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Vesicular mechanisms of drug resistance in apicomplexan parasites

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SUMMARY Vesicular mechanisms of drug resistance are known to exist across prokaryotes and eukaryotes. Vesicles are sacs that form when a lipid bilayer 'bends' to engulf and isolate contents from the cytoplasm or extracellular environment. They have a wide range of functions, including vehicles of communication within and across cells, trafficking of protein intermediates to their rightful organellar destinations, and carriers of substrates destined for autophagy. This review will provide an in-depth understanding of vesicular mechanisms of apicomplexan parasites, Plasmodium and Toxoplasma (that respectively cause malaria and toxoplasmosis). It will integrate mechanistic and evolutionarily insights gained from these and other pathogenic eukaryotes to develop a new model for plasmodial resistance to artemisinins, a class of drugs that have been the backbone of modern campaigns to eliminate malaria worldwide. We also discuss extracellular vesicles that present major vesicular mechanisms of drug resistance in parasite protozoa (that apicomplexans are part of). Finally, we provide a broader context of clinical drug resistance mechanisms of Plasmodium, Toxoplasma, as well as Cryptosporidium and Babesia, that are prominent members of the phyla, causative agents of cryptosporidiosis and babesiosis and significant for human and animal health.

KEYWORDS artemisinin, drug resistance mechanisms, apicomplexan parasites, vesicular trafficking

INTRODUCTION

Apicomplexa are a vast phylum of eukaryotic microbes with both free-living and parasitic forms that evolved from a photosynthetic ancestor (chromerids) and are closely related to free-living single-cell predators (colpodellids) (1). Research is primarily

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in mechanisms of disease-causing organisms that actively engage their target host cells. Here, we will review parasites that cause malaria, toxoplasmosis, cryptosporidiosis, and babesiosis that are important as historical and emerging diseases caused by apicomplexans.

Although global levels of malaria have declined, the disease continues to be a major threat in many tropical and subtropical regions. The World Health Organization estimated 249 million cases and 608,000 deaths due to malaria in 2022 (2). This reflects an increase compared to levels in 2021 and 2020. Malaria control programs are particularly concerned about the emergence of resistance to drugs and insecticides (as well as unexpected disruption of services due to natural disasters, such as the coronavirus disease 2019 pandemic, climate change, and social and political instability) (3, 4). In contrast to malaria, toxoplasmosis is found throughout the world. In the US, it is the leading cause of death due to illness caused by ingestion of food (5). The infection can remain dormant for long periods of time (as has been extensively reviewed [6]). But symptoms may arise in people with immune deficiencies (including pregnant women), and severe cases show neurological, ocular, and other organ damages (7). Infections occur principally in humans, birds, and other warm-blooded animals by consumption of food contaminated with oocysts shed in cat feces (8). The sexual part of the *Toxoplasma gondii* life cycle only unfolds in cats both in domestic and wild members of the Felidae family (9). Cryptosporidiosis causes frequent watery diarrhea in children with enormous burden in Asia and Africa (10). Contaminated water, animals, or people transmit infection, which worsens to chronic disease in the presence of immunosuppression: severe life-threatening diarrhea occurs in individuals with advanced acquired immunodeficiency syndrome (11). *Cryptosporidium* infections are endemic worldwide, but their prevalence is substantially higher in low- and middle-income countries (12). Babesiosis is an infection of red blood cells caused by infected tick , called *Ixodes scapularis* bites, typically at the nymph stage (13). The symptoms can resemble those of malaria (fever, chills, head, and body ache). Many cases of babesiosis are asymptomatic, but the infection can be fatal in people with weakened immune systems or without a spleen. Although its incidence is increasing worldwide (13), babesiosis is most prevalent in the US and Europe and is also an infection of cattle. Therefore, apicomplexans are not unified by their diverse biological and pathological niches or modes of transmission. All members share a common morphology bequeathed by a group of structures and organelles collectively termed as the apical complex that is a set of ultrastructural and functionally homologous components generally found at the anterior end of certain stages but most notably the infective stages. They also contain a non-photosynthetic apicomplexan plastid apicoplast (which, however, is absent in *Cryptosporidium* [14]) (Fig. 1).

Vesicular mechanisms of apicomplexan parasites and their implications for drug resistance

The apical complex comprises three sets of secretory organelles, namely, the micronemes, rhoptries, and the dense granules (Fig. 1A). A plasma membrane vesicular structure, called the micropore, although not part of the apical complex, is also conserved across apicomplexans (Fig. 1A). In the extracellular, invasive 'merozoite' stage, micronemes discharge secretory vesicles with parasite ligands that target host cell surface receptors to create molecular attachments to initiate the entry process. As a second step, secretory vesicles from the rhoptries deliver their content at the zone of engagement to create and enlarge a nascent parasitophorous vacuole (PV) in the host cell into which the parasite can enter and be separated from defense mechanisms of the host cytosol and trafficking processes. Discharge from the dense granules occurs after rhoptry release. Micropores are not known to discharge their content or play a role in apical secretion. PV formation requires active actin-driven processes of the parasite (16, 17), confirming that the cytoskeletal propulsion force needed to form the new vacuole is driven by the pathogen and not the host (which is in contrast to the entry of many

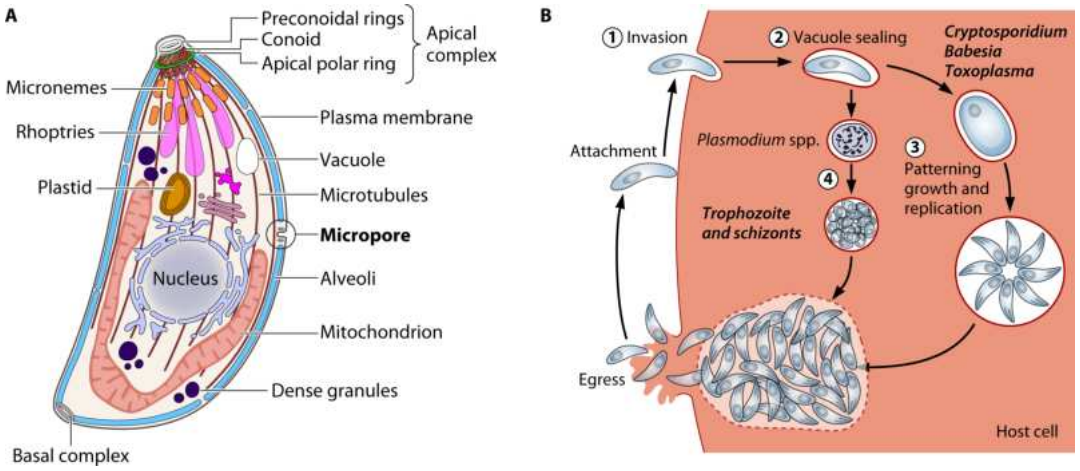


FIG 1 A representation of apicomplexan life cycle stages in mammalian hosts. (A) A model apicomplexan (a coccidian) demonstrating the structural features and apical secretory organelles and micropore discussed in this review. Apicomplexan demonstrates a significant variation in size, shape, and structural elements. In addition to *Plasmodium falciparum* and *Toxoplasma gondii*, these features are shared by *Cryptosporidium muris Babesia bovis*. (B) A representation of the lytic lifecycle of *Toxoplasma* tachyzoites *Cryptosporidium* and *Babesia* meronts and *Plasmodium* spp. asexual stages. (1) Invasion of the host cell driven by parasite cytoskeletal force. (2) At the end of invasion, the parasite helps to seal the vacuole. (3) During endodyogeny, daughter parasites grow through polymerization of the microtubules and the movement of the basal complex down the daughter cell. (4) During schizogony (seen only with *Plasmodium*), after a period of asynchronous nuclear division, the nuclei migrate to the nascent daughter cells, which require the basal complex to complete budding. Adapted from reference 15 (published under a CC BY license).

