Trends in Parasitology

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Spotlight

Plasmodium falciparum artemisinin resistance: something gained in translation

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Small-Saunders *et al*. uncovered a new facet of artemisinin resistance in *Plasmodium* in which parasites use a previously underexplored arm of stress response mechanisms. Through altered epitranscriptomic modifications on tRNA, changed translation patterns adapt resistant cells to facilitate entry into a quiescent-like state which provides the parasite an escape from many drugs.

Malaria continues to represent one of the major global health threats chiefly affecting the tropical and subtropical areas of the world. Despite over a century of research seeking to both understand and combat (eliminate) the disease, and some limited success, the rate of infection and mortality is currently on the rise [1]. The chief culprit is *Plasmodium falciparum*, an intracellular protozoan parasite transmitted by the females of a number of widely distributed anopheline mosquito species. The complex life cycle of the parasite offers many targets for intervention, with the chief avenues being vaccines and drugs. Whilst helpful and once (laboriously and expensively) licenced, neither have been hugely successful to date with vaccines facing the issues of efficacy and delivery, whilst drugs typically select for resistant parasites with distressing ease. Of the small number of currently employable drugs, the potent but swiftly metabolised active metabolite of artemisinin (dihydroartemisinin, DHA) is the frontline offering but nowadays always delivered in combination with one of a number of independent antimalarials that offer much longer bioavailability. Even these combinations are experiencing resistance [2] in part because artemisinin and all of its partners were initially used as monotherapies which unbolted the stable door to the development of resistance [3,4].

Artemisinin has been a wonderful drug, with many properties that are ideal. It has nanomolar potency, is activated *in situ* in the parasite through interaction with free haem, and the resulting free-radical form indiscriminately alkylates the first protein it encounters, leading to random and general proteotoxic and oxidative damage. Resistance occurs only in the young ring-stage asexual forms as a result of the relatively limited activation of the drug due to its equally relatively limited capacity for haemoglobin (Hb) uptake (and subsequent generation of free haem in the food vacuole of the parasite). The defining outward characteristic of an artemisinin-resistant parasite is a prolonged ring-stage period in which cells become either quiescent or grow slowly enough for long enough for the drug to be cleared from the bloodstream [5].

Artemisinin resistant (ART^R) parasites exhibit mechanisms that tend towards [6]:

(i) minimising the potential for artemisinin-induced damage through reduced activation of the drug;
(ii) facilitating repair of the damage caused through cell cycle arrest.

(i) Reduced activation of the drug is most straightforwardly achieved by reducing ring-stage Hb uptake and subsequent production of free haem. This is achieved by an accumulation of mutations in a Kelch domain protein, K13, which, through its location at the cytostome at the plasma membrane end of an engulfing vacuole, organises vacuolar delivery of membrane-engulfed Hb. Effective mutations destabilise K13 and thus the delivery complex, reducing uptake.

(ii) Upregulation of cellular damage-repair activities (unfolded protein response, UPR, proteasome degradation, and stress responses) can also enhance parasite recovery. In combination with reduced Hb uptake, enhanced repair facilitates sufficiently rapid degradation of damaged proteins and restoration of the normal protein complement and survival.

Thus, resistant parasites mount a complex but straightforward response, but this is also not the whole story. Only a proportion of the mutant K13 cells survive artemisinin challenge, and some appear to have entered true quiescence, a phenomenon known as persistence that is well established in bacterial genetics [7]. In essence, true persistence requires no heritable mutation at all; however, quiescence combined with direct resistance mechanisms provides an even more effective route to survival. But what does quiescence/persistence look like? An obvious mechanism that leaves no heritable fingerprint could involve epigenetic means. Epigenetics is an established feature of the *Plasmodium* lifestyle that permits bet-hedging which contributes to antigenic variation, commitment to sexual development, and variation of nutrient channels [8]. These outcomes are a result of the reshaping of the chromosomal environments surrounding the relevant genes yet leave no trace when genomic DNA is characterised in isolation. An underappreciated arm of epigenetics is epitranscriptomics – the chemical modification and functional modulation of RNA [9]. The first study of epitranscriptomics in *Plasmodium* looked at the role of adenosine methylation (M^6A) that was shown to be developmentally regulated, negatively associated with mRNA stability and translational efficiency. Furthermore, M^6A specific binding proteins effect translational inhibition, clearly demonstrating the potential of yet another level of (programmed) developmental regulation in *Plasmodium*.

