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Current Opinion in Pharmacology

Mechanisms of artemisinin resistance in *Plasmodium falciparum* malaria

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Artemisinin-based combination therapies (ACTs) have substantially reduced worldwide malaria burden and deaths. But malaria parasites have become resistant to artemisinins. Prior studies suggested two different molecular pathways of artemisinin-resistance. Here we unify recent findings into a single model, where elevation of a lipid, phosphatidylinositol-3-phosphate (PI3P) results in vesicle expansion that increases the engagement with the unfolded protein response (UPR). Vesicle expansion (rather than increasing individual genetic determinants of the UPR) efficiently induces artemisinin resistance likely by promoting 'proteostasis' (protein translation coupled to proper protein folding and vesicular remodeling) to mitigate artemisinin-induced proteopathy (death from global abnormal protein-toxicity). Vesicular amplification engages the host red cell, suggesting that artemisinin resistant malaria may also persist by taking advantage of host niches and escaping the immune response.

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Current Opinion in Pharmacology 2018, **42**:46–54

This review comes from a themed issue on **Anti-infectives**

Edited by **Timothy Egan** and **Digby Warner**

https://doi.org/10.1016/j.coph.2018.06.003

Introduction

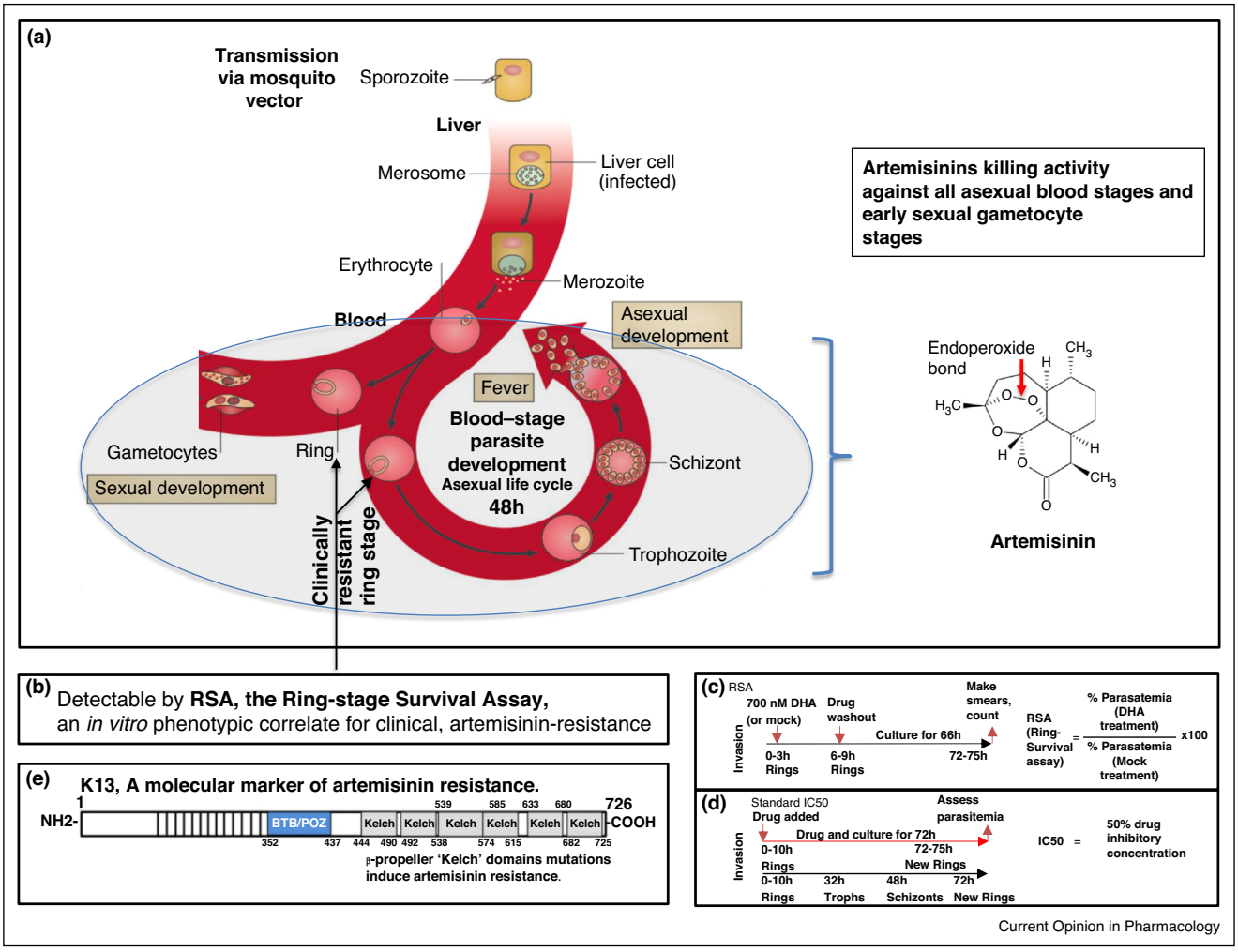
World-wide levels of malaria have substantially decreased over the last decade. Notably, twenty-one countries are expected to have eliminated the disease by 2020 [1]. Yet a substantial burden remains. In 2016, 445 000 deaths and 216 million new cases were caused by *Plasmodium falciparum*, the most virulent of human malaria parasites [1]. Artemisinin-based combination therapies (ACTs) are frontline, fast acting drugs that have been key to reducing the malaria burden and deaths. However, *P. falciparum* resistance to artemisinins and ACTs has emerged, threatening global malaria control and elimination.

