

Recent highlights in antimalarial drug resistance and chemotherapy research

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This review summarizes recent investigations into antimalarial drug resistance and chemotherapy, including reports of some of the many exciting talks and posters on this topic that were presented at the third Molecular Approaches to Malaria meeting held in Lorne, Australia, in February 2008 (MAM 2008). After surveying this area of research, we focus on two important questions: what is the molecular contribution of *pfcr* to chloroquine resistance, and what is the mechanism of action of artemisinin? We conclude with thoughts about the current state of antimalarial chemotherapy and priorities moving forward.

The search for new antimalarials

For decades, malaria chemotherapy has relied on a limited number of drugs. However, the acquisition and spread of drug resistance has led to an increase in morbidity and mortality rates in many malaria-endemic regions. This increasing burden caused by drug-resistant parasites has stimulated investigators to seek out novel antimalarial inhibitors and drug targets, and to define the genetic basis of resistance to existing drugs, as a means to facilitate detection and develop novel strategies to overcome resistance.

Targeting chromatin-modifying enzymes

The post-genomic era has ushered in a renewed focus on mechanisms of gene regulation in *Plasmodium*, driven largely by investigations into the process of allelic exclusion in the *var* gene family [1]. These have revealed a key role for the *Plasmodium falciparum* histone deacetylase (HDAC) Sir2, the function of which in dynamic chromatin remodeling is intimately associated with *var* gene silencing [2,3]. Earlier studies had identified HDAC inhibitors (Table 1) that had moderate potency against cultured *P. falciparum* parasites *in vitro* and rodent malaria parasites *in vivo* (see, for example, Ref. [4]). At MAM 2008, Kathy Andrews (Queensland Institute of Medical Research, Australia) and colleagues reported highly potent *in vitro* inhibitors, with IC₅₀ values as low as 3 nM, which possess high selectivity for *P. falciparum* compared to mammalian cells [5]. These inhibitors hyperacetylate parasite histones and produce an altered RNA-expression profile for some *P. falciparum* genes. Zbynek Bozdech (Nanyang Technological University, Singapore) reported similar findings with

the HDAC inhibitor apicidin (abstracts for these and other studies reported at MAM 2008 appear in a Supplement to the *Int. J. Parasitol.*)*.

The apicoplast – how good a target?

Additional novel drug targets are thought to reside within the parasite apicoplast, an organelle ancestrally related to chloroplasts [6]. In describing these, Geoff McFadden (University of Melbourne, Australia) discussed the apicoplast type II fatty acid synthesis pathway, which has been postulated to be the target of several microbicides including triclosan and thiolactomycin [6]. Groups led by David Fidock (Columbia University, New York, NY) and Stefan Kappe (Seattle Biomedical Research Institute, Seattle, WA) have recently found that, unexpectedly, this pathway is non-essential in asexual blood stages but is key to the development of infectious liver-stage forms. A more promising apicoplast pathway for the development of an effective blood-stage antimalarial is that of isoprenoid biosynthesis. Proof of principle has been achieved already with the demonstration that fosmidomycin, an inhibitor of the isoprenoid enzyme 1-deoxy-D-xylulose 5-phosphate (DOXP) reductase, can clinically cure *P. falciparum* infection either with extended monotherapy or as a component of shorter-duration combination therapies [7]. The challenge is to identify fosmidomycin analogs that have a more suitable pharmacokinetic profile. For now, the most clinically useful apicoplast-specific antimalarials are the antibiotics, such as clindamycin, tetracycline and azithromycin, that target protein translation. These antibacterials, which produce a ‘delayed death’ phenotype with *Plasmodium* parasites, have proven to be fairly effective components of antimalarial combinations [6,8]. *In vitro* resistance to azithromycin, however, can be acquired readily with *P. falciparum* asexual blood-stage cultures [9]. To make these antibiotic classes more effective as antimalarials will require analog synthesis programs that can identify compounds with greater potency and a reduced propensity to succumb to resistance.

