International Journal for Parasitology: Drugs and Drug Resistance 5 (2015) 149–162

Contents lists available at ScienceDirect

International Journal for Parasitology: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijppaw

Invited review

The malaria parasite cation ATPase PfATP4 and its role in the mechanism of action of a new arsenal of antimalarial drugs

Natalie Jane Spillman [a, b, *], Kiaran Kirk [a]

[a] Research School of Biology, The Australian National University, Canberra, Australian Capital Territory 2601, Australia
[b] Department of Medicine (Infectious Diseases), Washington University School of Medicine, Saint Louis, MO 63110, USA

ARTICLE INFO

Article history:
Received 20 May 2015
Received in revised form
3 July 2015
Accepted 8 July 2015
Available online xxx

Keywords:
Plasmodium
Antimalarial
PfATP4
Resistance selection
Drug discovery
Cipargamin

ABSTRACT

The intraerythrocytic malaria parasite, *Plasmodium falciparum*, maintains a low cytosolic Na^+ concentration and the plasma membrane P-type cation translocating ATPase 'PfATP4' has been implicated as playing a key role in this process. PfATP4 has been the subject of significant attention in recent years as mutations in this protein confer resistance to a growing number of new antimalarial compounds, including the spiroindolones, the pyrazoles, the dihydroisoquinolones, and a number of the antimalarial agents in the Medicines for Malaria Venture's 'Malaria Box'. On exposure of parasites to these compounds there is a rapid disruption of cytosolic Na^+. Whether, and if so how, such chemically distinct compounds interact with PfATP4, and how such interactions lead to parasite death, is not yet clear. The fact that multiple different chemical classes have converged upon PfATP4 highlights its significance as a potential target for new generation antimalarial agents. A spiroindolone (KAE609, now known as cipargamin) has progressed through Phase I and IIa clinical trials with favourable results. In this review we consider the physiological role of PfATP4, summarise the current repertoire of antimalarial compounds for which PfATP4 is implicated in their mechanism of action, and provide an outlook on translation from target identification in the laboratory to patient treatment in the field.

1. Introduction

Malaria is a serious global health problem. In the most recent 2014 World Malaria Report it was estimated that 3.2 billion people are at risk of infection, with some 584,000 deaths in 2013 (World Health Organization, 2014). In the absence of a vaccine, malaria control relies primarily on chemotherapy and vector control using insecticide-treated mosquito nets. Malaria parasites, including the deadly *Plasmodium falciparum*, have developed some degree of resistance to all of the antimalarial drugs currently in use. Recent reports of reduced *in vivo* susceptibility to the first-line treatment artesunate (Ashley et al., 2014) give cause for increasing concern, and underpin an urgent need to develop new antimalarial therapies.

Although target-based drug discovery approaches are still being pursued (Coteron et al., 2011; Chatterjee and Yeung, 2012), recent efforts to identify new candidate antimalarials have focussed on high-throughput 'phenotypic' screening of large chemical libraries for compounds that kill the parasite in 'whole-cell' assays (Plouffe et al., 2008; Gamo et al., 2010; Guiguemde et al., 2010). Determining the target and the mechanism of action for compounds identified in such screens is challenging. One approach that has been used successfully involves the generation of resistant parasites by prolonged exposure of parasites to the compound of interest, then recovery and analysis of the genomic DNA of resistant parasites to identify resistance-associated mutations. Using this technique, multiple recent studies have implicated the *Plasmodium falciparum* P-type ATPase 4, PfATP4 (PFL0590c; PF3D7_1211900), in resistance to, and hence in the action of, a diverse range of new antimalarials (Rottmann et al., 2010; Flannery et al., 2014; Jimenez-Diaz et al., 2014; Lehane et al., 2014; Vaidya et al., 2014).