bacterial pathogens, where remodeled, host cytoskeletal processes effect entry). Nonetheless, components of the host lipid bilayer, including host proteins anchored by lipid anchors, are detected in the PVM (18) (Fig. 1B). During intracellular development, parasite-driven vesicular processes continue to remodel the PVM and the host cell environment to sustain nutritional requirements and avoid the immune system (19). The apicomplexan plastid provides essential biosynthetic and metabolic functions, including fatty acid, *de novo* heme, and isoprenoid precursor biosynthesis, and also harbors energy metabolism usually found in the mitochondria of mammals (20). *Toxoplasma gondii*, *Cryptosporidium*, and *Babesia* are replicated in their human host cells by a process, called endodyogeny, where organellar replication is immediately followed by division and daughter cell budding. In contrast, *Plasmodium* is divided by schizogony, which comprises multiple rounds of nuclear replication and division (along with apical organelle replication and assembly), followed by the final segmentation into individual daughter 'merozoites.' Soon after merozoite invasion into the red blood cell, the apical complex of *Plasmodium* disintegrates, and the parasite adopts an ameboid 'ring stage' (that looks like a signet ring). The ring stage lasts for about half of the parasite's intracellular life. In subsequent 'trophozoite' and 'schizont' stages, rapid parasite growth occurs concomitant with active digestion of host hemoglobin, followed by replication of nuclei and apical organelles, and, finally, the assembly of infectious daughter merozoites.

Vesicular mechanisms driven by parasitic apicomplexans are critically important to every aspect of host engagement, intracellular parasite survival, and proliferation. As indicated earlier, their entry into a target cell is controlled by the sequential discharge of parasite lipids and proteins from apical organelles, such as micronemes and rhoptries (two or 6–12 rhoptries/parasite in *Plasmodium* and *Toxoplasma gondii*, respectively) to form the PV without substantial dependence on host endocytic machinery, thereby enabling a diverse host range. Interestingly, rhoptries are detected only during the invasive stages in *Plasmodium*, and both rhoptries are discharged, while they are present throughout the intracellular replicative stages in *Toxoplasma gondii*, and only a few rhoptries shed their contents during the formation of PV. Nonetheless, both *Plasmodium* and *Toxoplasma gondii* rhoptries participate in the formation of PV with the help of lipids derived from the host cell (18, 21–23). A detailed discussion on the formation and

maintenance of PV is beyond the scope of this review and has been recently reviewed elsewhere (24, 25)

Plasmodium and *Babesia* primarily invade mature erythrocytes that lack the intrinsic capacity to form endocytic vesicles or vacuoles. *Toxoplasma gondii* and *Cryptosporidium* reside and proliferate in vacuoles protected from the active phagosomes of the wide range of host cells (particularly immune cells), which would ordinarily kill an engulfed pathogen. In addition to a nucleus, apicomplexan parasites contain an endoplasmic reticulum, Golgi, mechanisms of endocytic feeding, and secretory expansion of plasma membrane, as well as destinations beyond the vacuole and the host milieu (26–28). Hence, their vesicular mechanisms directly regulate the biogenesis and function of parasite's secretory and endocytic organelles, as well as the parasite's surrounding vacuolar and host cell milieu.

Vesicles broadly implicated in drug resistance across apicomplexan (as well as more broadly, protozoan) parasites may be grouped under 'extracellular vesicles' or EVs. EVs are found across kingdoms in most organisms and engage a wide range of functions (29). In microbial infections, they are best characterized in bacterial pathogenesis and well described in several excellent reviews (30, 31). They also exist in eukaryotic parasitic and apicomplexan infections (32–34). However, unlike bacterial EVs, parasite and apicomplexan EVs have not yet been characterized for complex functions, such as the ability to present decoys to bind or encapsulate drugs, degrade them via enzymatic functions, possess intrinsic natural anti-parasite activity, and be used to deliver drugs or assess drug permeability, present targets to mitigate drug resistance, shield resistance genes from degradation or the immune response, or disseminate resistance genes to widely amplify drug resistance (30, 35). In parasitic infections, EVs have been studied for transfer of resistance genes to sensitive counterparts. Early studies suggested that *P. falciparum* had the capacity to spread drug-resistant plasmids without physical contact but via 70 nm EVs (36), and there is evidence that EVs promote intercellular communication between parasites (37). But, perhaps, the most robust evidence for EV in transfer of parasite resistance genes comes from *Leishmania* (38). EVs delivered resistance genes to sensitive parasites between same and distinct species of *Leishmania* and gain of resistance conferred growth advantage and adaptability. Endosomal Sorting Complex Required for Transport (ESCRT) is known to be important in the formation of EVs (33). The ESCRT system is also needed for plasma membrane remodeling and maintenance, compartmentation of cell organelles, sorting of ubiquitinated proteins, fission and repair of damaged cell membranes, and cytokinesis in apicomplexan parasites (39). A recent review provides excellent in-depth analyses of EVs across all pathogenic single-cell microorganisms (30).

Historically, there have been many fewer drugs developed to treat toxoplasmosis, babesiosis, and cryptosporidiosis, relative to malaria. This is likely because infection by the human malaria parasite, *Plasmodium falciparum*, is associated with febrile and fatal disease (but individuals who live in endemic countries may present different levels of protection either because of host polymorphisms that prevent severe disease or repeated infections that result in immunity) (40–42). In contrast, most people with healthy immune systems remain asymptomatic for infections by *Toxoplasma* and *Babesia* or recover from *Cryptosporidium* infections without treatment. However, all four pathogens can be killed by clindamycin (which targets the apicoplast [43]) in *Plasmodium*, *Toxoplasma*, and *Babesia*, but, notably, *Cryptosporidium* lacks an apicoplast (44). Atovaquone, which targets the malarial mitochondria (45), is also used to treat babesiosis (46). Similarly, pyrimethamine (which targets folate utilization) and sulfadiazine (a sulfonamide antibiotic) are used to treat active toxoplasmosis (47) (and also incorporated into an antimalarial combination). Inflammatory eye disease caused by *Toxoplasma* is treated with glucocorticosteroids. Nitazoxanide, a general anti-infective drug, treats *Cryptosporidium* infections in patients with an intact immune system but not those who are immunocompromised (48). None of the indicated drugs target vesicular mechanisms of apicomplexan parasites.