The recent article by Jennifer Small-Saunders and colleagues sheds yet more light on both the biology of RNA modification and the complexities of drug-resistance mechanisms [10]. The work further highlights the innate flexibility of parasite (organismal) biology that makes successful drug discovery and implementation so difficult. The working RNA species involved in protein production (rRNA and tRNA) are highly modified after transcription (hundreds of modifications have been identified) which is essential for their healthy function [11]. In *P. falciparum*, it has been demonstrated that cytosine methylation of $tRNA^{Asp\ (GTC)}$ is critical for parasite homeostasis, and a gene knockout-induced inability to make that modification rendered the parasite both more sensitive to stress and more likely to commit to gametocytogenesis [12]. Furthermore, earlier work from the Preiser group had established that blood-stage asexual *P. falciparum* exhibits a developmentally co-ordinated pattern of tRNA modifications, cataloguing 22 synchronous modifications [13]. Here, the authors examine the modifications of tRNA species in *P. falciparum* resulting from sustained artemisinin challenge in both ART^R and ART^S backgrounds. There were already clues that tRNA biology might shape the parasite response to artemisinin as an increase in U34 tRNA-modifying enzymes was observed following drug challenge [14]. The authors first established that ART^R parasites (strain $Dd2^{R359T}$) do indeed manifest a globally reduced tRNA modification profile following artemisinin challenge when compared with ART sensitive (ART^S) isogenic Dd2 parasites. Interestingly, the two changes that reached significance, mcm^5s^2U and mCm on position 34, also have biosynthetic pathways, which implies that a regulated response of ART^R parasites to a specific drug challenge might be occurring. Furthermore, the proteome of ART^R and ART^S parasites also differed in the absence of drug challenge, implying rewiring of the parasite as a consequence of evolving ART^R status. ART^R parasites upregulated UPR-associated proteins and in the presence of drug demonstrated a proportional reduction in general translational capacity. This implies that damaged proteins could be removed rapidly whilst the cell simultaneously limits the production of new targets for drug-induced damage hastening recovery. Furthermore, it should result in slow growth and possibly promote entry into quiescence, a helpful outcome to avoid short-lived ART. The *P. falciparum* genome is an overtly (93%) AT-rich eukaryotic genome and so codon bias is inherent in gene composition. Pairs of codons that differ by A/G or C/T in their third position typically exhibit a ratio of 4 or 5 to1 in *P. falciparum*, and any search must look for extreme bias beyond that base level. Remarkably, examination of codon usage in the upregulated and downregulated proteins in these datasets still identified three codons that were significantly associated with the affected proteins and, in particular, $Lys^{AAA/AAG}$ with the former associating with upregulated proteins and the latter with downregulated ones. The circle between tRNA reprogramming and cell biological outcome was completed with the realisation that the differentially regulated mcm^5s^2U modification occurs on the U34 (Figure 1) wobble position of $Lys^{AAA/AAG}$ codons and ensures translational fidelity. For a subset of these regulated proteins, further analysis eliminated the remaining possibility that transcriptional alterations might be influencing protein abundance. Indeed, in some cases transcript and cognate protein abundance were inversely related, highlighting the potential power and specificity of