2. Na^+ regulation in *P. falciparum*-infected erythrocytes

The maintenance of a low cytosolic Na^+ concentration ($[Na^+]_{cyt}$) is a fundamental characteristic of all cells. Cells maintain a low $[Na^+]_{cyt}$ by limiting Na^+ influx, by sequestering Na^+ and through the action of Na^+ efflux transporters. Human erythrocytes maintain a low $[Na^+]_{cyt}$ through the extrusion of Na^+ via a well-characterised, ouabain-sensitive P-type Na^+/K^+-ATPase. Thus, upon parasite invasion of a host erythrocyte, the *Plasmodium*

* Corresponding author. Department of Medicine (Infectious Diseases), Washington University School of Medicine, Saint Louis, MO 63110, USA.
E-mail address: Spillman@wusm.wustl.edu (N.J. Spillman).

http://dx.doi.org/10.1016/j.ijpddr.2015.07.001

merozoite faces a dramatic change in ionic environment, moving from the high-[Na^+]/low-[K^+] environment of the blood plasma, to the low-[Na^+]/high-[K^+] environment of the host cell cytosol (Lee et al., 1988). Some 12–18 h after invasion (i.e. in the 'ring' stage) there is a significant increase in the permeability of the erythrocyte membrane to a wide range of low molecular weight solutes, including Na^+ and K^+ (Ginsburg et al., 1985; Kirk et al., 1994; Staines et al., 2001). Na^+ enters the infected erythrocyte, down its inward concentration gradient, via parasite-induced "New Permeability Pathways". The increased influx of Na^+, and the consequent increase in [Na^+] in the erythrocyte cytosol, stimulates the Na^+/K^+-ATPase; its activity increases more than twofold in an effort to maintain a low erythrocytic [Na^+] (Staines et al., 2001). However this is insufficient to counter the increased influx of Na^+. Furthermore, as the intraerythrocytic parasite matures, the Na^+/K^+-ATPase activity subsequently decreases (Staines et al., 2001), perhaps as a result of a reduction in the Mg^{2+}/ATP ratio in the erythrocyte cytosol, a key determinant of Na^+/K^+-ATPase activity (Atamna and Ginsburg, 1997; Mauritz et al., 2009). The overall result of these changes is that the erythrocyte $[Na^+]_{cyt}$ progressively increases, eventually reaching a concentration similar to that in the extraerythrocytic plasma (Lee et al., 1988; Lew et al., 2003; Pillai et al., 2013). Under normal physiological conditions this is around 130 mM; in patients with malaria it may fall below this as malaria-associated hyponatremia is common (English et al., 1996; Hanson et al., 2009; van Wolfswinkel et al., 2010).

The intraerythrocytic parasite is enclosed within a parasitophorous vacuole membrane which, at the mature, metabolically active, trophozoite stage, is thought to be freely permeable to low molecular weight solutes (including inorganic ions) due to the presence in this membrane of high-conductance broad-selectivity channels (Desai et al., 1993; Desai and Rosenberg, 1997). The [Na^+] in the parasitophorous vacuole is therefore expected to be similar to that in the erythrocyte cytosol.

Despite a high-extraparasitic [Na^+] in the host erythrocyte cytosol (and, presumably, the parasitophorous vacuole space), the parasite itself maintains a low $[Na^+]_{cyt}$ (Ginsburg et al., 1986; Lee et al., 1988; Wunsch et al., 1998; Mauritz et al., 2011; Spillman et al., 2013a; Flannery et al., 2014; Jimenez-Diaz et al., 2014; Vaidya et al., 2014). There is therefore an inward [Na^+] gradient across the plasma membrane of the mature parasite. This combines with the parasite's high inwardly-negative membrane potential (approximately −95 mV (Allen and Kirk, 2004)) to constitute a large inwardly directed Na^+ electrochemical gradient. The Na^+ electrochemical gradient provides a source of energy for the active uptake by the parasite of the essential nutrient inorganic phosphate, which is transported across the parasite plasma membrane via a Na^+/phosphate symporter (Saliba et al., 2006). Initial experiments suggested that maintenance of an inwardly directed Na^+ gradient was essential for parasite growth (Brand et al., 2003). More recently it has been shown that parasites can be cultured in a low-Na^+ medium, under which conditions the increase in [Na^+] in the host erythrocyte cytosol is prevented (Pillai et al., 2013). This argues against an essential role for the increased erythrocyte [Na^+] under the particular experimental conditions tested. However, as has been pointed out (Kirk and Lehane, 2014), for parasitised erythrocytes in low Na^+ medium there is still likely to be both an inward [Na^+] gradient and an inwardly negative membrane potential, and therefore an inward Na^+ electrochemical gradient. It therefore remains untested and unknown to what extent this gradient is important for parasite development.