In contrast, many prominent antimalarials target the food vacuole (fv) of malaria parasites. Parasite vesicular intermediates underlie the biogenesis and functions of this organelle that are principally the digestion of hemoglobin and export of nutrients to the parasite. Resistance mechanisms in transporters, such as Pfmdr1, the homologue of mammalian P-glycoprotein (P-gp), and part of the ATP-binding cassette, mediate plasmodial food vacuole resistance to a broad range of drugs (49). Similarly, mutations in chloroquine resistance transporter (Pfcrt) in the food vacuole mediate resistance to chloroquine, quinine, as well as piperaquine (a partner drug in artemisinin-based combination therapies; ACTs) (50). Additionally, over the last decade, evidence has accrued on *P. falciparum* vesicles enriched for the resistance determinant PfKelch13 as major drivers of resistance to a leading class of antimalarials, called the artemisinins (51–60). Although much has been learned about the properties of PfKelch13's repeat propeller domains that integrate vesicular, proteostatic, and epigenetic regulation of cell survival, substantial gaps remain in terms of understanding the mechanisms of artemisinin resistance (ART-R). In the next section, we evaluate the prevailing models of ART-R and integrate concepts from conserved vesicular functions of apicomplexan 'micropore' organelles, as well as shared properties of Kelch repeat propeller (KRP) domains across pathogenic eukaryotes, to propose a new model for malarial ART-R.

VESICULAR MECHANISMS OF ARTEMISININ RESISTANCE IN *PLASMODIUM*

The protozoan *Plasmodium falciparum* is the most virulent human malaria parasite that infects erythrocytes to cause all the symptoms and pathologies associated with acute infection, as well as severe disease (61, 62). Artemisinins are a front-line therapy for both acute and severe diseases. They are based on the active ingredient of the qinghao (*Artemisia annua*) plant used to treat maladies (including malaria) in ancient China (63). These rapidly acting drugs are short lived and, therefore, combined with less potent but longer acting antimalarials to develop ACT whose worldwide deployment contributed significantly to reducing the global burden of malaria infection and severe disease (64–66). Hence, the emergence of artemisinin (partial) resistance, ART-R, threatens malaria control and eradication (67, 68).

The low nanomolar activity of artemisinins is dependent on the cleavage of their endoperoxide bond and creation of carbon free radicals that indiscriminately insert into protein, lipids, and nucleic acids in the cytoplasm and subcellular organelles, and thereby kill the parasite (69). Therefore, mechanisms of resistance to these drugs were not expected to arise from mutations in a single enzyme to confer catalytic resistance to the drug (as occurs in folate resistance arising from mutations in *dhps*). Reverting the action of one (or a few) transporters that expel the drug (such as seen with resistance to chloroquine) was also an unlikely mechanism since artemisinins cross cellular membranes. Over several years, multiple candidate genes for artemisinin resistance emerged from genome-wide association studies (GWAS), including *mdr1*, *cg10*, *tctp*, *atp6*, and *ubp1* (70, 71), but they clearly were not primary mechanisms of resistance. By combining artemisinin-induced *in vitro* selection (over 5 years) with GWAS, as well as genetic editing, the gene *pfkelch13* emerged as a major marker of ART-R (51, 52). PfKelch13 has been localized to vesicular structures at all stages of blood-stage *P. falciparum* malaria parasites (54–56, 72). It contains WD40 domains that have a highly conserved β-propeller structure with six blades predicted to act as a scaffold for protein–protein interactions (51). Other parasite WD40 proteins, Pfcoronin and PfAtg18, that associate with vesicles may also contribute to ART-R (although the levels of resistance achieved are lower than those seen with Cambodian PfKelch13 mutations) (73, 74).

As the first major causal determinant of ART-R, PfKelch13 has been intensely investigated to develop mechanistic models of resistance. In addition to the WD40 domain, the structure of PfKelch13 suggests it functions as a cullin E3 ubiquitin ligase substrate adapter with the potential to effect global cellular changes (i.e., large-scale processes that prevent or revert cytoplasmic or organellar degradation and/or modulate the epigenetic landscape to promote persistence through a multigenic survival

response) (75, 76). PfKelch13 has been localized to vesicles secreted by ER, as well as the digestive food vacuole of trophozoites (54–56, 72). One study reported that PfKelch13 was found to concentrate in a structure known as the 'cytostome' (72), an apicomplexan micropore seen only in *Plasmodium* (also called the Type II micropore [77] prominent at the trophozoite stage; Type II micropores and their relationship to Type I micropores that are conserved across all apicomplexans are further discussed in the next section).

The PfKelch13 function at the Type II micropore cytostome and its regulation of the ingestion of hemoglobin into the digestive food vacuole are the prevailing mechanisms of ART-R (72, 78) (Fig. 2). A large number of PfKelch13-interacting complex proteins (KICs) (72) have also been localized to the cytostome, and a few of which have been shown to play a role in ART-R, as measured *in vitro* laboratory assays (72) but not in clinical ART-R. These findings strongly support that PfKelch13 and KICs play a role in regulating the food vacuole's uptake of hemoglobin, whose digestion releases heme (72, 78), which in turn activates the killing action of artemisinins at the trophozoite stage (69, 79). ART-R mutations can reduce levels of PfKelch13 by 50%, which is proposed to reduce the degradation of hemoglobin, heme release, and the killing activity of artemisinins (Fig. 2B). But, what this prevailing model does not capture well is that neither clinical ART-R nor *in-vitro* ART-R are manifested in trophozoites. PfKelch13 mutant trophozoites are killed just as effectively as their wild-type counterparts. ART-R is prominent only in very early ring parasites (at 0–3 h) at least 20 h before the trophozoite stage. To account for this deficiency, it has been proposed that hemoglobin uptake and digestion may also occur in early ring stages (72, 80).

Stage-specific clinical resistance in young rings

As previously indicated, the potent action of artemisinins against malaria parasite depends upon heme because it cleaves the endoperoxide bond of this class of drugs and triggers their killing action (69, 79). Ferrous heme plays a major role in this process. The digestion of hemoglobin in the food vacuole leads to the release of ferrous heme bound to molecular oxygen. Deoxygenation and oxidation of ferrous to ferric heme yield superoxide that cleaves an artemisinin–endoperoxide bond to generate free radicals and reactive oxygen species that indiscriminately form adducts with a wide range of cellular targets to kill the parasite. Consistently, knockdown of the hemoglobinase falcipain 2a reduces the production of ferrous heme and parasite sensitivity to artemisinins (81). Ferric heme in the food vacuole rapidly crystallizes into hemozoin, the black pigment of malaria (actively produced from 24 to 48 h of the *P. falciparum* life cycle). This pigment is also seen in late rings (18 h+), suggesting they also engage in hemoglobin digestion and release of ferrous heme. Recent studies suggest that hemoglobin digestion also occurs (at low levels) in young rings, and this has been forwarded as a rationale by which released heme could activate killing of young parasites, which, during its conversion to ferric heme, can trigger artemisinin activation at these stages (72, 78). However, free heme alone is sufficient to activate artemisinin, regardless of the PfKelch13 mutation status. It is, therefore, conceivable that just as seen in trophozoites, the release of free heme in rings may enable artemisinins to bypass ART-R conferred by PfKelch13 resistance mutations.

