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Feature Review

Artemisinin Action and Resistance in *Plasmodium falciparum*

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The worldwide use of artemisinin-based combination therapies (ACTs) has contributed in recent years to a substantial reduction in deaths resulting from *Plasmodium falciparum* malaria. Resistance to artemisinins, however, has emerged in Southeast Asia. Clinically, resistance is defined as a slower rate of parasite clearance in patients treated with an artemisinin derivative or an ACT. These slow clearance rates associate with enhanced survival rates of ring-stage parasites briefly exposed *in vitro* to dihydroartemisinin. We describe recent progress made in defining the molecular basis of artemisinin resistance, which has identified a primary role for the *P. falciparum* K13 protein. Using K13 mutations as molecular markers, epidemiological studies are now tracking the emergence and spread of artemisinin resistance. Mechanistic studies suggest potential ways to overcome resistance.

Trends

Heme-activated artemisinin indiscriminately targets parasite biomolecules.

Stage- and exposure time-dependence of artemisinin killing underpins differential parasite response rates.

Mutations in the propeller domain of the *P. falciparum* Kelch-like protein K13 are central to artemisinin resistance.

K13 mutations appear to be associated with an enhanced cell stress response.

Targeting the *P. falciparum* proteasome might thwart K13-mediated artemisinin resistance and presents a strategy to block its spread.

Artemisinins: Front-Line Antimalarials under Threat

In 2015, the malaria parasite *Plasmodium falciparum* killed over 400 000 people, most of whom were children under the age of 5 years [1]. Thus, it is of acute concern that resistance to the **artemisinin** derivatives (ARTs; see Glossary), the first-line drug class used to treat malaria, emerged several years ago, and is now evident in six countries in Southeast Asia. If resistance spreads to India and Africa, a major health crisis is feared. The WHO has warned: 'There is a limited window of opportunity to avert a regional public health disaster, which could have severe global consequences'. This review discusses recent insights into how ARTs kill malaria parasites, and how *P. falciparum* achieves resistance. We also examine potential ways to improve treatment outcomes in regions where resistance is established and to slow the spread of resistance.

ART (also known as Qinghaosu; Figure 1) is a sesquiterpene lactone produced by the Chinese medicinal herb *Artemisia annua*. Activation of the **endoperoxide bridge** of this drug is essential for antimalarial activity [2]. Semisynthetic **lactol derivatives** such as **dihydroartemisinin** (DHA), artesunate, and artemether (Figure 1) exhibit improved bioavailability and efficacy [3]. DHA is the active *in vivo* metabolite of all clinically used ARTs [4]. ARTs are exceptionally fast-acting against intraerythrocytic asexual blood-stage malaria parasites, effecting up to 10 000-fold reductions in parasite burden every 48 h. This pharmacodynamic hallmark is of critical benefit in treating severe malaria and reversing its otherwise lethal course [5,6]. An inherent disadvantage of ARTs is their very short *in vivo* half-lives (typically ~1 h in humans [5,7]). As a result, ARTs are coadministered with longer half-life partner drugs, such as lumefantrine, amodiaquine, piperaquine, mefloquine, sulfadoxine–pyrimethamine, or pyronaridine, in **ART-based combination therapies** (**ACTs**). These combinations help prevent recrudescence (which can occur even after 5 days of ART **monotherapy**) and are employed to slow the

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development of parasite resistance (Box 1) [8,9]. An important goal in the malaria chemotherapy field is to develop compounds that benefit from longer plasma half-lives yet achieve the same rapid parasite killing as ART derivatives through a similar mode of action. These objectives appear to have been achieved with the ozonides (or 1,2,4-trioxalanes), a class of fully synthetic endoperoxide antimalarials (Figure 1). Two members of this class, namely OZ277 and OZ439, have been evaluated in humans [10–14]. The combination of OZ277 (arterolane) and piperaquine, known as Synriam™, is available in India. The usefulness of OZ277 may be limited however, as its half-life is only 2- to 3-fold longer than that of DHA [15], and it is reported to have lower plasma exposure in malaria patients than in uninfected volunteers [16]. OZ439 (artefenomel) is currently being assessed either alone or as a **combination therapy** in Phase II human clinical trials ([12,17,18]; ClinicalTrials.gov NCT02083380). Ongoing studies are also investigating whether OZ439 (whose terminal half-life is 46–62 h [17,19]) can overcome resistance to ARTs (see later) and provide an effective drug for new antimalarial combinations [12,14].

