“Snakes and Ladders” of drug resistance evolution

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Malaria is a major public health problem affecting 500 million people each year. Only few anti-malarial drugs are now available for fighting this deadly disease and their effectiveness is alarmingly dwindling because of the drug-resistant strains. Here we discuss recent findings on the evolutionary process behind the gain of a resistant gene. It was shown that for a protein to become resistant to an inhibitor, an intricate stepwise order of mutations must be followed. The projected evolutionary steps were compared with the field data, which reflects the natural history of drug resistant development.

Malaria Drug Resistance

The deadliest form of human malaria is caused by the parasitic protozoan Plasmodium falciparum. The parasite resides in red blood cells and uses hemoglobin as a food supply for producing actively dividing progenies. Their vigorous propagation has become an excellent target for anti-malarials. Dihydrofolate reductase (DHFR) is an enzyme in the folate pathway providing nucleotide building blocks. It exists together with thymidylate synthase (TS) as a bi-functional protein with a non-structural loop linking the DHFR and TS domains. The anti-folate pyrimethamine acts as a competitive inhibitor by replacing the endogenous substrate dihydrofolate from the active site of DHFR.

Like many other anti-malarials, pyrimethamine has now been considered ineffective in most parts of the world. Many P. falciparum strains have gained a series of dhfr-ts mutations abrogating the effectiveness of pyrimethamine. The most resistant form is the quadruple mutant consisting of four mutations located at the DHFR part of the protein, namely, changes of Asn51 to Ile (N51I), Cys59 to Arg (C59R), Ser108 to Asn (S108N) and Ile164 to Leu (I164L). These residues are a part of the active site or located nearby (for structural detail see ref. 1). The mutations block pyrimethamine from binding to the DHFR active site. In term of drug research, this seems to be an open-and-shut case of drug-resistant mutations. But, it also poses an important question from the aspect of evolutionary biology: how did the gene transform from the wild-type to the quadruple mutant? The transition requires four mutations that could follow twenty-four evolutionary pathways via fourteen possible intermediates. Although the issue of evolutionary trajectory has been addressed many times using directed mutagenesis, dhfr-ts evolution provides an unprecedented opportunity to prove the validity of the proposed evolutionary pathways because the data on natural alleles from around the world are available for comparison.

Tracing Protein Evolution

For the parasite to become resistant to pyrimethamine, the mutations need to (1) substantially reduce the binding of the drug to the active site and (2) maintain the substrate-product transition rate. This is a difficult task since both the inhibitor and the substrate share the same active site. Any mutation causing pyrimethamine resistance is likely to compromise enzymatic activity as well. A mutation could also perturb other properties of the protein, viz. stability, folding and synthesis. The spread of pyrimethamine-resistant
In addition, protein evolution is more dynamic, and the intrinsic robustness property for each protein is likely to create different outcomes in the mutational pathway. From the study, the malaria *dhfr-ts* gene can only evolve through three out of the twenty-four available pathways (Fig. 2A).\(^\text{10}\) To become a quadruple mutant (N51I/C59R/S108N/I164L), the first mutation is S108N, which is the only single mutation that slightly improves drug resistance. The second step then can be gained either via N51I/S108N or C59R/S108N mutant. Interestingly, both mutants do exist in nature, which successfully confirms that the evolutionary pathways could be achieved in more than one way. Then, the drug selection pressure would favor N51I/C59R/S108N or C59R/S108N/I164L before reaching the epitome of the resistant alleles, N51I/C59R/S108N/I164L. Even though these four mutations could exist together, certain combinations are not viable (Fig. 2B). For example, the S108N/I164L mutant was found to be extremely unfit indicating severe reduction in enzymatic activity or protein production by this combination.\(^\text{10}\) All three trajectories are consistent with *P. falciparum* *dhfr-ts* alleles collected from around the world, proving the success of this evolutionary analysis approach. Nevertheless, in this Snakes and Ladders game, there are two possible winning squares, high drug-resistant level and maximum fitness, which could be mutually exclusive from one another especially in the condition without drug selection. There is no doubt that the quadruple mutant gives the highest advantage in term of drug resistance, but it also causes reduced overall fitness as shown by a poor propagation rate.\(^\text{10}\) This finding might explain why the quadruple mutant has not yet taken hold in Africa. The quadruple mutant with I164L was recently identified in Kenya,\(^\text{11}\) which could be the result of an evolutionary process in action. But improved fitness and drug resistance do not need to be mutually exclusive. A good example is the S108N mutant, which showed slight improvement in drug resistance, but the change also affected its growth.\(^\text{12}\) The gain of extra mutations to become double mutants (N51I/S108N and C59R/S108N) could improve the

**Figure 1.** “Snakes and Ladders” of protein evolution. Starting from a wild-type protein, mutations that occur in random could either promote or compromise the gain of a new function. The game is a balance between adaptability (ability for protein to gain a new function) and robustness (ability to withstand mutation in order to preserve native function). The effect of epistasis (genetic interaction between mutations) could be compared to landing on Ladder and Snake squares. The winning goal will depend on selective pressure. If the parasites live constantly under the drug pressure, the resistance trait and high fitness will be desirable outcomes. On the contrary, high fitness will be the only goal when the drug pressure is removed. These topics were thoroughly reviewed in references 19 and 20.
growth and the level of drug resistance simultaneously in only one change.12

In addition to specific point mutations, malaria parasites were shown to have copy number polymorphism in short repetitive motifs of many proteins.13-15 The cause and effect of these extra copies still remain elusive. It will also be interesting to extend the trajectory analysis to malarial surface antigens which are highly diverged in order to avoid detection by the host immune.16

The Missing Link in Protein Evolution

With a thoroughly mapped DHFR evolutionary pathway, it is still too early to assume that we finally see the complete picture. Any change in flux caused by a mutation of one enzyme could influence a change at another enzyme. Recent evidence showed that extra copies of GTP cyclohydrolase gene (gch), a rate-limiting enzyme of the folate pathway, were found in many P. falciparum isolates.17,18 The gch gene amplification could be an attempt to boost the overall flux of the pathway. Sooner or later, with a cheaper and faster whole-genome sequencing technology, the mutation trajectories of multiple genes in an ongoing selection process can be mapped. A simple Snakes and Ladders game of drug resistance between one gene and one drug could become a game played on a multi-dimensional landscape.

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