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Mechanisms of resistance to the partner drugs of artemisinin in the malaria parasite

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The deployment of artemisinin-based combination therapies (ACTs) has been, and continues to be, integral to reducing the number of malaria cases and deaths. However, their efficacy is being increasingly jeopardized by the emergence and spread of parasites that are resistant (or partially resistant) to the artemisinin derivatives and to their partner drugs, with the efficacy of the latter being especially crucial for treatment success. A detailed understanding of the genetic determinants of resistance to the ACT partner drugs, and the mechanisms by which they mediate resistance, is required for the surveillance of molecular markers and to optimize the efficacy and lifespan of the partner drugs through resistance management strategies. We summarize new insights into the molecular basis of parasite resistance to the ACTs, such as recently-uncovered determinants of parasite susceptibility to the artemisinin derivatives, piperaquine, lumefantrine, and mefloquine, and outline the mechanisms through which polymorphisms in these determinants may be conferring resistance.

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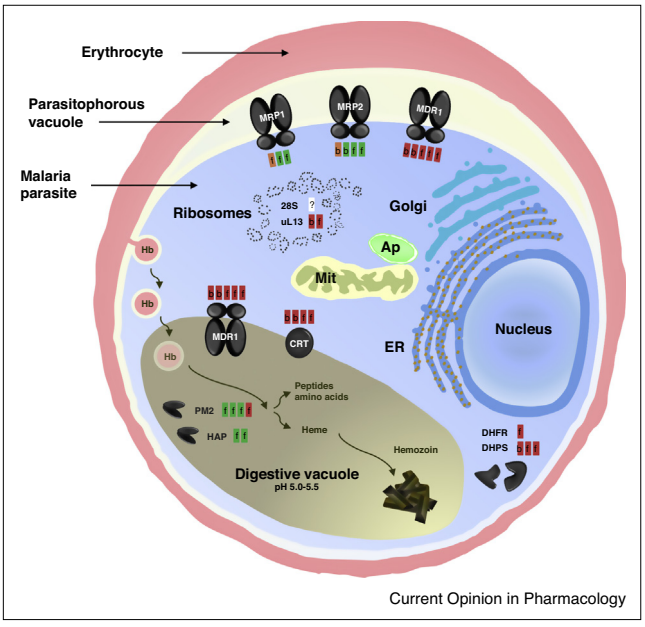
Introduction

Despite recent advances in the fight against malaria, the disease remains a leading global health problem that imposes enormous mortality, morbidity, and socio-economic burdens upon afflicted countries. An estimated 445 000 people died from malaria in 2016 and approximately 90% of these deaths were caused by the most lethal of the human malaria parasites — *Plasmodium falciparum* [1]. In the absence of an effective vaccine, the treatment and prevention of malaria relies heavily upon chemotherapy. The current frontline treatments pair an artemisinin derivative (which is metabolized into dihydroartemisinin) with either lumefantrine, mefloquine, amodiaquine, piperaquine, pyronaridine, or sulfadoxine–pyrimethamine. These ACTs combine the rapid parasiticidal action (but short half-life) of dihydroartemisinin with the slower action (but longer half-life) of the partner drug. However, the continuing emergence of *P. falciparum* parasites that are resistant to one or more of the current partner drugs, and/or which are partly resistant to dihydroartemisinin, poses a substantial threat to the control and elimination of malaria [2••,3,4,5•,6–11,12••].

