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Plasmodium falciparum malaria: Convergent evolutionary trajectories towards delayed clearance following artemisinin treatment

Prapon Wilairat [a], Krittikorn Kümpornsin [b], Thanat Chookajorn [b,*]

[a] Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand
[b] Genomics and Evolutionary Medicine Unit (GEM), Center of Excellence in Malaria, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

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ABSTRACT

Malaria is a major global health challenge with 300 million new cases every year. The most effective regimen for treating *Plasmodium falciparum* malaria is based on artemisinin and its derivatives. The drugs are highly effective, resulting in rapid clearance of parasites even in severe *P. falciparum* malaria patients. During the last five years, artemisinin-resistant parasites have begun to emerge first in Cambodia and now in Thailand and Myanmar. At present, the level of artemisinin resistance is relatively low with clinical presentation of delayed artemisinin clearance (a longer time to reduce parasite load) and a small decrease in artemisinin sensitivity in cultured isolates. Nevertheless, multiple genetic loci associated with delayed parasite clearance have been reported, but they cannot account for a large portion of cases. Even the most well-studied *kelch 13* propeller mutations cannot always predict the outcome of artemisinin treatment *in vitro* and *in vivo*. Here we propose that delayed clearance by artemisinin could be the result of convergent evolution, driven by multiple trajectories to overcome artemisinin-induced stress, but precluded to become full blown resistance by high fitness cost. Genetic association studies by several genome-wide approaches reveal linkage disequilibrium between multiple loci and delayed parasite clearance. Genetic manipulations at some of these loci already have resulted in loss in artemisinin sensitivity. The notion presented here is by itself consistent with existing evidence on artemisinin resistance and has the potential to be explored using available genomic data. Most important of all, molecular surveillance of artemisinin resistance based on multi-genic markers could be more informative than relying on any one particular molecular marker.

Introduction

> "Nothing in biology makes sense except in the light of evolution", quote from Theodosius Dobzhansky (Jan 25, 1900–Dec 18, 1975).

The global effort to eliminate malaria has been repeatedly thwarted by the spread of multidrug-resistant parasites. During the past half-century, malaria parasites have overcome the antimalarials chloroquine and pyrimethamine–sulfadoxine resulting in millions of lives succumbing to the disease [1]. At present, the current regimen for treating *Plasmodium falciparum*, the most virulent of the four canonical species causing human malaria, is a combination therapy based on the key antimalarial, artemisinin [2]. This drug was discovered in a traditional Chinese herbal medicine derived from sweet wormwood, *Artemisia annua* [3]. Antimalarial activity tests of extracted compounds revealed the active ingredient as a novel sesquiterpene lactone containing an endoperoxide bridge (Fig. 1A) [2].

Artemisinin has become the drug of choice because it kills every red blood cell stage of the parasites, resulting in rapid parasite clearance (within hours) [2]. In life-threatening severe malaria, infected red blood cells lodge inside the human body en masse, making rapid parasite clearance a matter of life or death [4]. Even though the mode of action of artemisinin is not well defined, the presence of the endoperoxide moiety is necessary for its antimalarial function (Fig. 1A) [5]. Scission of the endoperoxide bond allows formation of artemisinin adducts with target molecules, such as those involved in hemoglobin degradation and heme detoxification [6]. Malaria parasites consume red blood cell globins by digesting the protein into short peptides, and the released free heme is packed together into an inert semi-crystalline complex known as hemozoin [7].

Evidence of emerging artemisinin-resistant *P. falciparum* has grown during the past five years. Cases with delayed clearance following artemisinin treatment were first reported in Western

* Corresponding author. Tel.: +66 2 354 4086.
E-mail address: thanat.cho@mahidol.edu (T. Chookajorn).

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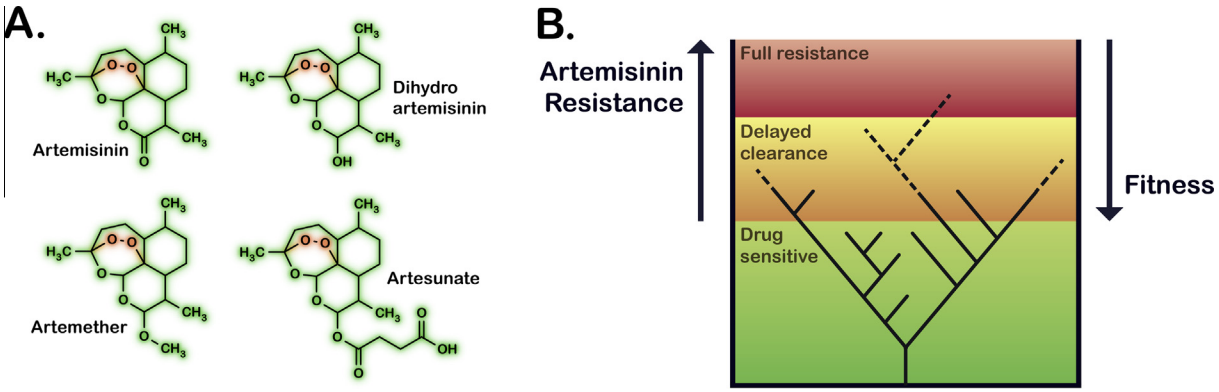