Parasite metabolism

Inhibitors that arrest parasite metabolism or transport processes are now attracting considerable interest. One area is vitamin biosynthesis, which occurs in *P. falciparum* but is absent in humans. Carsten Wrenger (Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany) presented the development of prodrugs that

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Table 1. *P. falciparum* drug targets with active small molecule inhibitors currently under investigation

Cellular localization	Target	Refs
Apicoplast	DOXP reductoisomerase	[7]
	Apicoplast gyrase	[8]
	Apicoplast protein synthesis	[8]
	Apicoplast transcription	[8]
Cytosol	Fatty acid synthesis	[76]
	Choline synthesis	[17]
	Coenzyme A biosynthesis	[77]
	Gamma-glutamylcysteine synthetase	[76]
	Hypoxanthine-guanine phosphoribosyl transferase	[76]
	Leucine aminopeptidase	[78]
	Methionine aminopeptidases	[79]
	Protein farnesyltransferase	[80]
	Protein N-myristoyl transferase	[81]
	Shikimate pathway	[76]
	Thioredoxin reductase	[76]
	Thymidylate synthase	[82]
	Vitamin B6 synthesis	[83]
Dense granules	Subtilisin-like proteases	[84]
Digestive vacuole	Cysteine/aspartic proteases	[76]
	Heme detoxification	[85]
Membrane	Folate-biopterin transport	[86]
	Hexose transport	[12,13]
	V-type H ⁺ -ATPase	[14]
	Host-parasite interactions during invasion	[26]
Mitochondria	Dihydroorotate dehydrogenase	[21]
	Mitochondrial bc1 complex	[23]
Nucleus	Cyclin-dependent protein kinases (<i>Pfmrk</i>)	[76]
	DNA	[87]
	Histone acetyltransferase	[88]
	Histone deacetylase	[5]
Red blood cell	Heterotrimeric G protein	[89]

upon activation by pyridoxine kinase can inhibit pyridoxal phosphate. Another pathway currently being targeted is the biosynthesis of coenzyme A from the precursor pantothenate (vitamin B₅). Kevin Saliba and his group (Australian National University, Canberra, Australia) have now identified analogs of pantothenate that possess selective antimalarial activity, with IC₅₀ values of 15 μM or higher [10].

Parasite transporters

Transporters involved in nutrient acquisition from the host are also under investigation as candidate targets. Enrique Salcedo from the group of Patrick Bray (Liverpool School of Tropical Medicine, UK) reported that the parasite-encoded folate-biopterin transporters PFFBT1 and PFFBT2 can be inhibited by the dihydrofolate reductase inhibitor methotrexate and the organic anion transporter inhibitor probenecid. Folic acid uptake can also be inhibited by the folate precursor para-amino benzoic acid, indicating a wide spectrum of substrates for these two parasite transporters. An association between energy-dependent uptake of folates and mitochondrial processes has also been observed by Ping Wang in the group of John Hyde (University of Manchester, UK), based on studies with compounds known to inhibit mitochondrial processes in other systems (see Ref. [11] and data reported at MAM 2008)*. Sanjeev Krishna (St. George's Hospital Medical School, London,

UK) and Kevin Saliba are also exploring inhibitors of the hexose transporter PfHT [12,13] and the *P. falciparum* V-type H⁺-ATPase [14], respectively.

Choline biosynthesis

Unquestionably, the most advanced antimalarial drug development program targeting parasite metabolism relates to inhibition of *de novo* phosphatidylcholine biosynthesis (Figure 1). This program, spearheaded by Henri Vial (CNRS and University of Montpellier, France), has identified choline analogs that inhibit *P. falciparum* asexual blood stages at single-digit nanomolar concentrations [15]. Proof of fully curative antimalarial activity with short-course treatments has been obtained in rodents and non-human primates infected at high parasitemias [16,17]. These compounds are thought to inhibit choline transport and also exert an effect on parasite phospholipid biosynthesis. These multiple modes of action, distinct from current antimalarial agents, are a major strength of these inhibitors [18] because they could help delay the development of resistance. Human clinical trials with this exciting new class of compounds are currently under way, operated by Sanofi-Aventis (H. Vial, personal communication).