All of the ACT partner drugs, as well as dihydroartemisinin, act against the blood stages of the parasite. In this phase of its lifecycle, the parasite invades an erythrocyte and supports its growth by digesting hemoglobin within a lysosomal-type organelle known as the 'digestive vacuole' (DV) (Figure 1) [13]. This process results in the release of heme monomers, which are detoxified via conversion into the inert crystal hemozoin. Many antimalarials accumulate and/or act within the acidic environment of the DV, and the heme released from hemoglobin is a key activator of dihydroartemisinin [14•]. Indeed, all of the ACT partner drugs bar sulfadoxine–pyrimethamine are known (or thought) to concentrate within the DV via 'weak-base trapping' [15] (Figure 2a), and some exert their main antimalarial effect therein by preventing the detoxification of heme, and/or through interfering with another step in hemoglobin digestion (reviewed in [16]). The propensity for diverse pharmacons to access, and accumulate within, the DV likely underlies the associations of polymorphisms in two DV residents — the *P. falciparum* 'multidrug resistance protein 1' (*pfmdr1*) and the 'chloroquine resistance transporter' (*pfcrt*) — with changes in the parasite's sensitivity to almost all of the current antimalarial drugs [4,8,9,17–21,22••,23] (Table S1). PfMDR1 is a putative ABC transporter (i.e., a pump that expends ATP to move solutes against their electrochemical gradients; Figure 2b), whereas PfCRT belongs to the drug/metabolite transporter superfamily (i.e., it is a 'carrier' that mediates the transport of a solute down its electrochemical gradient; Figure 2c) [24,25].

PfMDR1 and PfCRT are located at the DV membrane and were originally identified for their roles in conferring resistance to the former 'wonder drug' chloroquine [25,26]. Mutations in PfCRT are both necessary and sufficient to confer chloroquine resistance. They enable the transporter to efflux chloroquine out of the DV (Figure 2c) and thus away from the drug's antiplasmodial

Figure 1



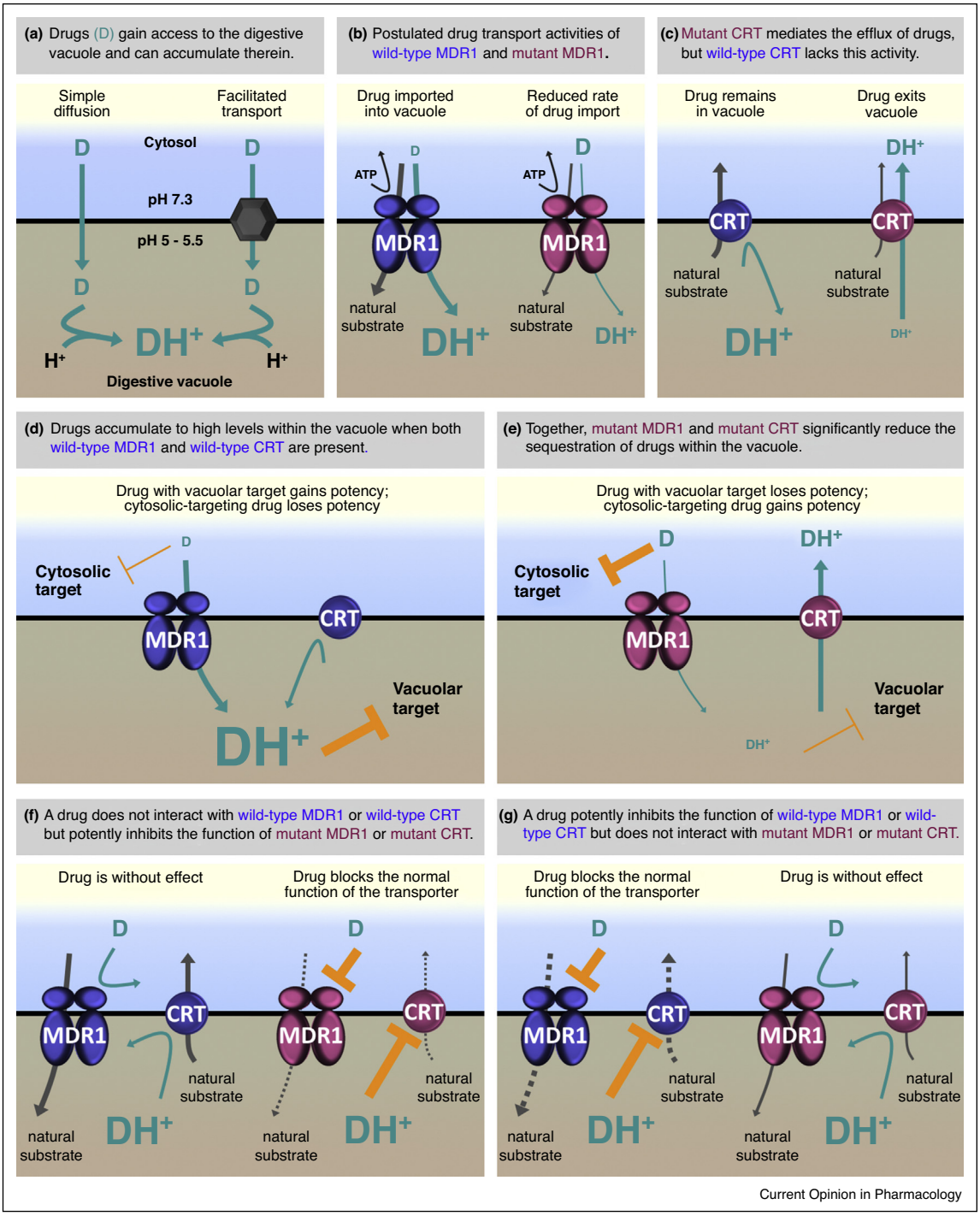
Determinants of resistance to ACT partner drugs in *P. falciparum*. The schematic shows a human erythrocyte (red) infected with an asexual-stage parasite (blue) that is surrounded by its parasitophorous vacuole (cream). The known determinants of resistance to sulfadoxine–pyrimethamine, amodiaquine, pyronaridine, piperaquine, mefloquine, and lumefantrine are depicted as grey cartoons and are shown at their respective location(s) within the parasite. The adjacent rectangles each represent the outcome of a study that investigated the knock-out or gene-disruption phenotype of the gene. Green, orange, red, and white correspond to dispensable, slow growth, essential, or undetermined phenotypes, respectively; 'b' and 'f' indicate that the study was performed in *P. berghei* or *P. falciparum*, respectively. Refer to Table S1 for gene identifiers, gene acronyms, and references. *Abbreviations*: Ap, apicoplast; ER, endoplasmic reticulum; Golgi, Golgi apparatus; Hb, hemoglobin; Mit, mitochondrion.

