultanachai *et al*. 2002), amino acid replacements at up to 60 sites in beta lactamase appear to contribute to resistance to first, second, third, or fourth generation cephalosporins, monobactams, and beta lactamase inhibitors such as amoxicillin, aztreonam, ceftazidime, cephalosporin, cefotaxime, cefuroxime, and cefepime (Salverda *et al*. 2010). Further, while experimental evolution of PfDHFR follows only a few evolutionary pathways with high probability, more pathways are available in the experimental evolution of beta lactamase, especially when the mutational landscape is opened wide by error-prone PCR amplification (Salverda *et al.* 2011). Even in that case, however, the initial mutations largely determine which of the alternative pathways remain accessible (Salverda *et al.* 2011).

All mutations are not created equal: compensatory mutations and the cost of antifolate resistance

In the evolution of drug resistance in prokaryotes, there is often a pattern of initial loss of fitness due to drug-resistance mutations arising early, which is followed by compensatory mutations restoring fitness even in the absence of drug (Andersson 2006). In PfDHFR, there is no necessary pattern of kinetic or catalytic trade-off between attaining antifolate resistance and maintaining fitness in the absence of drug pressure. Mutations leading to an increase in drug-resistance may have neutral, beneficial, or detrimental effects on enzyme function on the native substrate (Sirawaraporn *et al.* 1997; Sandefur *et al.* 2007).

In determining the evolutionary trajectories most often followed under pyrimethamine pressure, both bacterial and yeast assays yielded no evidence that attaining drug resistance necessarily entails a loss of fitness in the absence of drug (Lozovsky *et al.* 2009; Brown *et al.* 2010). Early steps in the evolutionary trajectory sometimes do increase drug resistance at the cost of fitness in the absence of drug. Later steps in the pathway, however, incorporate compensatory mutations that restore the growth rate in the absence of drug to near wildtype levels while greatly increasing resistance to the pyrimethamine (Brown *et al.* 2010).

The major implication of these findings is clear. The alleles present on the most likely pathways in computer simulations correlate to those observed in clinical settings. This finding

implies that the highly resistant triple and quadruple alleles are likely to be stably maintained in the population even in the absence of drug pressure, as there is no discernible cost associated with maintaining drug resistance. Field observations have hinted that this may be the case, with parasites carrying the resistant genotype being present even after drug pressure is relaxed (Walliker *et al.* 2005).

Interestingly, the evolutionary trajectories followed on these adaptive landscapes also explain some observations that show resistant alleles to decrease in frequency once drug pressure is removed. Two examples from field studies in different parts of the world serve to illustrate this point. The single mutant S108N is often the first step in resistance in the field and the most often accessed first mutation in computer simulations. In the Sudan, S108N was observed to decline in frequency in the absence of drug pressure (Abdel-Muhsin *et al.* 2004), which is explained by the lower relative fitness of the single mutant to the wildtype in the absence of drug and the fact that compensatory mutations have not yet arisen. Similarly, a decline in the major allele N51I-S108N-I164L, which is associated with pyrimethamine resistance in Peru, was observed concomitantly with a rise in frequency of S108N after pyrimethamine was withdrawn in that country (Zhou *et al.* 2008). S108N ranks higher than N51I-S108N-I164L in terms of fitness in the absence of drug, although both mutants do worse than the wildtype, and S108N may thus be expected to out-compete the triple mutant in the absence of drug pressure. N51I-S108N-I164L is more resistant to pyrimethamine than S108N, thus maintaining a higher frequency while drug pressure was being applied. Examination of simulated pathways that contain this particular triple mutant show that S108N is unlikely to be the first step in these pathways. This observation correlates well with the fact that the S108N single mutant occurs on a different haplotype from N51I-S108N-I164L and is thus unlikely to be merely a reversion.

Resistance to chlorcycloguanil may similarly be maintained with compensatory mutations ameliorating the cost of drug resistance, although this finding is less ubiquitous than in the case of pyrimethamine (Costanzo *et al.* 2011). In chlorcycloguanil, the clearest example is that of the double mutant A16V-S108T, also a prime example of sign epistasis, where the initial S108T mutation loses relative fitness in the absence of drug. Fitness is restored by the subsequent addition of A16V. More commonly observed pathways enable the acquisition of resistance to chlorcycloguanil with fewer concessions in fitness in the absence of drug than observed with pyrimethamine, as progression towards chlorcycloguanil resistance frequently follows paths that differ from those followed for pyrimethamine resistance.

An antifolate by any other name: overlapping landscapes reveal distinctive pathways

Antibiotic cross-resistance to drugs of the same class is perhaps an expected occurrence, explained by the similar target, structure, and mode of action in closely related drugs. And, in fact, the description of malaria cross-resistance to antifolates in a mouse system (Thompson and Bayles 1968) is only one interesting aspect of the findings in that early study. An important and unexpected feature of antifolate cross-resistance is that cross-resistance is asymmetrical. Malarial strains selected for chlorcycloguanil resistance showed broader resistance to pyrimethamine than strains selected for pyrimethamine resistance.