Inhibition of mitochondrial enzymes

Compounds that inhibit pyrimidine *de novo* biosynthesis also provide promise for future development. Major efforts in this direction are being applied to dihydroorotate dehydrogenase (DHODH), a key flavin mononucleotide-dependent mitochondrial enzyme that utilizes coenzyme Q as the final electron acceptor [19,20]. Margaret Phillips (UT Southwestern Medical Center, Dallas, TX) and colleagues recently have identified several potent, species-selective inhibitors of *Pf*DHODH [21]. These inhibitors display IC₅₀ values in the range 20–600 nM and Exhibit 200–20 000-fold selectivity for the malarial over the human enzyme. These completely block the Q-dependent oxidation of flavin mononucleotide, indicating that they act upon the electron transfer pathway between flavin mononucleotide and coenzyme-Q [22]. Giancarlo Biagini and Steve Ward (Liverpool School of Tropical Medicine, Liverpool, UK) have also identified a new class of mitochondrial inhibitors, known as dihydroacridinediones. These inhibitors target the quinol oxidation site (Q_o) of the parasite mitochondrion bc1 complex, causing a collapse of the mitochondrion membrane potential and cell death, and display selectivity against the parasite enzyme that is ~5000-fold higher than for human liver bc1 [23].

Inhibition of parasite invasion of red blood cells

Drugs could also potentially be designed that inhibit parasite invasion of host red blood cells (RBCs). One mechanism of parasite invasion involves the microneme protein EBA-175, which recognizes host-cell sialic-acid-containing glycophorin A [24–26]. Using NMR spectroscopic techniques and molecular modeling, Mark von Itzstein and colleagues (Griffith University, Queensland, Australia), are exploring the possibility of using neuraminic acid derivatives to compete for sialic acid recognition by EBA-175. Perturbation of this interaction might decrease or prevent parasite invasion of host RBCs.

* MAM 2008, Lorne, Australia, *Int. J. Parasitol.* 38, Suppl. 1, S1–S138.