target (the detoxification of heme) [27–30]. The resistance-conferring isoforms of PfCRT can likewise efflux several other antimalarials from the DV, including quinine, quinidine, quinacrine, and methylene blue [27,31]. By contrast, wild-type PfCRT lacks significant drug transport activity (Figure 2c). Polymorphisms in *pfmdr1* are not sufficient to confer chloroquine resistance but can alter the level of resistance exhibited by parasites harbouring a resistance-conferring isoform of PfCRT [20,22••,23,32]. Efforts to characterize PfMDR1 in heterologous systems have not delivered robust results and/or direct measurements of its transport activity [33,34]. However, whilst the full nature of PfMDR1's role in conferring antimalarial resistance remains to be resolved, the location of its ATPase domains at the cytosolic face of the membrane [35] suggests that it pumps drugs into the DV (Figure 2b). Aside from conferring drug resistance, PfCRT and PfMDR1 fulfil essential (but currently unresolved) functions in the intraerythrocytic parasite [21,36•,37,38•]. It is therefore possible that PfCRT and PfMDR1 are themselves the target of drugs (reviewed in [39]). Experimental support for this hypothesis comes from two recent studies [19,40]. In the first, 'quinine dimers' were found to be more active against chloroquine-resistant parasites than their chloroquine-sensitive counterparts and the extra killing effect in the chloroquine-resistant parasites was shown to be attributable to the potent ability of these drugs to inhibit chloroquine-resistance-conferring isoforms of PfCRT (which would likely block the transport of the protein's natural substrate (s); Figure 2f) [40]. In the second study, the phenomenon of chloroquine-resistant parasites exhibiting hypersensitivity to quinine when T76I is introduced into PfCRT was likewise shown to be due to quinine gaining the ability to bind extremely tightly to the transporter [19]. The additional mutations in PfCRT (e.g., C72R, Q352K, or Q352R) that suppressed quinine hypersensitivity abolished the interaction between the transporter and quinine. These changes also abrogated the ability of the transporter to mediate chloroquine efflux from the DV, thus explaining why rescue from this PfCRT-induced hypersensitivity to quinine simultaneously restored the parasite's sensitivity to chloroquine [19]. In the case of PfMDR1, it has been proposed that mefloquine may target the wild-type transporter and that amplification and/or mutation of *pfmdr1* alleviates this inhibitory effect and thereby confers mefloquine resistance (Figure 2g; [41,42]).