The parasite's ability to maintain a large inwardly directed Na^+ gradient (even in the presence of supra-physiological extracellular [Na^+] (Spillman et al., 2013a)), is consistent with the parasite having an active Na^+ extrusion mechanism. The presence of such a mechanism was confirmed by the demonstration of the ability of the parasite to extrude Na^+ from parasites pre-loaded with Na^+, against a steep inward Na^+ gradient (Spillman et al., 2013a). The maintenance of a low cytosolic [Na^+] is disrupted by the P-type cation-ATPase inhibitor orthovanadate and it has been postulated that the active extrusion of Na^+ from the parasite is via PfATP4 (Spillman et al., 2013a), one of seven putative cation-transporting P-type ATPases encoded by *P. falciparum* (Martin et al., 2005, 2009a). PfATP4 is expressed at all stages of the asexual erythrocytic cycle (Rottmann et al., 2010), and is localised to the parasite plasma membrane (Dyer et al., 1996; Rottmann et al., 2010).

PfATP4 was initially annotated as a Ca^{2+}-transporting ATPase, on the basis of sequence similarities to known Ca^{2+} pumps (Trottein and Cowman, 1995, Trottein et al., 1995, Dyer et al., 1996; Krishna et al., 2001). Initial experiments involving the heterologous expression of PfATP4 in *Xenopus laevis* oocyte membranes gave results consistent with PfATP4 having a Ca^{2+}-dependent ATPase activity (Krishna et al., 2001); however these results were not replicated in a subsequent study (Rottmann et al., 2010) and, in any case, a Ca^{2+} transport function has not been demonstrated directly. Ca^{2+}-ATPases are closely related to another class of P-type Na^+-ATPases, the type IID ENA-ATPases (***exitus natrus***, translating to exit of salt; (reviewed in Rodriguez-Navarro and Benito, 2010)), which efflux Na^+ from cells of lower eukaryotes including fungi, bryophytes and several protozoan parasites (including *Trypanosoma* (Caruso-Neves et al., 1999; Iizumi et al., 2006), *Leishmania* (Stiles et al., 2003; de Almeida-Amaral et al., 2008) and *Entamoeba* (De Souza et al., 2007)).

The observation that the presence of extracellular Na^+ imposes a significant 'acid load' has led to the hypothesis that PfATP4-associated Na^+ efflux is coupled to the influx of H^+ equivalents (Spillman et al., 2013b, 2013a). A Na^+/H^+ countertransport mechanism has been proposed for members of the ENA-ATPase family (Rodriguez-Navarro and Benito, 2010) and cation countertransport is a feature of many other P-type ATPases (Niggli and Sigel, 2008). The acid load arising from the influx of H^+ equivalents is countered by a plasma membrane V-type H^+-ATPase (Saliba and Kirk, 1999; Hayashi et al., 2000). PfATP4 is thus proposed to be (at least to a significant extent) responsible for the "abundant proton pumping" conundrum (i.e. the presence of a powerful plasma membrane H^+-ATPase, without there being an obvious metabolic requirement for it) posited by Ginsburg (2002). Although not discussed by Rottmann et al. (2010), parasites with mutations in *pfatp4* were found to show a significant (albeit small, almost two-fold) cross resistance to concanamycin A (an inhibitor of V-type H^+-ATPases; (Rottmann et al., 2010)). One possible explanation for any such cross-resistance is that the mutant PfATP4 has reduced activity relative to the wild type protein, with the result that there is reduced H^+ countertransport in the PfATP4 mutant line and hence a reduced requirement for H^+-pumping by the V-type H^+-ATPase.

Fig. 1 shows a schematic of the proposed mechanisms of Na^+ regulation in malaria parasite infected erythrocytes.