Clinical clearance of parasites with $t_{1/2}$ of >5.5 h signifies ART-R. Clinical ART-R is maximal in very young (0–3 h) rings (82). Since artemisinins are rapidly cleared from the blood stream (in 1–3 h), clinically, after a single drug dose, all parasite stages (rings, trophozoites, and schizonts) are exposed to maximal drug levels for a very short window, during which their life cycle stage remains largely the same. Thus, unsurprisingly, rings with presumably lesser levels of heme survive to gain resistance. The first report on the detection of clinical resistance was reported as delay in clearing of ring-stage parasites from circulation (and this still remains as the gold standard for measuring ART-R) (83–85). However, since rings are not the major metabolically active stage of parasite growth, ART-R cannot be measured *in vitro* by standard IC_{50} assays (that, by definition, detect

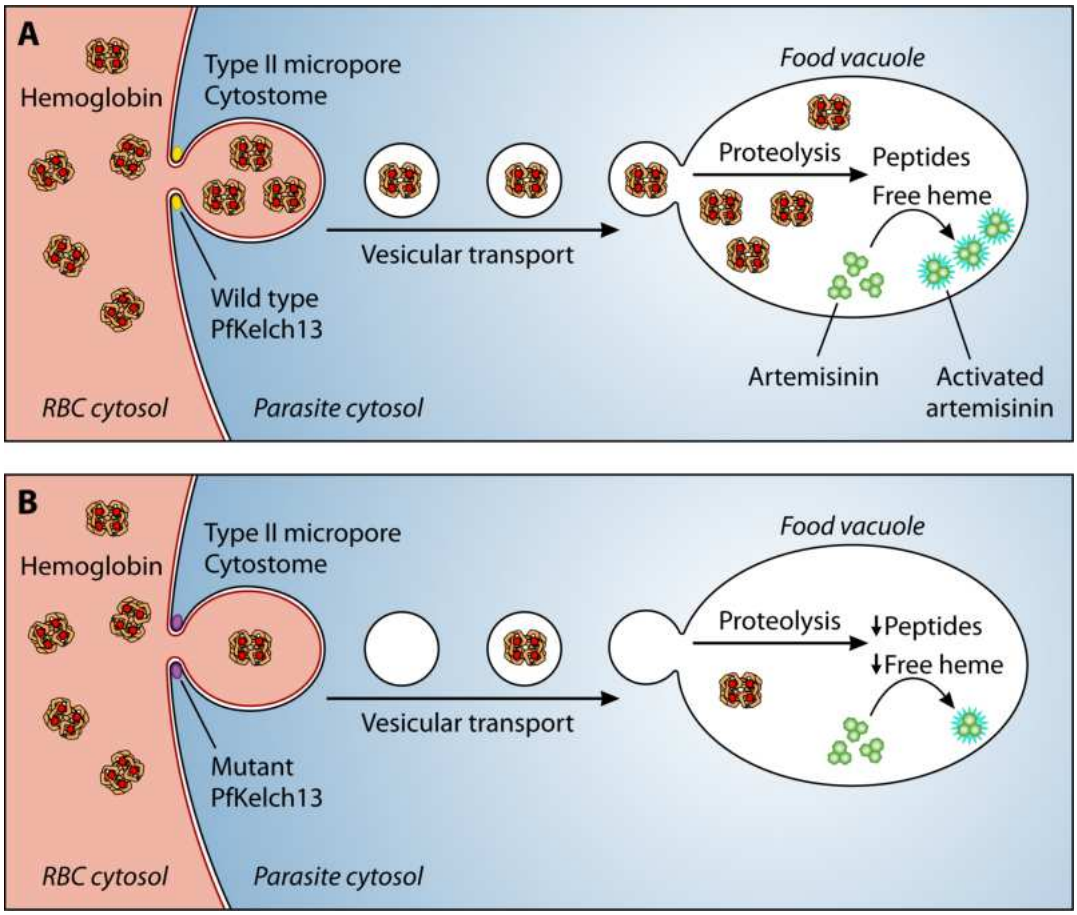


FIG 2 Prevailing model for PfKelch13 mediated ART-R in *P. falciparum* malaria. (A) Model for the mechanism of PfKelch13's activation of artemisinin. (B) Its antagonism by mutant PfKelch13 that confers ART-R. PfKelch13 localizes to cytostomes, which are Type II micropore organelles that mediate the endocytosis of hemoglobin prior to its vesicular transport to the food vacuole where its hydrolysis releases heme, which activates artemisinins in drug-sensitive *Plasmodium falciparum* (A). In parasites with ART-R, mutant PfKelch13 reduces reduced hemoglobin uptake, digestion, and free heme release, resulting in decreased artemisinin activation (B). This model is proposed in both trophozoite- and ring-stage parasites. Notably, its limitations are that it does not explain the following. (i) Why does mutant PfKelch13 not confer ART-R to trophozoite stage parasites? (ii) Why does a resistance mutation expressed throughout a clonal parasite population fail to protect the vast majority of the mutant ring parasites exposed to artemisinins? (iii) It does not consider that the generation of high levels of free heme in ring-stage parasites with PfKelch13 mutations may render them sensitive to killing by artemisinins. Based on reference 78.

concentrations of drugs required to inhibit pathogen replication by 50%). Instead, a ring-stage survival assay (RSA) using very early rings (0–3 h of the 48 h cycle) assesses *in vitro* ART-R in the laboratory (82).

The use of RSA *in lieu* of standard IC_{50} assays has posed some unique challenges. To begin with, a threshold RSA value of >1 percentage (proportional to a control value) was proposed as ART-R (82). More recently, RSA > 2 was accepted as a more robust index of resistance (86). RSA values in certain parasite strains can range greater than a log higher, but this is also dependent on the genetic background of the parasite (87). There is, however, a general agreement that high RSA reflects a higher level of resistance. Yet, it is important to note that high RSA is not necessarily equated with a greater *spread* of resistance. PfKelch13 mutations C580Y and R539T with high RSA prevail in Cambodia and South East Asia (88, 89). But, elsewhere, such as Africa, mutations with lower RSA have shown a greater spread (68, 90, 91). RSA evaluation alone is not sufficient to claim the presence of resistance mutations. It must be coupled with clinical clearance with $t_{1/2}$ of >5.5 h to signify ART-R. Gene mutations associated with one or the other are considered likely candidates of AR, while those showing both properties are accepted (by WHO) as validated markers of AR (2).