Another two ABC transporters that are thought to influence the parasite's susceptibility to several of the ACT drugs are the 'multidrug resistance-associated proteins' 1 and 2 (PfMRP1 and PfMRP2) (Table S1). Their roles in drug resistance remain unclear, but based on their similarities to human MRP1, it seems likely that these proteins function to export glutathione-conjugated drugs from the parasite's cytosol. Consistent with this hypothesis, parasites lacking *pfmrp1* displayed heightened sensitivities to several antimalarials (including chloroquine and artemisinin), whereas increases in the expression of *pfmrp2* reduced the parasite's sensitivity to drugs such as chloroquine and mefloquine [43,44].

The mechanism underlying chloroquine resistance appears to be fairly straightforward, insofar as it entails — at least for the most part — a reduction in the accumulation of chloroquine within the DV (Figure 2d,e). The mechanistic basis of *P. falciparum* resistance to sulfadoxine–pyrimethamine is likewise relatively uncomplicated. Resistance is conferred by mutations in the 'dihydropteroate synthase' (*dhps*) and 'dihydrofolate reductase' (*dhfr)* genes, which encode the targets of sulfadoxine and pyrimethamine, respectively [45,46] (Table S1). However, it is becoming increasingly apparent that resistance to several of the other ACT partner drugs, and to dihydroartemisinin itself, is multifactorial. This review summarizes our current understanding of the molecular determinants of resistance to the ACTs in *P.*

Figure 2



Mechanisms by which PfMDR1 and PfCRT could modulate the parasite's sensitivity to drugs. **(a)** An uncharged drug (D) can enter the digestive vacuole (DV) by diffusing across the membrane (simple diffusion). Alternatively, or in addition, the drug accesses the DV via a carrier or a pump (facilitated transport). Upon entering the acidic environment of the DV, weak-base drugs such as chloroquine, amodiaquine, pyronaridine, piperaquine, mefloquine, and lumefantrine will become protonated and thereby accumulate via 'weak-base trapping'. Dihydroartemisinin may also enter the DV and become activated by the heme generated from hemoglobin digestion. **(b)** Wild-type PfMDR1 (blue) may be a key route by which various drugs enter the DV, whereas certain mutant isoforms of PfMDR1 (maroon) may possess reduced capacities for drug import. Mutation of PfMDR1 can also incur a fitness cost, possibly because the natural function of the protein is diminished. **(c)** Certain mutant isoforms of PfCRT (maroon) are known to transport a number of different drugs (including chloroquine) out of the DV, whereas wild-type PfCRT (blue) lacks significant drug transport activity. In the case of chloroquine, this difference in transport properties underpins the parasite's mechanism of resistance. Most mutant isoforms of PfCRT impart a fitness cost, presumably because they have reduced capacities for the transport of the natural substrate(s). **(d)**

falciparum, as well as the mechanisms by which polymorphisms in these genes may be reducing the parasite's susceptibility to these drugs.