3. Multiple inhibitors show an association with PfATP4

PfATP4 has been implicated in the mechanism of action of a chemically diverse array of antimalarial drug candidates, many of which have been shown to disrupt parasite Na^+ regulation. The chemical structures of representatives of the different chemical classes identified to date are shown in Fig. 2, and relevant data for parasite EC_{50} values (i.e. the concentration of a drug that gives half-maximal inhibition of parasite growth) and PfATP4 mutations are summarised in Table 1 and Fig. 3. Prolonged culturing of parasites in the presence of these compounds gives rise to parasites showing some degree of resistance, associated in each case with mutations

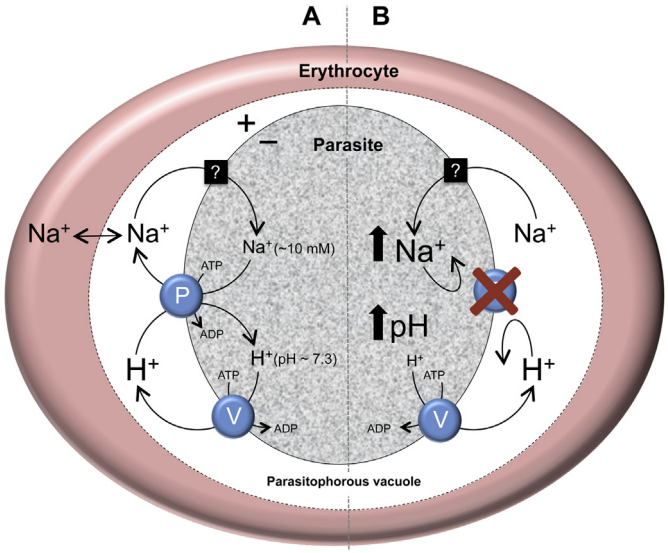


Fig. 1. The putative role of PfATP4 in Na^+ homeostasis in the malaria parasite, *P. falciparum*. PfATP4 is indicated by the circle labelled 'P' and the V-type H^+-ATPase is indicated by the circle labelled 'V'. The parasite resides within the parasitophorous vacuole, the membrane of which is thought to be freely permeable to low molecular weight solutes, including inorganic ions such as Na^+. The Na^+ concentration within the parasitophorous vacuole is therefore likely to be similar to that in the erythrocyte cytosol. A) PfATP4 is postulated to function as a Na^+-ATPase, actively extruding Na^+ from the intraerythrocytic parasite, countering the inward leak of Na^+ (via unknown pathways) and maintaining a low $[Na^+]_{cyt}$ (approximately 10 mM). The PfATP4-mediated efflux of Na^+ is proposed to be accompanied by an influx of H^+ ions and this constitutes a significant acid-load on the parasite. H^+ ions entering the parasite via the putative Na^+-ATPase are extruded by the plasma membrane V-type H^+-ATPase, with the parasite pH_{cyt} maintained at approximately 7.3. B) Inhibition of PfATP4 results in an increase in $[Na^+]_{cyt}$ as Na^+ moves into the cell, down its electrochemical gradient, via the Na^+ leak pathways. There is a concomitant increase in pH_{cyt}, attributed to the lifting of the PfATP4-mediated acid load.

in PfATP4. Why so many recently-identified antimalarial chemotypes should have converged on PfATP4 is unclear; however the convergence potentially reflects the critical biological role of Na^+ regulation, and/or limitations in the number of accessible drug targets in *Plasmodium*, noting that the whole-cell screening approach is limited in discovering targets that are essential, inhibitable and accessible in *in vitro* culture.

3.1. Spiroindolones

An initial spiroazepineindole lead compound was identified in a high-throughput screen of approximately 12,000 compounds for the ability to inhibit the growth of asexual blood-stage malaria parasites. The screen was carried out at the Genomics Institute of the Novartis Research Foundation (GNF) (Plouffe et al., 2008). Several spiroindolone (a term describing spiroazepineindoles and spirotetrahydro-β-carbolines) derivatives were synthesised (Yeung et al., 2010), with the most active compound, NITD609 (renamed KAE609 and now to be rebranded as cipargamin; Fig. 2A), killing culture-adapted and field isolates of *P. falciparum* and *Plasmodium vivax* with an EC_{50} of <10 nM (Rottmann et al., 2010) (Table 1). KAE609 also inhibits gametocyte development (in an *in vitro* growth assay) and oocyst development in mosquitoes (van Pelt-Koops et al., 2012). KAE609 displayed no significant cytotoxicity against mammalian cells, and a single dose (100 mg/kg) was shown to completely cure a mouse *Plasmodium berghei* infection (Rottmann et al., 2010). The early promise of KAE609 in both *in vitro* and *in vivo* assays, led to it being awarded the Medicines for Malaria Venture Project of the Year in 2009 (http://mmv.org/research-development/project-year-award/project-year-award-2009; Last accessed May, 2015).