CONSERVED APICOMPLEXAN MICROPORE VESICLE TARGETS IN ARTEMISININ RESISTANCE

As indicated earlier, young 0–3 h ring stages have not yet fully initiated vesicular remodeling to either the red cell or the food vacuole, which in turn is expected to severely limit endocytic digestion of hemoglobin (particularly compared to the late ring- and trophozoite-stage parasites). Furthermore, since clinical ART-R is only mimicked *in vitro* by 0–3 h ring-stage parasites, it is important to consider the mechanisms of these rings and their capacity for vesicular ART-R. These early intra-erythrocytic parasite stages are poorly understood because large numbers of pure populations of 0–3 h rings are technically challenging to generate and study. The principal function of a young ring is to remodel the red cell to establish nutrient and membrane transport pathways and survive for the next 18–24 h to reach the trophozoite stage when robust uptake and digestion of hemoglobin in the fv generate high levels of nutrients to support parasite growth. Thus, while studies suggest that young rings digest hemoglobin (72, 80), the fv, as well as the PfKelch13 function at the cytostome, are most prominent at the trophozoite stage. This is at considerable odds with the PfKelch13 mutations conferring ART-R in rings. Knockdown studies also suggest an essential ring-stage function for PfKelch13 (72). An additional confounder is that, even in monogenetic backgrounds, not all 0–3 h ring parasites with PfKelch13 ART-R mutations survive drug exposure in the RSA. Indeed, even in highly resistant strains (RSA > 10), 90% of the parasites die, suggesting there is substantial heterogeneity in the PfKelch13 function in 0–3 h rings.

Type I and II micropore Kelch13 functions

Apicomplexans contain two stable types of endocytic 'micropore' structures at their plasma membranes (77). 'Type I' micropores are found in both *Plasmodium and Toxoplasma gondii* at their respective extracellular 'merozoite' and 'tachyzoite' stages (Fig. 1 and 3). The Type I micropore is associated with electron-dense rings (EDRs) and flanked by the inner membrane complex (IMC). It is a single-plasma membrane (PM) invagination linked to endocytosis (92) (Fig. 3). As indicated earlier, Type II micropores are cytostomes of food vacuoles that become prominent in the late ring/trophozoite stages of *Plasmodium*. Compared to Type I, Type II micropores are double-membrane invaginations of the PVM and the parasite plasma membrane (Fig. 3) that engage in the engulfment and digestion of hemoglobin. They lack the IMC but contain EDRs where PfKelch13 has been shown to localize and function (Fig. 3). Type II micropores are not found in *Toxoplasma gondii*. However, recent studies show that TgKelch13 localizes to Type I micropores in *T. gondii* (93). Notably, TgKelch13 localizes to EDRs of Type I micropores (while PfKelch13 localizes to EDR of Type II micropores; Fig. 3). Furthermore, although Type I and II micropores are distinct endocytic structures, the Kelch-interacting protein complexes (KICs) associated with *Tg*Kelch13 in Type I micropores show high levels of molecular similarity to those associated with PfKelch13 in Type II micropores of *P. falciparum* (93, 94), suggesting Kelch13 and KICs underlie conserved functional properties in both Type I and II micropores. Type I micropores are conserved across apicomplexans, including *Plasmodium*. *P. falciparum* merozoites and 0–3 h-young rings express single foci of PfKelch13 (53, 72), and we propose these are Type I micropores with PfKelch13. As in *T. gondii*, we suggest that PfKelch13 in Type I micropores may function in essential plasma membrane endocytic recycling. PfKelch13 function at double-membraned cytostomes (invaginations of the PVM and parasite plasma membrane) in the ingestion of host cell hemoglobin occurs only with the emergence of Type II micropores (as summarized in Fig. 3).

Heme binding

To undergo active killing by artemisinins, young rings need heme to activate these drugs. Furthermore, for a parasite to become ART-R in a manner dependent on PfKelch13, heme engagement must be proportionally antagonized by the severity of PfKelch13 resistance

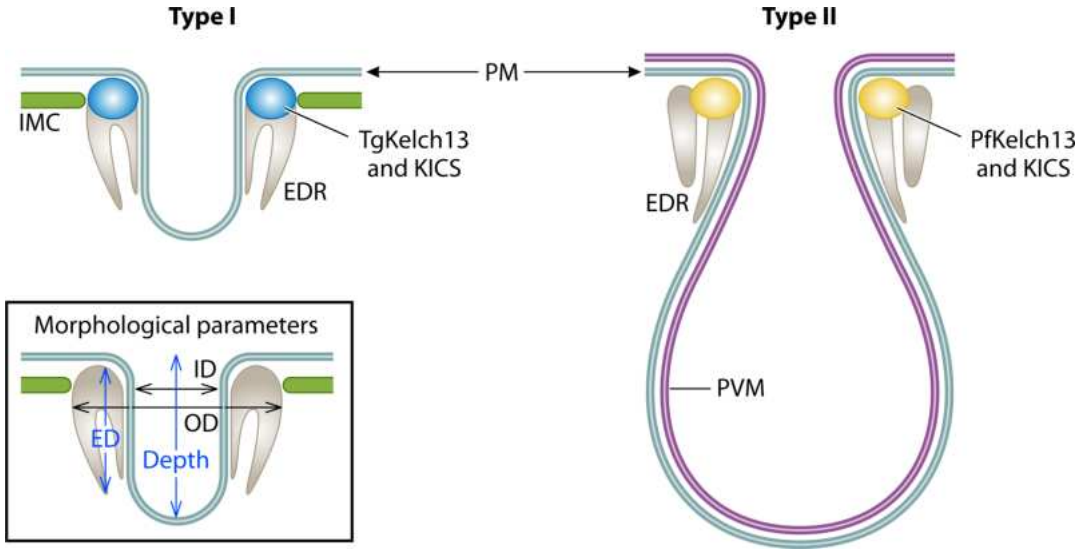
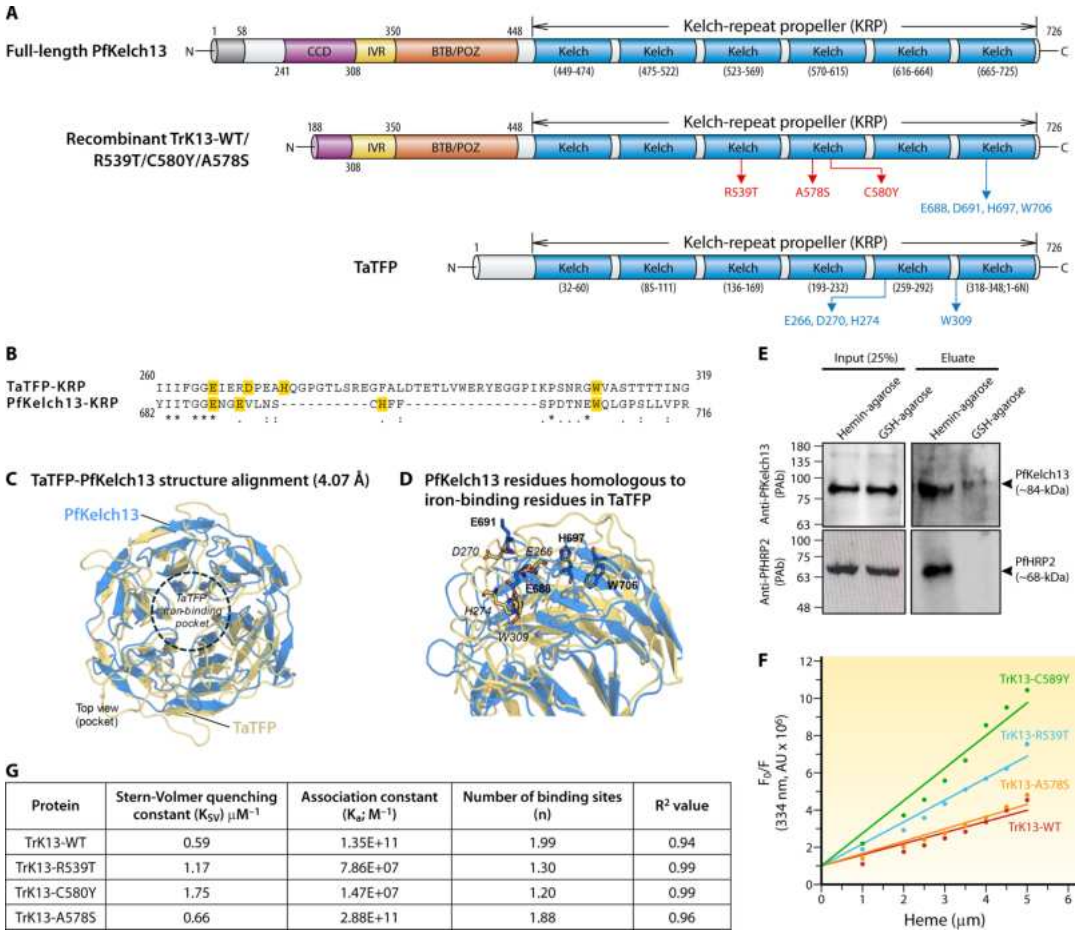