Dihydroartemisinin

Partial resistance to dihydroartemisinin causes delayed parasite clearance following ACT treatment and has been linked to mutations in the *pfkelch13* gene [3,10]. The mutations in *pfkelch13* appear to confer resistance by enhancing a cellular stress response that engages the unfolded protein response [47] and the ubiquitin-proteasome system [47], and may also result in a reduced rate of hemoglobin digestion [14•]. These adaptions are thought to enable the parasite to cope with the otherwise lethal oxidative stress and protein damage caused by dihydroartemisinin. Several additional genes have been implicated in the dihydroartemisinin resistance phenotype, including certain isoforms of PfCRT and PfMDR1 [9,18,21,22••,43,48•,49,50•]. It is currently unclear how these two transporters contribute to *pfkelch13*-dependent dihydroartemisinin resistance, nor is it known what role they play in the phenomenon of *pfkelch13*-independent dihydroartemisinin resistance [49,50•,51] — which has been observed both *in vitro* and in recent treatment failures [5•,52•]. The former has been linked to elevated levels of phosphatidylinositol-3-phosphate (a signalling molecule produced by the parasite's phosphatidylinositol-3-kinase) [49,51]. It is possible that dihydroartemisinin exerts its antiplasmodial effects primarily within the cytosol, and that the transport activities of certain isoforms of PfMDR1 and PfCRT contribute to resistance by causing the drug to become sequestered within the DV (Figure 2d), whereas expression of other isoforms may result in the drug gaining increased access to the cytosol (Figure 2e) [22••,53•]. Alternatively, it may be that neither transporter interacts with dihydroartemisinin and that the polymorphisms in *pfcrt* and *pfmdr1* are compensatory — that is, they contribute to the dihydroartemisinin resistance phenotype by improving the underlying fitness of the parasite.

Amodiaquine and pyronaridine

Amodiaquine is structurally related to chloroquine and likewise accumulates within the DV, wherein it exerts its antiplasmodial effect by binding (with greater affinity than chloroquine) to hemozoin crystals [54]. Consistent with these commonalities, mutations in PfCRT appear to be the main determinant of amodiaquine resistance, with certain mutations in PfMDR1 causing further decreases in the parasite's sensitivity to the drug [20,22••,55]. However, it is worth noting that chloroquine-resistant parasites from South America and the Philippines (e.g., the 7G8, Ecu1110, and Ph1 strains, all of which display low-to-moderate levels of chloroquine resistance) generally exhibit high levels of amodiaquine resistance, whereas many of the chloroquine-resistant strains from Africa and South-East Asia (e.g., GB4, Dd2, and K1, which exhibit high-level resistance to chloroquine) display low-level amodiaquine resistance [20,26,56,57]. Chloroquine-resistant strains expressing GB4-type isoforms of PfCRT and PfMDR1 have nevertheless increased in prevalence over chloroquine-sensitive parasites (harbouring wild-type PfCRT and PfMDR1) in regions of Africa where amodiaquine-artesunate has been deployed [58].

In vitro studies performed with live *P. falciparum* parasites further suggest that a common mechanism underpins resistance to amodiaquine and chloroquine. High levels of amodiaquine resistance have been correlated with low levels of amodiaquine accumulation [55], and indirect measurements of drug efflux from the DV using a 'proton-efflux' assay have detected amodiaquine transport via chloroquine-resistance-conferring isoforms of PfCRT (whereas the wild-type protein lacked this activity) [27]. Direct measurements of amodiaquine transport, such as can be achieved with the *Xenopus* oocyte expression system [28], are needed to determine the capacities of clinically-relevant isoforms of PfCRT and PfMDR1 for amodiaquine transport. Such analyses would identify the transport properties that correlate with high-level amodiaquine resistance.

Pyronaridine is an aza-acridine derivative that is structurally-related to quinacrine and amodiaquine, and which has only recently been deployed as an ACT partner drug. Little is known about the determinants of pyronaridine resistance, or indeed of its mode-of-action (which is

(Figure 2 Legend Continued) When both wild-type PfMDR1 and wild-type PfCRT are present, a given drug may be sequestered within the DV and would therefore attain relatively low concentrations within the cytosol. In this scenario, a drug with a DV target (e.g., chloroquine) would exhibit increased potency, whereas the potency of a cytosolic-acting drug (e.g., amantadine; [19]) would be reduced. **(e)** Together, the mutant isoforms of PfMDR1 and PfCRT cause a significant decrease in the sequestration of a given drug within the DV and thereby increase its cytosolic concentration. A drug with a vacuolar target (e.g., chloroquine) will exhibit reduced potency, whereas the potency of a cytosolic-acting drug (e.g., amantadine; [19]) will be increased. Whilst not yet observed, it is possible that wild-type PfCRT possesses a greater capacity than mutant PfCRT for the transport of certain drugs. Such a scenario would further complicate the decipherment of resistance mechanisms in the parasite. **(f)** A given drug may have little or no interaction with the substrate-binding sites of wild-type PfMDR1 and PfCRT and thus has little effect on the normal physiological functions of these transporters. By contrast, the drug could be a potent inhibitor (but not a substrate) of mutant PfMDR1 and/or mutant PfCRT and thereby kill the parasite by blocking the essential function of the transporter. The quinine dimers [40] are an example of such a phenomenon, as is quinine in the case of parasites harbouring chloroquine-resistance-conferring isoforms of PfCRT that carry the T76I mutation [19]. **(g)** In an alternate scenario, a given drug may exert an antiplasmodial effect by blocking the natural function of wild-type PfMDR1 or wild-type PfCRT, which the parasite could overcome through amplification and/or mutation of the respective gene. It is possible that mefloquine targets wild-type PfMDR1 in this manner, such that overexpression of the transporter (resulting from multiple copies of *pfmdr1*) alleviates this inhibitory effect and thereby confers mefloquine resistance [41,42].