The nature of the asymmetry in cross-resistance can be understood in the light of the underlying adaptive landscapes. The adaptive landscapes for pyrimethamine and chlorcycloguanil do overlap, but they are not congruent. For instance, in both systems, the triple mutant N51I-C59R-S108N and the quadruple mutant N51I-C59R-S108N-I164L show high resistance, although the relative degrees of resistance differ. The triple mutant N51I-C59R-S108N is observed in many different parts of the world and pyrimethamine is nearly ineffective against this strain (Wang *et al.* 1997; Nzila-Mounda *et al.* 1998). Chlorcycloguanil is effective against the triple mutant N51I-C59R-S108N, the same combination of mutations that renders pyrimethamine ineffective, while the addition of the mutation I164L leads to failure to clear parasites when treated with either compound (Nzila-Mounda *et al.* 1998; Kublin *et al.* 2002). I164L is observed to often be incorporated as the last mutational step leading to high pyrimethamine resistance (Sibley *et al.* 2001), which corresponds to a high likelihood of it being incorporated last or second to last in simulated trajectories (Lozovsky *et al.* 2009; Brown *et al.* 2010). In contrast, the I164L may be observed at earlier steps in the chlorcycloguanil landscape (Costanzo *et al.* 2011), thus leading to an allele generally more resistant to a broader spectrum of antifolates. Based on the knowledge of evolutionary trajectories for each drug, this type of situation could thus be explain asymmetry in resistance such as that observed in the Thompson and Bayles (1968) study.

Conclusion: transgenic systems reveal evolutionary trends

A theoretical work published in *Journal of Genetics* (Agur and Slobodkin 1986) looked at the interplay between the biological parameter of interest (in our case the *pfdhfr* allele) and the environmental parameter of interest (in our case the specific drug used) and found that the environmental changes affect the shape of the adaptive landscape and thus the pattern of evolution. The adaptive landscapes for pyrimethamine and chlorcycloguanil hold to this supposition and underscore the transcendence of evolutionary principles when properly applied to different systems. While transgenic systems cannot and should not replace *in vivo* experimentation, the tractability and flexibility of such systems should be exploited to inform work done in the parasites. Perhaps most importantly, these systems allow exploration of the patterns and constraints of evolution in such a way as to provide

a glimpse into processes governing the much more complex phenomenon that is drug resistance in nature.

Acknowledgement

This work was supported by NIH grant GM079536 to DLH. MSC was supported by the Mustard Seed Foundation Harvey Fellows Programme.

References

Abdel-Muhsin A. A., Mackinnon M. J., Ali E., Nassir E. A., Suleiman S., Ahmed S. *et al.* 2004 Evolution of drug-resistance genes in *Plasmodium falciparum* in an area of seasonal malaria transmission in eastern Sudan. *J. Infect. Dis.* **189**, 1239–1244.

Agur Z. and Slobodkin L. 1986 Environmental fluctuations - how do they affect the topography of the adaptive landscape. *J. Genet.* **65**, 45–54.

Andersson D. I. 2006 The biological cost of mutational antibiotic resistance: any practical conclusions? *Curr. Opin. Microbiol.* **9**, 461–465.

Brown K. M., Costanzo M. S., Xu W., Roy S., Lozovsky E. R. and Hartl D. L. 2010 Compensatory mutations restore fitness during the evolution of dihydrofolate reductase. *Mol. Biol. Evol.* **27**, 2682–2690.

Chusacultanachai S., Thiensathit P., Tarnchompoo B., Sirawaraporn W. and Yuthavong Y. 2002 Novel antifolate resistant mutations of *Plasmodium falciparum* dihydrofolate reductase selected in *Escherichia coli. Mol. Biochem. Parasitol.* **120**, 61–72.

Costanzo M. S., Brown K. M. and D. L Hartl 2011 Fitness tradeoffs in the evolution of drug resistance in dihydrofolate reductase in *Plasmodium falcipaurm. PLoS ONE* **6**, e19636.

Cowman A. F., Morry M. J., Biggs B. A., Cross G. A. and Foote S. J. 1988 Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum. Proc. Natl. Acad. Sci. USA* **85**, 9109–9113.

Diggens S. M., Gutteridge W. E. and Trigg P. I. 1970 Altered dihydrofolate reductase associated with a pyrimethamine-resistant *Plasmodium* berghei berghei produced in a single step. *Nature* **228**, 579–580.

Gregson A. and Plowe C. V. 2005 Mechanisms of resistance of malaria parasites to antifolates. *Pharmacol. Rev.* **57**, 117–145.

Kublin J. G., Fraction K. Dzinjalamala, Kamwendo D. D., Malkin E. M., Cortese J. F. *et al.* 2002 Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria. *J. Infect. Dis.* **185**, 380–388.