ARTs Are Activated to Cytotoxic Species *In Situ*

ART activation is thought to involve iron-catalyzed reductive scission of the endoperoxide bond, generating carbon-centered radicals that react with susceptible groups in parasite proteins and other biomolecules [20,21] (Figure 2). The major source of iron in parasites, which develop inside a parasitophorous vacuole inside infected erythrocytes, is thought to be in the form of heme species that are liberated as a result of plasmodial protease-mediated degradation of imported host hemoglobin. Most of this proteolysis occurs during the **trophozoite stage** when parasites endocytose large quantities of hemoglobin from the erythrocyte into an acidic digestive vacuole [22–24]. Although most of this pool of highly reactive heme is sequestered via incorporation of Fe^{3+}-heme dimers (known as β-hematin) into chemically inert hemozoin crystals [25], some heme is available in the reduced Fe^{2+} form to activate ARTs [2,25,26] (see later). ARTs are less active against mid-**ring-stage parasites** than against trophozoites [27], and are inactive against mature (stage V) gametocytes [28] and liver stages [29], consistent with decreased or absent hemoglobin digestion at these stages. However, ARTs are active against very early asexual ring-stage parasites, just after erythrocyte invasion [27,30]. This finding suggests that early rings might already import and digest host hemoglobin, even before formation of the digestive vacuole where most of the hemoglobin is degraded during the trophozoite stage. In support of this concept, ART action can be substantially mitigated in very early rings as well as trophozoites by inhibiting falcipains, which proteolytically cleave host hemoglobin and release heme-iron [30]. Recent evidence suggests that biosynthetic heme produced by the parasite might also contribute to ART activation in very early rings [31–33].

Another secondary activator of ARTs might be reduced (Fe^{2+}) iron that is not heme-bound. *P. falciparum* maintains a low steady-state labile iron pool [34]. Additional iron may be released via hydrogen peroxide-mediated degradation of heme in the digestive vacuole [35] or by reaction with reduced glutathione in the parasite cytoplasm [36]. Iron chelators weakly antagonize ART activity [30,37,38]. However, this antagonism is much less potent than that observed with hemoglobinase inhibitors, providing further evidence that heme-iron rather than free iron is the main activator. At present, the heme-iron hypothesis seems more compelling than earlier reports suggesting alternative mechanisms for activation, such as cofactors involved in maintaining redox homeostasis [39,40]. Nonetheless, it remains possible that factors in addition to heme-iron or other iron sources might also contribute to ART activation.

ART-Mediated Killing is Stage- and Exposure Time-Dependent

The level of potency of short pulses of ARTs against *P. falciparum* asexual intraerythrocytic parasites *in vitro* is notably impacted by the stage of parasite development [27,41]. Interestingly, this stage specificity appears to reflect altered temporal responses to ARTs rather than differences in intrinsic sensitivity. When exposed to short (physiologically relevant) pulses of ARTs, mid-ring-stage 3D7

Glossary

Artemisinin: also known as Qinghaosu; a constituent of the Chinese medicinal herb *Artemisia annua*. Its semisynthetic derivatives generally possess improved pharmacokinetic characteristics.
Artemisinin-based combination therapies (ACTs): these drugs pair a short-lived ART derivative with a second drug with a longer *in vivo* half-life to maximize treatment efficacy. Appropriate combinations should also delay the acquisition of parasite resistance compared with isolated use of either drug. Triple combination ACTs are currently in development.
Combination therapy: employing multiple drugs (normally two for malaria) to maximize treatment efficacy, delay emergence of drug-resistant parasites, and in some mixtures, kill dormant or transmissible forms of parasites.
Dihydroartemisinin (DHA): the active *in vivo* metabolite of clinically used ARTs.
Endoperoxide bridge: a peroxide (-O-O-) group that bridges two carbons that are both part of the ART molecule.
K13: *P. falciparum* Kelch protein K13 (gene ID: PF3D7_1343700), the primary determinant of *P. falciparum* ART resistance in Southeast Asian parasites.
Lactol derivatives: DHA is the reduced lactol derivative of ART, formed when the cyclic carbonyl group of ART is reduced to a hydroxyl group. The semisynthetic derivatives (artemether, arteether, artesunate, and artelinate) are ethers or esters of the lactol.
Monotherapy: employing a single drug as a disease treatment.
Parasite clearance: the disappearance of parasites from blood circulation, as measured by a given assay (e.g., PCR, blood smear). Clearance is often reported in terms of its half-time. The clearance half-time is the number of hours required for the parasitemia to decrease by half during the log-linear phase of parasite reduction.
Pyknotic morphology: characterized by chromatin condensation in the nucleus.
Ring-stage parasites: the early phase of the intraerythrocytic asexual life cycle, defined as the period after invasion of red blood cells, but prior