thought to include inhibition of hemozoin formation [59]). The involvement of known resistance mediators, such as *pfcrt*, *pfmdr1*, and *pfmrp1*, is yet to be resolved. For example, some studies have observed an association between mutations in PfCRT and decreases in pyronaridine susceptibility, whereas others have not [7,60]. It seems likely that resistance to pyronaridine will be multifactorial.

Piperaquine

Piperaquine is a bis-aminoquinoline analogue of chloroquine that was initially thought to display no cross-resistance with chloroquine [61,62], and to lack significant interactions with PfCRT and/or PfMDR1 [63,64]. However, these conclusions are being re-evaluated following the realization that standard *in vitro* growth assays do not provide reliable assessments of the parasite's susceptibility to piperaquine [4]. Indeed, it is becoming increasingly apparent that piperaquine resistance in *P. falciparum* is a multifactorial phenomenon, and that polymorphisms in *pfcrt* and *pfmdr1* do contribute to the underlying mechanisms (see below) [4,6,65,66,67•]. Two recently-identified determinants of high-level piperaquine resistance in Cambodian isolates are *plasmepsin 2* and *plasmepsin 3*, which encode DV-localized aspartate proteases that are involved in the degradation of host hemoglobin [2••,12••,67•] (Table S1). The amplification of *plasmepsin 2* and/or *plasmepsin 3* was strongly correlated with dihydroartemisinin–piperaquine treatment failures, and the inactivation of either gene resulted in parasites that were hypersensitive to piperaquine [68••]. Piperaquine resistance in Cambodian isolates has also been associated with single-copy *pfmdr1* [2••,12••,67•] as well as mutant isoforms of PfCRT [4], but none of these polymorphisms were by themselves sufficient to confer high-level resistance. Elsewhere, the deployment of dihydroartemisinin–piperaquine has been observed to select for the chloroquine-resistance-conferring alleles of *pfcrt* and *pfmdr1* in Uganda and Papua New Guinea [4,6,65,69,70], but did not appear to exert this effect in Burkina Faso [71,72]. There is also some evidence of a role for *pfmrp2* in the piperaquine resistance phenotype [44].

The knock-out of *plasmepsin 2* or *plasmepsin 3* (or of both genes) does not affect parasite growth [68••,73,74], which means it is unlikely that these enzymes are the antiplasmodial targets of piperaquine. Nevertheless, piperaquine evidently inhibits one or more steps in the degradation of hemoglobin; it causes the accumulation of both free heme and undigested hemoglobin, and inhibits the formation of hemozoin [17]. Given the current knowledge, the simplest mechanistic explanation for piperaquine resistance would entail a reduction in its ability to inhibit hemoglobin digestion (through the amplification of *plasmepsin 2* and/or *plasmepsin 3*) and a decrease in the accumulation of the drug within the DV (mediated primarily by polymorphisms in *pfcrt* and *pfmdr1*; Figure 2d,e).