Fig. 1. Artemisinin derivatives and convergent evolution trajectories towards artemisinin resistance. (A) Artemisinin and its derivatives have a sesquiterpene lactone scaffold (light green). The endoperoxide bridge (orange) is a vital moiety for their antimalarial activity. The derivatives have different pharmacokinetic parameters, making them suitable for specific routes of administration. (B) Projection of convergent trajectories. Multiple evolutionary paths are taken to change from wild-type (green area) to resistance, resulting in delayed clearance (yellow area). The evolution trajectories can cross from the green area to the yellow area at multiple points, reflecting the evolutionary process involving multiple alleles. Some paths might incur too much fitness cost and cannot evolve further. The dashed lines represent projections that these parasites can evolutionarily change towards full resistance (red area), but they must overcome fitness tradeoff from the additional resistant mutations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Cambodia in 2009 [8]. The latest epidemiological survey has revealed the spread of parasites with delayed artemisinin clearance phenotype to other parts of Southeast Asia, as far as Western Myanmar [9]. Long-term artemisinin selection of *P. falciparum in vitro* shows a series of genetic mutations being accumulated during the gain of reduced artemisinin sensitivity, one of which is located at *kelch 13*, which, interestingly, was detected in a genome-wide association study as well [10].

P. falciparum kelch 13 appears to be highly polymorphic, with a strong degree of diversification in parasites from Southeast Asia [11–13]. Although transgenic *kelch 13* mutants confer reduced artemisinin sensitivity, parasites from the field containing *kelch 13* mutants do not always show the delayed clearance phenotype, and vice versa [9,12,14]. In addition, SNPs at several other loci are associated, but to a lesser degree, with delayed clearance [12,13].

Change in artemisinin sensitivity is specific to certain stages of malaria parasite intra-erythrocytic maturation. Fluctuation in levels of artemisinin sensitivity specifically occurs during the ring stage [15,16]. Test for artemisinin resistance can be conducted only in young ring-stage parasites. This phenomenon could be interpreted as a form of 'dormancy' that allows the parasites to remain in a metabolic stupor during the relatively short period of drug exposure [17].

Emerging artemisinin resistance has not reached the levels of chloroquine and antifolate resistance [18]. The increase in IC_{50} value is still small, consistent with the delayed clearance phenotype and not that of complete treatment failure. The terms "emerging artemisinin resistance" and "artemisinin tolerance" were coined to describe low but consistent reduction in sensitivity to artemisinin. Because artemisinin resistance is an issue of great importance to global health, the lack of clear resistance phenotype instigated a scientific discourse on the true nature of artemisinin resistance, the most notable being a witty comparison to Aesop's fable of 'the boy who cried wolf' [19]. Nevertheless, both spectrums of the debate share the same concern that outright artemisinin resistance will be catastrophic to the global malaria control and eradication efforts [1,19]. It is undeniable that *P. falciparum* malaria with delayed clearance following artemisinin treatment has been observed in several regions of Southeast Asia [9], but the level of resistance is still relatively low even after more than a decade of clinical use; *P. falciparum* parasites are eventually cleared from infected individuals and small shifts in parasite dose–response growth curve are restricted, as indicated above, to certain stages of parasite development [9,18]. Here we present a hypothesis that could explain the nature of artemisinin resistance in malaria parasites.

Hypothesis

Artemisinin should not be considered as an enzyme inhibitor or poison (as in the case of DNA topoisomerase inhibitors), but as a cause of cell stress [20]. In this "light", the question becomes how cells respond to and survive the stress onslaught. Malaria parasites are exposed to artemisinin in the human body for only a short period of time (a few hours at most), and ring-stage parasites that have evolved strategy(ies) to weather the storm will survive. A delay in ring maturation suggests a state of dormancy, an inherent feature of malaria parasites (e.g. sporozoite in mosquito salivary gland and possibly hypnozoite in liver) [21]. Shutting down metabolism leads to minimal protein biosynthesis, and thereby reduction in protein targets for artemisinin adduct formation, and which can readily be replaced once normal cellular activity resumes.

The evolutionary pathways that malaria parasites have to take in order to arrive at the desired phenotype (delayed clearance) will be many, and thus it would be premature to settle on a particular genetic marker as being "the" marker of emerging artemisinin resistance in malaria parasites (Fig. 1B). For the sake of analogy, it is comparable to the situation of beta-thalassemia [22], where the beta-thalassemia phenotype is easily identified: reduced biosynthesis of beta-globin relative to alpha-globin. However, this stems from a plethora of mutations in beta-globin gene (in exons, introns and promoter), reflecting the various evolutionary pathways taken to produce the same phenotype, and different populations have their own basket of mutations. A predominant beta-thalassemia mutation in a given population is unlikely to be a global genetic marker of beta-thalassemia.

Evaluation of the hypothesis

Supporting evidence for the possibility that emerging artemisinin resistance in malaria parasites is the result of convergent evolutionary pathways aimed at reducing stress from artemisinin can be divided into two groups: lack of selective sweep and additional genetic mutations conferring reduced artemisinin sensitivity.