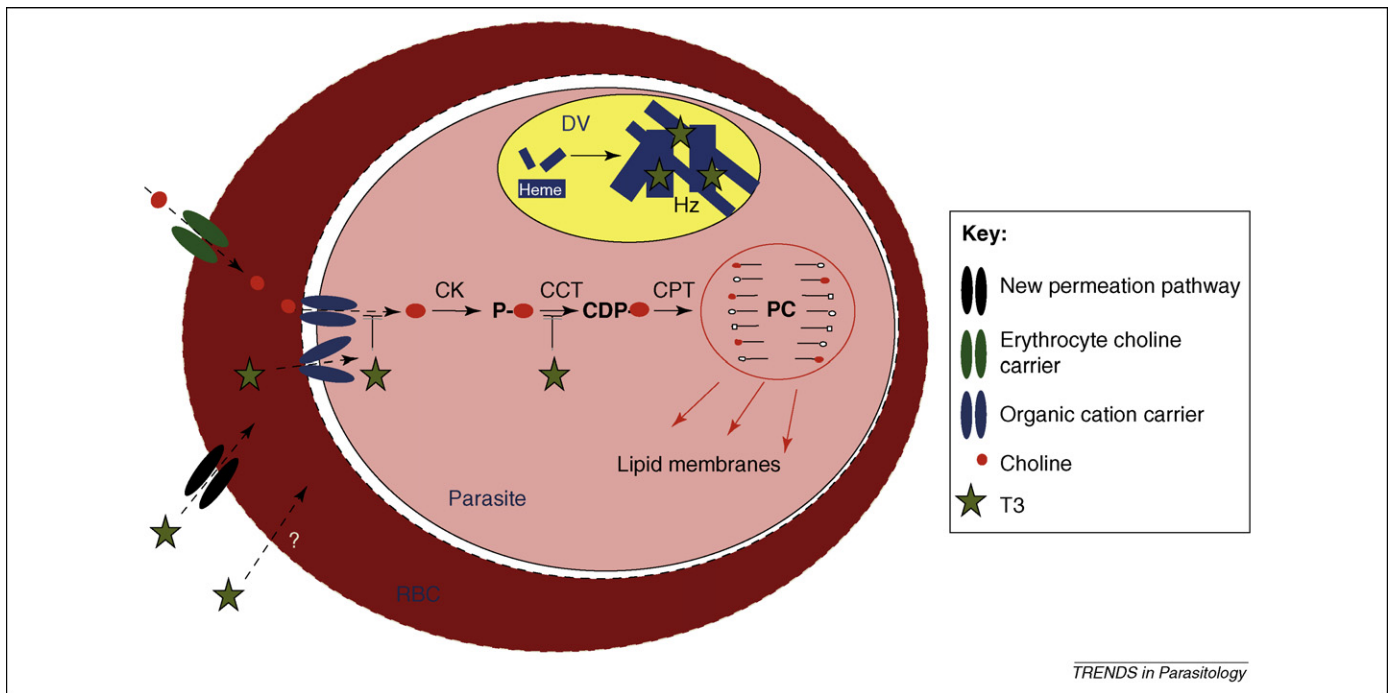


Figure 1. Mechanism of the antimalarial activity of the choline analog T3. This representative bis-quaternary ammonium compound is thought to enter the infected erythrocyte through the new permeation pathway or another (unknown) mechanism (represented by the question mark near the RBC membrane). This choline analog is then taken up into the intraerythrocytic parasite via an organic cation transporter present on the parasite plasma membrane. This perturbs choline uptake from the host cell compartment, thus disrupting the synthesis of the predominant membrane lipid, phosphatidylcholine. Additional effects on phosphatidylcholine biosynthesis enzymes have also been proposed [16]. T3 and other biscationic compounds also accumulate within the parasite, mostly in the digestive vacuole (DV, yellow) where they bind heme (ferriprotoporphyrin IX). This heme interaction has also been hypothesized to contribute to their antiplasmodial activity [18]. These compounds, therefore, might have multiple sites of action within the parasite. CCT, phosphocholine cytidylyltransferase; CDP, cytidine diphosphate; CK, choline kinase; CPT, choline phosphate transferase; Hz, hemozoin; P, phosphate; RBC, red blood cell.

Inhibition of parasite proteases

The falcipain and plasmepsin families of cysteine and aspartic proteases, respectively, which are involved in parasite-mediated hemoglobin digestion in the digestive vacuole (DV) of intra-erythrocytic parasites, have long been defined as promising drug targets [27,28]. In collaboration with Philip Rosenthal (University of California, San Francisco, CA), scientists at GlaxoSmithKline (Tres Cantos, Spain) have identified inhibitors of falcipains 2 and 3 that prevent *in vitro* *P. falciparum* growth at low nanomolar concentrations. These inhibitors can also cure *P. falciparum* infections propagated *in vivo* in human RBCs in a new immunocompromised mouse model [29]. Drug discovery efforts focused on the plasmepsin enzymes have lessened after the discovery of ten plasmepsin paralogs in the *P. falciparum* genome and the finding that parasites can survive, albeit with a reduced growth rate, without all four plasmepsins of the DV [30,31]. Recent studies by Dan Goldberg (Washington University School of Medicine, St. Louis, MO) now reveal substantial redundancy within and between these two hemoglobinase families and show that the plasmepsin zymogens can be cleaved and activated by falcipains or autoactivated in the presence of falcipain inhibitors [32]. Interestingly, HIV protease inhibitors, which are believed to target one or more parasite aspartic proteases, have been found to be effective against malarial infection. Kathy Andrews and Tina Skinner-Adams (Queensland Institute of Medical Research, Australia) presented their data with HIV protease inhibitors tested against *P. falciparum* *in vitro* or *Plasmodium chabaudi* *in vivo* (reviewed in Ref. [33]). These findings are particularly