Mefloquine and lumefantrine

The amplification of the wild-type *pfmdr1* gene reduces the parasite's sensitivity to mefloquine and the structurally-related drug lumefantrine [11,21,23,41,75–77], but simultaneously increases sensitivity to chloroquine and amodiaquine [32,41,78] (Table S1). Furthermore, chloroquine-resistance-associated alleles of *pfmdr1* and *pfcrt* typically hypersensitize parasites to lumefantrine, mefloquine, artemisinin, and dihydroartemisinin [8,9,53•,56,79–85]. Indeed, multiple clinical studies have observed that artemether-lumefantrine selects for wild-type *pfmdr1* (and/or for multiple copies of *pfmdr1*) as well as wild-type *pfcrt* (when present in the parasite population) over the chloroquine-resistance-associated alleles [8,9,65,69,71,72,86–90]. This phenomenon, whereby resistance to a drug causes hypersensitivity to another drug, is known as 'collateral drug sensitivity' and it is thought that the opposing selection forces at play could be exploited to combat existing and emerging drug resistance [19,91]. However, much remains to be understood about the mechanisms that underlie collateral drug sensitivity in *P. falciparum* parasites.

Although mefloquine and lumefantrine can interfere with hemozoin formation [92], it has long been suspected that both drugs target other processes [16,93]. For example, it was proposed that these drugs may exert their main antimalarial effect by inhibiting the normal function of PfMDR1 (Figure 2g; [41,42]). Whilst the main target of lumefantrine remains unresolved, the cytoplasmic ribosome (Pf80S) of the asexual blood-stage parasite has recently emerged as a potential key target of mefloquine (Table S1 and Figure 1) [94•]. It is therefore possible that amplified *pfmdr1* and wild-type *pfcrt* contribute to the mefloquine resistance phenotype by enabling the drug to sequester within the DV, whereas the chloroquine-resistance-associated isoforms increase the parasite's sensitivity to mefloquine by redirecting the drug back to its cytosolic target (Figure 2d,e). Polymorphisms in *pfmrp1* and *pfmrp2* [11,43,44] could further enhance mefloquine resistance by increasing the export of the glutathione-conjugated drug out of the parasite cytosol.

Conclusions

Whilst recent breakthroughs have significantly advanced our understanding of the genes and molecular mechanisms underpinning the malaria parasite's resistance to ACT partner drugs, much remains to be resolved. It is becoming increasingly apparent that some, if not most, of these drugs have elicited multifactorial mechanisms of resistance in *P. falciparum* and that we are yet to elucidate all of the key determinants. Nor do we have an adequate grasp of the underlying biological processes — both in regard to drug modes-of-action and mechanisms of

resistance — which in several cases are evidently quite complex. Avenues for overcoming these shortcomings could include genetically-manipulating parasites from a broad range of backgrounds to either gain or lose known resistance-associated polymorphisms, followed by *in vitro* assays of drug susceptibility as well as whole-genome sequencing. The resulting datasets would enable correlations to be made between changes in the parasite's sensitivity to a drug and other potential determinants of resistance.

A recurring theme across many of the ACT partner drugs was the involvement of polymorphisms in transporters, particularly *pfcrt* and *pfmdr1*, in the resistance phenotype. This observation underscores the significant role drug redistribution plays in conferring, or contributing to, antimalarial resistance. It also raises the possibility that some of these transporters may instead (or additionally) be targets of one or more of the ACT partner drugs. Distinguishing between these scenarios, and elucidating the transport properties of the proteins involved (with and without resistance-associated mutations), would provide valuable insights into the physiological adaptions being employed by the parasite to evade the antiplasmodial effects of the ACT partner drugs. Achieving these outcomes will require the combined use of robust heterologous expression systems (to undertake direct measurements of transport) and live parasite assays (to correlate the transport properties determined *in vitro* with indirect — but *in situ* — measurements of transporter function).

Ultimately, detailed knowledge of the parasite's mechanisms of resistance to the ACT partner drugs is needed to reap full benefit from any interventions seeking to limit the evolution and impact of resistance. Examples of such approaches would be the re-optimization of dose regimens, the rational design of triple combination therapies that consist of drugs which impose conflicting evolutionary pressures, or the concurrent (or rotational) deployment of two therapies that likewise exert opposing forces upon the parasite.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.coph.2018.07.010.

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