FIG 3 Type I and II micropores of apicomplexans. Type I micropores are found in both *Toxoplasma* and *Plasmodium*. In *Plasmodium falciparum*, Type I micropores are found in the merozoite and early ring stages. The Type I micropore is characterized by the presence of electron-dense rings (EDRs) on an inner membrane complex (IMC) pore and a plasma membrane (PM) invagination with functions in nutrient uptake and plasma membrane recycling. Type II micropores are only found in the *Plasmodium* in late ring/trophozoite stages and characterized by the additional invagination of the parasitophorous vacuolar membrane (PVM; but without the IMC) as a double-membrane 'cytostome' that participates in the digestion of hemoglobin. *P. falciparum* Kelch13 (PfKelch13) and proteins of Kelch interacting complexes (KICs) have been localized to Type II micropores/cytostomes. *Toxoplasma gondii* Kelch13 (TgKelch13) and KICs have been localized to Type I micropores in *T. gondii*, suggesting the conservation of molecular functions between Type I and II micropores in apicomplexans. Conservation of Type I micropores across all apicomplexans leads to the proposal of a model, in which PfKelch13 is also localized in Type I micropores of *P. falciparum*. Additional abbreviations: ED, electron dense ring depth; ID, inner diameter; OD, outer diameter. Adapted from reference 77 (published under a CC BY license).

mutation. Levels of heme directly engaged with PfKelch13 are likely to be much lower than levels of heme produced by digestion of hemoglobin (in either the nascent food vacuoles of rings or mature food vacuoles of trophozoites). Our recent studies suggest that PfKelch13's β-propeller domain has the intrinsic capacity to directly bind iron and heme (ferrous protoporphyrin IX; FPIX) (95, 96) at nanomolar concentrations (Fig. 4). This propensity to bind heme is based on PfKelch13's mimic of the KRP domain with iron-binding properties from the field pennycress *Thlaspi arvense* thiocyanate-forming protein belonging to the *Brassicaceae* family (97) (Fig. 4). Heme binding is manifested by both native PfKelch13 and *in vitro*-produced recombinant protein. Furthermore, heme-binding capacity was reduced in the two PfKelch13 mutations of ART-R (C580Y and R539T) but unaffected by A578S, a polymorphism that fails to confer ART-R. Since PfKelch13 shows nanomolar affinity for heme, we propose a new model (Fig. 5) to suggest that heme directly bound to PfKelch13 in Type I micropores may encounter and activate artemisinins in 0–3 h rings prior to hemoglobin digestion in nascent Type II micropores. Moreover, the extent of heme biding maybe reduced by PfKelch13 mutations of ART-R.

A NOVEL APICOMPLEXAN TYPE I MICROPORE MODEL FOR RING-STAGE ART-R

In a new mechanistic model of ring-stage ART-R (Fig. 5), we propose that PfKelch13 bound to heme localizes to Type I micropores of daughter merozoites emerging within schizonts (Fig. 5A). When merozoites invade erythrocytes, PfKelch13 continues to sequester heme in young rings (Fig. 5A), which in turn cleaves the endoperoxide bridge of internalized artemisinins and activates free radical formation to effect ring-parasite killing (Fig. 5A and B). Mutants of ART-R PfKelch13 bind less heme and are, therefore, less prone to catalyzing endoperoxide cleavage when encountering artemisinin, providing a mechanism of resistance in young rings (Fig. 5B). It has been suggested that PfKelch13



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Protein	Stern-Volmer quenching constant (K_{SV}) μM^{-1}	Association constant (K_a; M^{-1})	Number of binding sites (n)	R^2 value
TrK13-WT	0.59	1.35E+11	1.99	0.94
TrK13-R539T	1.17	7.86E+07	1.30	0.99
TrK13-C580Y	1.75	1.47E+07	1.20	0.99
TrK13-A578S	0.66	2.88E+11	1.88	0.96

FIG 4 Conserved heme and iron-binding properties of *Thlaspi arvense* thiocyanate-forming protein (TaTFP), PfKelch13, and the consequences of AR mutations. (A) PfKelch13 (top), recombinant TrK13-WT (middle), and TaTFP (bottom) share the Kelch repeat propeller (KRP) domain. Red indicates ART-R mutations R539T, C580Y, and the non- pathogenic polymorphism, A578S. Blue indicates iron-binding residues in TaTFP and homologous residues in TrK13. *Abbreviations*: CCD, coiled-coil domain; IVR, intervening region; BTB/POZ, bric-à-brac/poxvirus and zinc-finger domain. (B) Sequence alignment between KRP domains of TaTFP and PfKelch13 with positional conservation of residues of Fe^{2+} binding (yellow). (C) Structural alignment of the PfKelch13 propeller (blue) and TaTFP (gold) domains. (D) Stick model shows iron-binding amino acid residues in TaTFP (italics) and their homologous sites in PfKelch13 (bold). (E) Native *P. falciparum* Kelch13 binds hemin–agarose beads but not GSH–agarose. PfHRP2, a known heme-binding protein, was used as a positive control. Molecular weight standards are indicated in kDa. (F) Heme binding as measured by tryptophan fluorescence intensity by wild type (WT) and mutations R539T, C580Y, and A578S. (G) Stern–Volmer quenching constants (K_{SV}), association constants (K_a), and the number of binding sites (N) calculated from the equations $F_0/F = K_{sv}[Q] + 1$ and $\log[(F_0 - F/F] = \log K_a + n\log[Q]$, where F_0 = fluorescence intensity in the absence of heme, F = fluorescence intensity in the presence of heme, and [Q] = concentration of quencher (heme). The figure is adapted from reference 96 (published under a CC BY license), with the blots in E from references 95 (bioRxiv preprint) and 96.