promising in light of the growing use of these protease inhibitors for the treatment of HIV in malaria-endemic regions.

Emerging technologies

The ability to identify new drug targets and antimalarial compounds through large-scale screens is rapidly improving with emerging high throughput technologies. Vicky Avery (Griffith University, Queensland, Australia) and colleagues are using a DAPI nuclear dye-based high-throughput confocal imaging assay to screen natural product libraries for whole-cell antimalarial activity and have identified several plant extracts with micromolar activities [34]. Whole-cell assays using fluorescence-activated cell sorting analysis of a green fluorescence protein-expressing *P. falciparum* line are also being developed by Chairat Uthairibull (National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand) in collaboration with Brendan Crabb (the Burnet Institute, Melbourne, Australia) and Alan Cowman (The Water and Eliza Hall Institute, Melbourne, Australia), as part of the WHO TDR-supported Transfection Network. Elizabeth Winzeler (Genomics Institute of the Novartis Research Foundation and The Scripps Research Institute, La Jolla, CA) and colleagues have also developed a 1536-well, SYBR green I fluorescence-based parasite proliferation assay that was used to screen a Novartis collection of 2.6 million chemically diverse compounds, producing more than 9000 active antimalarial hits [35].

Powerful new 'omic' technologies are also being developed to explore drug mode of action and resistance. A

the parasite cytoplasm. This drug translocation is sensitive to verapamil. When the proton pump of the parasite is inhibited by concanamycin A, CQ causes a verapamil-sensitive increase in the rate of DV alkalization of CQR strains, indicating that CQ efflux is associated with protons [47] (Figure 2).

However, PfCRT mutations are not the full story. Using a transfection-based approach, Stephanie Valderramos and David Fidock (Columbia University, New York) recently have obtained definitive evidence that the genetic background of the host strain determines the extent to which mutant *pfert* exerts CQR. This finding confirms earlier indications obtained with genetically diverse field isolates or culture-adapted lines (reviewed in Ref. [48]). The most unusual response was observed with the transfected D10 clone engineered to express mutant *pfert*, in that its decreased susceptibility to CQ was most pronounced at the IC₉₀ level (i.e. the concentration of drug that inhibits 90% of growth relative to untreated control) and not the IC₅₀ level. Further experiments revealed that depending on the strain, mutant *pfert* confers either resistance or a state of tolerance, implying that other factors must contribute to enable certain strains to acquire high-level resistance in the presence of mutant *pfert*.

Point mutations or changes in the levels of expression of the *pfmdr1* gene product Pgh1 can also affect the *P. falciparum* CQR phenotype in a strain-dependent manner [49–52]. Recent studies by David Johnson and Steve Ward have provided evidence that Pgh1 expression can be increased upon parasite exposure to the anticonvulsant phenobarbitone (Figure 2), which causes decreased *in vitro* CQ susceptibility in both CQR and CQS parasites [53]. This effect is distinct from other studies in which Pgh1 overexpression has been associated with increased CQ susceptibility [54]. In the Johnson study, putative nuclear-receptor-binding sites with homology to mammalian sequences activated by phenobarbitone were identified in the *pfmdr1* and *pfert* promoter regions, and several putative nuclear receptor genes were identified. These findings raise the novel hypothesis that nuclear-receptor-mediated responses to drug exposure might be a mechanism of gene regulation in *P. falciparum* [53].