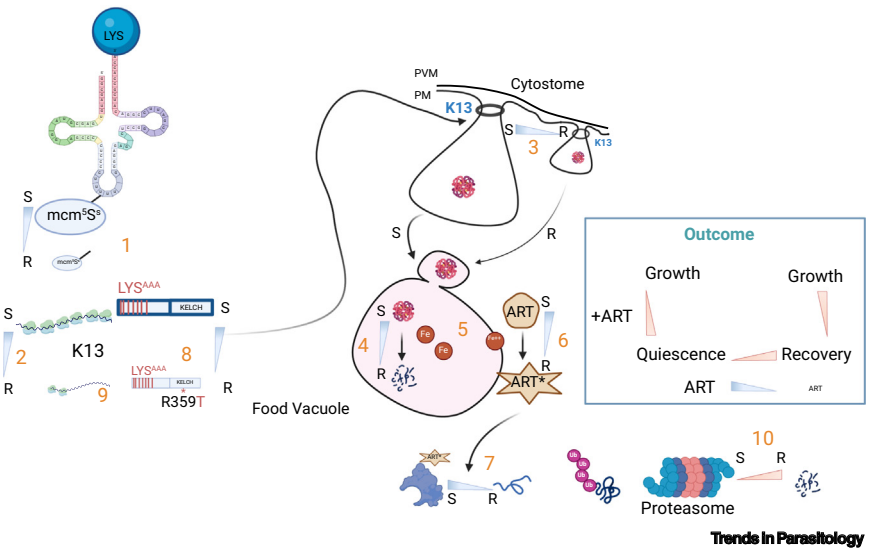

Figure 1. Potential impact of the epitranscriptomic $mcm^5S^stRNA^{LYS}$ insufficiency on the artemisinin resistance profile of parasites bearing the K13 R359T mutation. In the Dd2 SE Asian background of *Plasmodium falciparum* the selection (possibly through pressure of artemisinin exposure) of $mcm^5S^stRNA^{LYS}$ insufficiency is favourable to the entry into quiescence (1). Insufficiency reduces the ability to synthesise K13 (2) due to a preponderance of Lys^{AAA} codons (red bars, that contain the mcm^5S^s modification) towards the N terminus of K13, leading to slower growth. Slower growth results from reduction in the ability to form a cytostome (3) and engulf haemoglobin (Hb) which is digested to liberate amino acids for parasite protein synthesis (4). Consequently, there is reduced free Fe^{2+} (5) which, in turn, leads to reduced activation of artemisinin (6) and cellular damage (7) accompanied by an increased tendency to enter quiescence. The superimposition of K13 R359T amplifies this tendency due to the resulting increase in K13 instability (8) which is further amplified by a downregulation of *k13* transcription (9). Work independent of the study under discussion has shown that a metabolic rewiring can accompany the acquisition of artemisinin resistance – which results, amongst other features, in an upregulation of proteasomal capacity (10) that allows the parasite to reset protein homeostasis relatively rapidly whilst the quiescent cell rides out the short-lived artemisinin challenge [14]. The red asterisk indicates ART resistance conferring mutation in K13. The black asterisk indicates Fe^{++}-activated artemisinin. Abbreviations: ART, artemisinin; PM, plasma membrane; PVM, parasitophorous vacuolar membrane. Figure created with BioRender.

translational regulation. Pleasingly, K13 was one of the proteins that fitted all criteria of being downregulated at the transcriptional level, upregulated in the proteome, AND also exhibited overt codon bias for Lys^{AAA}. This would allow a coordinated increase in K13 levels on demand, for example at the (promotion of the) cessation of quiescence.

The biosynthetic machinery for tRNA modification that is known to affect the levels of Lys codon-biased proteins in yeast is also present in *Plasmodium*. The authors then addressed the effect of disrupting the apparatus that generates s^2U modifications which is upregulated in DHA-treated ART^R $Dd2^{R359T}$. Conditional knock down (cKD) of tRNA 2-thiourilydase (PfMnmA) in an independent (NF54) ART^S background demonstrated the essentiality of this modification and consequent reduction in mcm^5s^2U modified tRNA species. The authors were still able to test ART sensitivity in the slowly dying parasites and confirmed, as expected, that an increasing lack of mcm^5s^2U-modified tRNA contributed to increasing resistance to DHA exposure. The authors also tested additional drugs in the $PfMnmA^{cKD}$ background which gave nuanced outcomes ranging from increased sensitisation of apicoplast targeting drugs (e.g., fosmidomycin), and increased resistance to mitochondrial targeting drugs (atovaquone). Critically, they also tested ART combination therapy (ACT) partner drugs which showed no change in survival rates, validating the concept of ACT.

In summary, the authors have exposed another layer of the onion that factors into ART resistance. In ART^R parasites, codon bias and manipulation of the availability of fully formed specific tRNA species are combined to reduce the synthesis of mutated, more labile K13. This in turn 'starves' the parasite by reducing Hb uptake while simultaneously reducing the capacity of ART to be activated and cause damage. The starved parasite also grows more slowly and future work may reveal a greater tendency of such parasites to enter quiescence. However, this mechanism of translational adaptability has to be set in terms of the natural history and evolution of *P. falciparum*. If we focus on K13 presumably this mechanism evolved long before it offered a means to escape challenge by artemisinin. Global comparisons of the plethora of *P. falciparum* genomes confirm that the K13 $Lys^{AAA/AAG}$ codon bias is universal and therefore translational regulation of K13 is possible regardless of ART^R status. Perhaps there are other circumstances where regulation of ingestion of Hb might need flexibility – for example, later on in the asexual cycle and the natural transition to growth in the trophozoite. More generally, the implications of the mechanism are exciting in terms of our broader understanding of proteome composition. The ability to selectively regulate protein production through the combination of codon bias and tRNA modification distribution and frequency might be applied to many biological situations and provide a further mechanism to achieve bet-hedging. The apparatuses to generate these many modifications could be subjected to many layers of well-known gene regulation, resulting in a complex network that modulates translational efficiency according to situational expedience, generating yet more phenotypic plasticity. The battle continues.

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Declaration of interests

The authors declare no competing interests.

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