mutations of ART-R reduce protein levels, and this may yield a mechanism of resistance (72) (analogous to mutations that underlie genetic disorders) (98). However, a recent study comparing the relative abundance of PfKelch13 mutant proteins and RSA values in isogenic (*P. falciparum* 3D7) backgrounds (87) showed that three resistance mutants, namely, C580Y, R539T, and R561H, had similar abundance (51–52%) compared to wild-type protein but showed a wide variation in parasite survival (16, 38, and 11%, respectively) (87). This strongly suggests that factors other than abundance influence ART-R. In addition, that resistant NF54^{C580Y}attB parasites (initial RSA: 4.8%) became more sensitive after *in trans* co-expression of wild type rather than mutant PfKelch13 (RSA 1% versus 1.6%) may be due to higher affinity of the former for heme, thereby effecting greater artemisinin activation and parasite killing. Further, in most genetic disorders, a two-fold reduction in protein levels are not associated with disease: rather reductions of 75–90% are required for significant functional detriment (98–100). Finally, even if in some

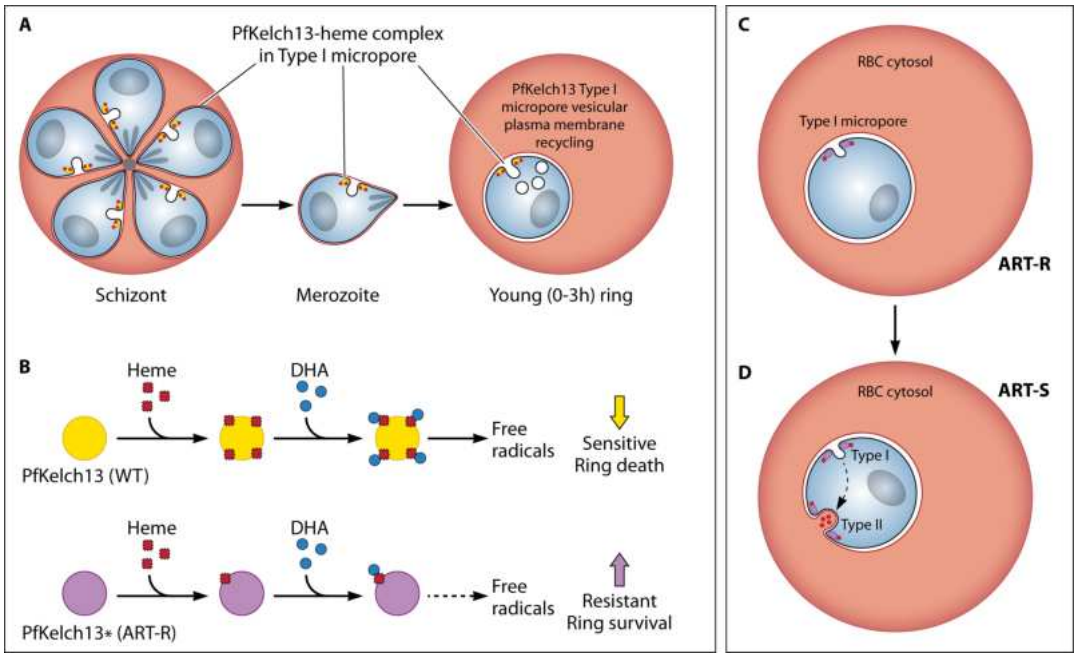

FIG 5 Novel Type I micropore model of ART-R in *P. falciparum* malaria. (A) PfKelch13–heme complexes (yellow filled circles with red squares) assemble at Type I micropores of *P. falciparum* merozoites as they develop in schizonts. Released merozoites invade new red blood cells to form rings with associated Type I micropores and PfKelch13–heme complexes. (B) In wild-type rings, PfKelch13–heme complexes promote adduct formation with dihydroartemisinin (DHA, blue dots) that activate carbon free radicals that destroy essential Type I micropore functions (of plasma membrane endocytosis), resulting in ring death and conferring sensitivity to artemisinins. PfKelch13 ART-R mutations (purple filled circles) show reduced heme binding, adduct formation, and activation of free radicals, enabling ring survival and ART-R. Abbreviation: DHA, dihydroartemisinin. (C) PfKelch13 mutant Type I micropore confer ART-R in young (0–3 h) rings. (D) PfKelch13 mutant Type II micropores emerge, enabling hemoglobin digestion in rings, which release free heme to activate artemisinin and render ART-R parasites in C, to become ART-S, thereby explaining why only a population of 0–3 h rings survived exposure to artemisinins in the RSA.

cases, 50% reduction in PfKelch13 mutations were sufficient to induce clinical ART-R, this does not explain young ring-stage specificity of resistance.

Thus, in Fig. 5, we propose a separate, essential ring-stage Type I micropore function for PfKelch13, where mutations that antagonize heme binding provide a resistance mechanism that fulfills all the criteria of both clinical and *in vitro* ART-R. This is compared to the prevailing model of Type II micropore function, where the digestion of hemoglobin to release free heme would in fact revert PfKelch13 mutant parasites from ART-R to ART-S both in ring and trophozoite stages. Our model in Fig. 5 also predicts that a substantial proportion of young rings may progress to from Type I to Type II micropores. Thus, as PfKeclh13 mutant rings progress from **5C** to **5D**, free heme generated by Type II micropore digestion of hemoglobin will convert their status from ART-R to ART-S in the RSA. In contrast, the prevailing model of Type II micropore function (Fig. 2) does not explain why a resistance mutation expressed throughout a clonal parasite population fails to protect most mutant ring parasites in the RSA. Indeed, even the most widespread resistant mutation, C580Y Cambodia, which results in clinical ACT failure, confers only 5–10% of parasites across different genetic backgrounds resistant in RSA. In Type I model of ART-R, these data could be explained by the emergence of Type II in 0–3 h rings that confers ART-S to an ART-R population (Fig. 5C and D). Other sources of heme iron in rings may also affect ART-R. In this regard, it is interesting to note that chemical selection experiments suggest that artemisinins can kill *T. gondii* by targeting their mitochondrion where heme synthesis occurs (albeit very high concentrations of artemisinin were used in these studies [101]). Mitochondrial targets have also been implicated in *P. falciparum* ring-stage resistance (59). Mitochondria are present in 0–3 h young ring stages and in addition to synthesizing heme *de novo*, recent studies suggest that they are also essential for blood-stage parasite survival (102).