Whereas CQ-resistant strains of *P. falciparum* have spread throughout virtually all malaria-endemic areas, CQR in *Plasmodium vivax* remains relatively rare and is restricted mostly to Indonesia, Irian Jaya and other countries in the Pacific region. The genetic basis of *P. vivax* CQR has remained elusive and does not seem to involve *pvcg10*, the *P. vivax* ortholog of *pfert* [55]. Simon Kang'a and others in the group of Jane Carlton (New York University Medical Center, New York, NY) recently surveyed variation across 36 microsatellite markers in 19 CQ-resistant and 21 CQS *P. vivax* samples from patients in Indonesia. Their preliminary analysis found no evidence of a selective sweep in the resistant isolates and revealed a low level of genetic diversity overall. Ric Price (Menziess School of Health Research, Darwin, Australia) adopted an alternative approach that employed an optimized *P. vivax* *in vitro* schizont maturation assay applied to *P. vivax* isolates from Papua and Thailand. Genotyping analysis of *pvmr1* revealed an association between CQR and a

novel Y976F mutation [56]. Their data also supported an association between *pvmr1* amplification and reduced mefloquine susceptibility, reminiscent of the well-established association between *pfmdr1* amplification and reduced mefloquine susceptibility [57]. Kasia Modrzynska (University of Edinburgh, UK) has also been pursuing genetic linkage studies with CQ-resistant lines of *P. chabaudi*. These studies found no association with the orthologs of *pfert* or *pfmdr1* and resulted in the unexpected finding that mutations in the deubiquitinating enzyme UBP-1 occurred in parasites pressured extensively with chloroquine (or artesunate) [58]. Defining the alternative mechanisms of CQR that prevail in other *Plasmodium* species will be important for a more complete understanding of how resistance to this drug is acquired and mediated.

Many questions remain about CQR and CQ treatment failures in humans. Johan Ursing (Karolinska Institute, Stockholm, Sweden) recently reported findings from Guinea-Bissau that CQ, at double doses, was highly effective – curing 78% of uncomplicated malaria patients harboring *pfert* K76T genotypes [59]. This raises the question of the relative importance of pharmacokinetics vs genetics as causes of drug failure. Questions about the relationship between fitness costs imposed by mutant *pfert*, the dynamics of mixed infections and the level of local CQ use have also been raised recently by Qin Cheng (Australian Army Malaria Institute, Enoggera, Queensland), who found that mutant *pfert* was decreasing in prevalence far more slowly in Hainan Island, China [60] than in Malawi [61] after the cessation of CQ use to treat *P. falciparum* malaria. Importantly, the returning CQS parasites have wild-type *pfert* and are genetically diverse.

Identification of the PfCRT K76T mutation by PCR has been used widely to identify CQR and has been proven to be a highly sensitive marker [62]. However, results need to be interpreted with caution, especially in high-transmission settings, where individual patients tend to be infected with many genetically distinct *P. falciparum* variants. Steve Meshnick (University of North Carolina, Chapel Hill, NC) has applied heteroduplex tracking assays (HTAs) to describe this internal heterogeneity. HTAs can be used to quantify variants and detect minority variants that represent as little as 1% of the population in a single host, in contrast to classical PCR methods that can often be insensitive below 10–20%. Using HTA, his group was able to detect the PfCRT K76T-bearing minority variants in ~25% of Malawian patients – all of whom seemed (using classical PCR) to be infected only with wild-type parasites. HTA was also used to detect rare instances of the DHFR I164L mutation in *P. falciparum* infections in Malawi [63].