Lozovsky E. R., Chookajorn T., Brown K. M., Imwong M., Shaw P. J., Kamchonwongpaisan S. *et al.* 2009 Stepwise acquisition of pyrimethamine resistance in the malaria parasite. *Proc. Natl. Acad. Sci. USA* **106**, 12025–12030.

Mita T., Tanabe K., Takahashi N., Culleton R., Ndounga M., Dzodzomenyo M. *et al.* 2008 Indigenous evolution of *Plasmodium falciparum* pyrimethamine resistance multiple times in Africa. *J. Antimicrob. Chemother.* **68**, 413–415.

Nzila-Mounda A., Mberu E. K., Sibley C. H., Plowe C. V., Winstanley P. A. and Watkins W. M. 1998 Kenyan *Plasmodium falciparum* field isolates: correlation between pyrimethamine and chlorcycloguanil activity *in vitro* and point mutations in the dihydrofolate reductase domain. *Antimicrob. Agents Chemother.* **42**, 164–169.

Peterson D. S., Walliker D. and Wellems T. E. 1988 Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in *falciparum* malaria. *Proc. Natl. Acad. Sci. USA* **85**, 9114–9118.

Rastelli G., Sirawaraporn W., Sompornpisut P., Vilaivan T., Kamchonwongpaisan S., Quarrell R. *et al.* 2000 Interaction of pyrimethamine, cycloguanil, WR99210 and their analogues with *Plasmodium falciparum* dihydrofolate reductase: structural basis of antifolate resistance. *Bioorg. Med. Chem.* **8**, 1117–1128.

Salverda M. L. M., de Visser J. A. G. M. and Barlow M. 2010 Natural evolution of TEM-1 β-lactamase: experimental reconstruction and clinical relevance. *FEMS Microbiol. Rev.* **34**, 1015–1036.

Salverda M. L. M., Merijn L. M., Salverda E., Dellus F. A., Gorter A. J. M., Debets J. *et al.* 2011 Initial mutations direct alternative pathways of protein evolution. *PLoS Genet.* **7**, e1001321.

Sandefur C. I., Wooden J. M., Quaye I. K., Sirawaraporn W. and Sibley C. H. 2007 Pyrimethamine-resistant dihydrofolate reductase enzymes of *Plasmodium falciparum* are not enzymatically compromised *in vitro. Mol. Biochem. Parasitol.* **154**, 1–5.

Sibley C. H., Hyde J. E., Sims P. F. G., Plowe C. V., Kublin J. G., Mberu E. K. *et al.* 2001 Pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: what next? *Trends Parasitol.* **17**, 582–588.

Sirawaraporn W., Sathitkul T., Sirawaraporn R., Yuthavong Y. and Santi D. 1997 Antifolate-resistant mutants of *Plasmodium falciparum* dihydrofolatereductase. *Proc. Natl. Acad. Sci. USA* **94**, 1124–1129.

Sirawaraporn W., Sirawaraporn R., Cowman A. F., Yuthavong Y. and Santi D. V. 1990 Heterologous expression of active thymidylate synthase-dihydrofolate reductase from *Plasmodium falciparum. Biochemistry* **29**, 10779–10785.

Thompson P. E. and Bayles A. 1968 Reciprocal cross resistance between cycloguanil hydrochloride and pyrimethamine in *Plasmodium berghei* infections in mice. *J. Parasitol.* **54**, 588–593.

Walliker D., Hunt P. and Babiker H. 2005 Fitness of drug-resistant malaria parasites. *Acta Tropica* **94**, 251–259.

Wang P., Lee C., Bayoumi R., Djimde A., Doumbo O., Swedberg G. *et al.* 1997 Resistance to antifolates in *Plasmodium falciparum* monitored by sequence analysis of dihydropteroate synthetase and dihydrofolate reductase alleles in a large number of field samples of diverse origins. *Mol. Biochem. Parasitol.* **89**, 161–177.

Weinreich D. M., Delaney N. F., DePristo M. A. and Hartl D. L. 2006 Darwinian evolution can follow only very few mutational paths to fitter proteins. *Science* **312**, 111–114.

Wooden J. M., Hartwell L. H., Vasquez B. and Hopkins Sibley C. 1997 Analysis in yeast of antimalaria drugs that target the dihydrofolate reductase of *Plasmodium falciparum. Mol. Biochem. Parasitol.* **85**, 25–40.

Zhou Z., Griffing S. M., de Oliveira A. M., McCollum A. M., Quezada W. M., Arrospide N. *et al.* 2008 Decline in sulfadoxine-pyrimethamine-resistant alleles after change in drug policy in the amazon region of Peru. *Antimicrob. Agents Chemother.* **52**, 739–741.

Received 4 April 2011, in revised form 5 May 2011; accepted 9 May 2011
Published on the Web: 19 August 2011