P. falciparum infection in humans is initiated when an infected mosquito bites to release sporozoites that infect liver cells. In the liver, sporozoites develop into merozoites which emerge into the blood stream to invade erythrocytes (red blood cells) and develop through distinct ring, trophozoite and schizont stages over a 48 hour asexual life cycle (Figure 1a). The blood stages of infection cause all of the symptoms and pathologies of malaria (and are thus targeted by all antimalarials in current use). Parasite killing by artemisinins depends on cleavage of their endoperoxide bond (Figure 1a). Proteomic studies indicate that artemisinins alkylate hundreds of proteins [2••,3••] suggesting they may kill by inducing 'proteopathy' or global degeneration of the parasite's (proteinaceous) cytoplasm. Resistance to artemisinins observed in the clinic, is seen in ring parasites that show 'delayed clearance' from the circulation, in response to direct administration of drug to patients [4,5]. Trophozoite and schizont stages that sequester in tissues are not found in circulation and hence not detected in blood smears used to monitor parasites [6].

The parasite gene *pfkelch13* (K13) is the primary marker of artemisinin resistance as defined both clinically and *in vitro* using the Ring-stage Survival Assay (RSA; [6] Figure 1b). In the RSA, parasites are treated at maximal concentrations of artemisinins achieved in plasma (~700 nM) for 6 hours to mimic pharmacological drug exposure seen in patients (Figure 1c). Notably, only the earliest stages of rings (0–3 hour in the life cycle) manifest artemisinin resistance, while later stages (including 9–12 hour and 18–21 hour rings) do not [6]. Moreover, *in vivo* clinical artemisinin resistance cannot be gauged in a standard *P. falciparum* IC50 assay (Figure 1d, carried out over 72 hours of continuous drug pressure for effects on parasite proliferation through the trophozoite/schizont stages).

Based on its sequence and structure, K13 is predicted to a substrate adapter of an E3 ligase [7] (Figure 1e). Its mammalian orthologues (the best characterized of which is Keap1 [7]) target binding, ubiquitination and proteosomal degradation of select substrates, keeping their levels low to maintain proper cellular homeostasis. Mutations in the β-propeller 'Kelch' domain diminish targeting and raise substrate levels in a cell. In cancer, K13 orthologues confer resistance to drugs that kill by inducing 'proteopathy' in tumors [8]. In *P. falciparum*, two major K13 effector mechanisms have been proposed to overcome artemisinin-induced

Figure 1



Life cycle of *Plasmodium falciparum* and measures of artemisinin drug resistance. **(a)** (Adapted from Delves, M., Scheurer, C, *et al*. 2012 [48]). *P. falciparum* malaria infection in humans is initiated when an infected mosquito bites and releases sporozoites that infect liver cells and develop into merozoites. These merozoites emerge into the blood and invade erythrocytes (red blood cells), where they develop over a 48-hour asexual life cycle through morphologically defined ring, trophozoite and schizont stages. Schizont lysis leads to the release of daughter merozoites (which coincides with fever), to initiate a new asexual life cycle. A small subpopulation of ring-form parasites develops into sexual gametocyte stages which are taken up by the mosquito during a blood meal. The killing activity of artemisinins (against asexual and early sexual gametocyte stages) is dependent on the cleavage of their endoperoxide bond. Clinical resistance to artemisinins is seen at the parasite ring stage. **(b)** Clinical artemisinin resistance is measured *in vitro* by the Ring-stage Survival Assay (RSA). **(c)** In the RSA, ring-stage parasites 0–3 hour, are treated with maximal concentrations (700 nM) of dihydroartemisinin (DHA, the active form of all artemisinins) seen in plasma for 6 hours, after which the drug is washed out, mimicking pharmacological exposure seen in patients. Parasites are subsequently allowed to progress for another 66 hours, after which parasitemia is determined. The RSA value is calculated as shown. **(d)** The standard IC50 assay is carried out by exposing ring, trophozoite and schizont stages to continuous drug treatment over 72 hours. **(e)** *Plasmodium falciparum* K13 (*Pf*Kelch13) is a causal molecular marker of artemisinin resistance. It contains a single BTB domain (common to all Broad-complex, Tramtrack and Bric-a-bric domain (BTB) family proteins), which is present at the amino terminus, followed by multiple copies of β-propeller 'Kelch' repeats. K13 contains six β-propeller Kelch domains, mutations in which induce artemisinin resistance.

proteopathy and death. They are namely, first, proteostatic dysregulation of parasite phosphatidylinositol-3-kinase resulting in elevation of parasite PI3P [9] and second, upregulation of parasite oxidative stress and protein damage pathways via the unfolded protein response (UPR) [10]. In this review we discuss the most recent advances and convergence of mechanisms in a unified model of K13-dependent and independent states of artemisinin resistance, with implications for resistance to partner drugs as well as new emergent antimalarials. We conclude by deliberating on how understanding mechanisms of resistance sheds critical insights into challenges that need to be overcome to eliminate parasites from regions with high and low