The mechanism of action of artemisinin

Artemisinin derivatives are the most important new class of antimalarials and are in widespread use. Studies into the cytotoxic effects of artemisinin derivatives have indicated that their activity might be propagated by interactions between the endoperoxide bridge of the drug and heme-iron, produced during hemoglobin degradation inside the DV. This iron has been proposed to generate free radicals that alkylate and oxidize proteins and lipids within infected RBCs (see Ref. [64] and references therein). This hypothesis

is consistent with the finding that artemisinin activity can be potentiated by oxygen and oxidizing agents and attenuated by reducing agents [65]. However, this has been questioned by other studies. One proposed that artemisinins are activated via reductive cleavage of the peroxide bond by intracellular iron–sulfur redox centers, which are common to *Plasmodium* enzymes, and that alkylation of these enzymes could result in parasite death [66]. Interestingly, incubation of parasite lysates with radiolabeled artemisinin identified several interacting proteins, indicating that parasite death might result from the alkylation and inactivation of parasite proteins [67,68]. Additional support against the heme hypothesis is that artemisinins are effective against ring-stage parasites that do not seem to have high concentrations of heme [69]. Specific proteins that have been proposed to be the target of artemisinins include cysteine proteases [70], proteins of the electron transport chain [71], translationally controlled tumor protein [68] and PfATP6, a SERCA-type Ca^{2+} -ATPase [72].

PfATP6 has received the most attention because its activity in transfected *X. laevis* oocytes is abolished by artemisinin but is unaffected by other antimalarials. This includes the inactive compound desoxyartemisinin, which lacks the endoperoxide bridge [72]. Additional studies in *X. laevis* have reported that an L263E point mutation can abolish inhibition by artemisinins [73]. Sanjeev Krishna summarized evidence from field studies and heterologous expression systems that support a role for PfATP6 mutations in emerging artemisinin resistance [74]. To test the role of the L263E mutation in parasite response to artemisinins, allelic exchange experiments were carried out in the Fidock laboratory, in collaboration between the two groups. The presentation included Krishna's statistical analyses of IC_{50} values, which showed a skewed distribution of artemisinin and dihydroartemisinin responses in the mutant recombinant parasites expressing the L263E mutation, with some assays showing higher IC_{50} values in the L263E mutants when the data were normalized to wild-type controls. The analysis of these data is ongoing and the results are keenly awaited because the role of PfATP6 in artemisinin susceptibility remains a topic of considerable debate.

Additional work investigating the mechanism of action of artemisinins was presented by Maria del Pilar Crespo, working in the group of Leann Tilley (La Trobe University, Melbourne, Australia), who provided evidence that artemisinin derivatives cause early disruption of the parasite DV and do not seem to affect the structure of the endoplasmic reticulum (where PfATP6 is presumably located). Using a proteomic approach, Sumalee Kamchonwongpaisan (National Centre for Genetic Engineering and Biotechnology, Bangkok, Thailand) presented evidence that artemisinins might affect parasite endocytosis of host proteins. Whether artemisinin accumulation in the DV relates to its mode of action, and how PfATP6 might contribute to artemisinin action and potential gain of resistance by *Plasmodium* parasites, clearly requires further studies.

Concluding remarks

The increasing awareness of the problems posed by multi-drug-resistant malaria, and of the need to understand the

basis of resistance and develop new chemotherapeutic strategies and drugs, has spurred a rapid increase in the volume of research being applied to this area. The topics cited previously illustrate the tremendous gains that can be achieved through the application of genetic, genomic, proteomic, biochemical, synthetic or medicinal chemistry, or structural biology approaches. Today's optimism that artemisinin-based combination therapies provide a sustainable answer as to how to reduce malaria morbidity and mortality finds parallels in earlier campaigns founded on CQ, the efficacy of which was once absolute. The elaboration of a coordinated Worldwide Antimalarial Drug Resistance Network to identify emerging resistance, spearheaded by Carol Sibley (University of Washington, Seattle, WA), is an important initiative in this regard [75]. Ever more research in the area of antimalarial resistance mechanisms and new chemotherapies must be supported to sustain the continuous stream of discoveries needed to reduce the devastating impact of malaria across the inter-tropical regions of the world.

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