CONCLUDING COMMENTS

Emerging, cross-apicomplexan perspectives for drugs, targets, and resistance mechanisms in *Plasmodium*, *Cryptosporidium*, *T. gondii*, and *Babesia*

Babesia parasites that digest hemoglobin for amino acids are not sensitive to artemisinins (103). Notably, although they break down hemoglobin, they do not produce hemozoin, and their genomes lack the complete heme synthesis system of *Plasmodium* (103). This suggests that generation of superoxides from conversion of ferrous to ferric heme is needed for activation of artemisinins, rather than just the PfKelch13-dependent endocytosis of hemoglobin *per se* (72). PfKelch13 as a resistance target is not the norm for vesicular resistance mechanisms in apicomplexans or in EVs (which transfer resistance between two distinct parasite populations). Rather, PfKelch13 vesicles confer stage-dependent resistance in the same organism. However, identification of parasite phosphatidylinositol-4-kinase as a drug target in *Plasmodium* (104) and *Cryptosporidium* (105) strongly suggests a future expansion of the endocytic vesicular mechanisms of drug action and resistance in these two apicomplexans. Additional shared targets may be the lysyl–tRNA synthetase of interest for drug discovery and development in both *Cryptosporidium* (106) and *P. falciparum* (107). As for malaria, there is global recognition of the urgent requirement of better therapeutics to treat *Cryptosporidium* infections (108, 109). In toxoplasmosis, treatments have remained largely unchanged over 20 years, with no new drugs recently been put on the market (110). Immunomodulatory advances may prevent relapses in immunocompromised individuals but remain in early stages of development (111). Emergence of Babesia parasites resistant to drugs, such as atovaquone, azithromycin, quinine, and clindamycin (and combinations thereof), and the limited value of these drugs in immunocompromised populations highlight the need for new drugs to treat babesiosis. Here, advances with antimalarial tafenoquine (which was recently FDA approved) may be beneficial since tafenoquine appears active against babesiosis (112, 113).

New insights on substrates of apicomplexan Kelch13

Canonical 'substrates' of a Kelch protein are expected to bind to β-propeller WD domains in a mutation-dependent manner. This raises the question as to whether our model heme may function as a substrate for PfKelch13 (Fig. 5B). Our model also suggests that in young rings, endoperoxide cleavage and activation of free radicals may occur more locally (i.e., at molecular proximity to PfKelch13 and influence other potential substrates of PfKelch13. Prior work has suggested that PfPI3K) as a PfKelch13 substrate (53). DHA targets the active site of PfPI3K with low nanomolar specificity, as demonstrated by both *in silico* modeling and experimental studies (53, 114). Inactivation of PfPI3K by low nanomolar concentrations of DHA has been shown to be dependent on endoperoxide cleavage (as measured through PfPI3K–DHA adduct formation [114], as well as reduction of enzymatic activity seen in 0–4 h ring parasites [53]). Notably, PI3P has also been shown to play a key role in both endocytic and exocytic vesicular trafficking in *P. falciparum* (115, 116). Additional studies are required to determine whether activation of artemisinins in the PfPI3K active site is mediated by heme bound to PfKelch13 in young rings. More substrates of PfKelch13 may exist since individual Kelch proteins have been reported to control levels of four–five substrates (117, 118). In mammalian systems, the best delineated examples of Kelch substrates are regulators of transcription (119, 120). These substrate levels are kept low by their binding to Kelch domain proteins, followed by ubiquitination and degradation (121–123). Mutations that antagonize binding of either result in their elevation in cytoplasm and subsequent targeting to nucleus to completely transform a wide range of cellular programs that impact energy growth and tumorigenesis. It is recognized that artemisinin resistance is a complex phenotype (60). Genetic background, which is known to play a very important role in genetic diseases and proteostasis (98), appears to be shared by PfKelch13, as assessed by the *in vitro* assessment of ART-R in different *P. falciparum* malaria strains

(87). But, overall, in the context of ART-R, in the main proliferative and differentiation stages of trophozoites and schizonts in blood-stage parasite growth, the killing effect of artemisinins far outweighs the presence of the PfKelch13 mutation. Nonetheless, clinical ART-R eventually contributes to ACT drug failure, and there are yet no replacements for artemisinin. Therefore, a better understanding of the substrates of plasmodial and other apicomplexan Kelch13 proteins, their functions, and insights into mechanisms of action may be extremely important for future control and elimination of malaria.

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AUTHOR BIOS

Kasturi Haldar, Ph.D., is a Professor at University of Notre Dame, USA. Over the last 30 years Dr. Haldar's lab has led fundamental research on how Plasmodium falciparum, the most virulent of human malaria parasites, secretes proteins and lipids to extensively modify its host erythrocyte. The work revealed new targets for drug action as well mechanisms of pathogenesis. Most recently the studies have focused on mechanisms of artemisinin resistance (front line antimalarials) in Plasmodium falciparum. In collaboration with Dr. Souvik Bhattacharjee, Dr. Haldar's lab established that the active metabolite of artemisinins as a potent inhibitor of parasite phosphatidylinositol-3-kinase and that a molecular mechanism of artemisinin resistance involves a parasite synthesized lipid PI3P. They continue with mechanistic analyses of artemisinin resistance and extend them to predictions relevant for clinical malaria. In collaboration with International Center for Diarrheal Diseases, Bangladesh (icddr, b), the National Malaria Elimination Program (NMEP), Govt of Bangladesh we developed studies evaluating clinical artemisinin resistance in Bangladesh and laboratory adaptation of resistant and sensitive Bangladeshi parasite strains from a remote, population that contains a high number of Indigenous non-Bengali people in the poorest communities. They present the last frontier to eliminate malaria in Bangladesh. Consequently, the Haldar lab has expanded research to include both molecular and translational studies on the elimination of malaria.

Souvik Bhattacharjee, Ph.D., is a Professor at Jawaharlal Nehru University, India. The primary focus of our lab at the Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi, India is to understand the molecular mechanisms underlying artemisinin resistance in the human malaria parasite Plasmodium falciparum. The global emergence and spread of resistance to the frontline antimalarial artemisinin and its derivatives severely impacts the continued use of WHO-recommended artemisinin-based combination therapy. We use genetic manipulation, biochemical and biophysical techniques to reveal parasite changes that lead to the induction of this resistance. Another significant area of our interest is in dissecting the mechanistic aspects of virulence protein translocation in plant pathogenic oomycete Phytophthora infestans, which caused the Irish Potato famine in 1942. The protein trafficking mechanisms are highly conserved across diverse eukaryotic pathogens, and we investigate conserved pathways that mediate these sequential steps.