Parasites grown in incrementally increasing concentrations of KAE609 (or another spiroindolone, NITD678) developed resistance to the compounds, and a high-density tiling array using genomic DNA from these parasites revealed that all mutant parasite lines had at least one non-synonymous mutation in *pfatp4* (Rottmann et al., 2010) (Table 1). The EC_{50} values for inhibition of parasite growth increased 7- to 24-fold in the spiroindolone-resistant lines (Rottmann et al., 2010) (Table 1). Most (6/8) of the associated amino acid changes reside in the predicted transmembrane helices of PfATP4 (Rottmann et al., 2010) (Fig. 3A). The ability of these mutations to confer resistance to KAE609 was investigated by over-expressing mutant PfATP4 in wild-type parasites (Rottmann et al., 2010). Parasites overexpressing mutant PfATP4 showed increased EC_{50} values for parasite killing by KAE609, relative to the EC_{50} values seen for parasites overexpressing the wild-type form of the protein (Table 1). Resistant parasites generated against KAE609 were cross-resistant to other spiroindolones (Rottmann et al., 2010; Spillman et al., 2013a).

For two of the active spiroindolone derivatives (NITD246 and NITD139) it was shown that addition of the spiroindolones to parasites (functionally isolated from their host erythrocyte using saponin) resulted in an immediate-onset, time-dependent increase in $[Na^+]_{cyt}$ (Spillman et al., 2013a), consistent with the compounds inhibiting active extrusion of Na^+. At the same time NITD246 caused a cytosolic alkalinisation, increasing the transmembrane H^+ gradient (Spillman et al., 2013a). This alkalinisation was attributed to a lifting of the acid load associated with the proposed Na^+/H^+ countertransport mechanism for PfATP4 (Spillman et al., 2013b) (see Section 2; Fig. 1). Inhibition of the plasma membrane V-type H^+ ATPase, by concanamycin A, causes a significant cytosolic acidification (Saliba and Kirk, 1999). This concanamycin-A induced acidification was significantly reduced in the presence of NITD246, again attributed to inhibition of PfATP4-mediated acid loading by the spiroindolone (Spillman et al., 2013b). NITD246 did not affect the resting cytosolic $[Ca^{2+}]$, and neither NITD246 or NITD139 affected cellular ATP levels within the timescale of the experiments, arguing against general ion disruption or cell energy deficit as the mechanism of action (Spillman et al., 2013a). Both a KAE609-resistant selected parasite line and a transgenic parasite line over-expressing mutant PfATP4 showed significantly increased resting $[Na^+]_{cyt}$ levels, reduced Na^+ efflux rates, and an increased sensitivity to the growth-inhibitory effects of supra-physiological extracellular Na^+, compared to their wild type counterparts (Spillman et al., 2013a).

PfATP4 mutations conferring spiroindolone-resistance occur in regions of the protein that are highly conserved between malaria parasite species, with an evolutionary analysis concluding that these mutations play a role in drug resistance and are not present due to genetic drift (Gardner et al., 2011). Distinguishing resistance-associated mutations from non-adaptive polymorphisms will contribute to effective surveillance if the spiroindolones progress to the field. KAE609 was the "first molecule with a novel mechanism of action to successfully complete Phase IIa studies for malaria in the last 20 years" (http://www.mmv.org/research-development/project-portfolio/kae609; Accessed May, 2015) and is making rapid progress through the drug development pipeline. Further details on the clinical trials for KAE609 (i.e. cipargamin) are included in Section 6.

3.2. Antimalarial compounds from the Malaria Box

The Medicines for Malaria Venture's Malaria Box is a diverse set of 400 compounds, selected from 'hits' identified in various high-throughput phenotypic screens of large chemical libraries for

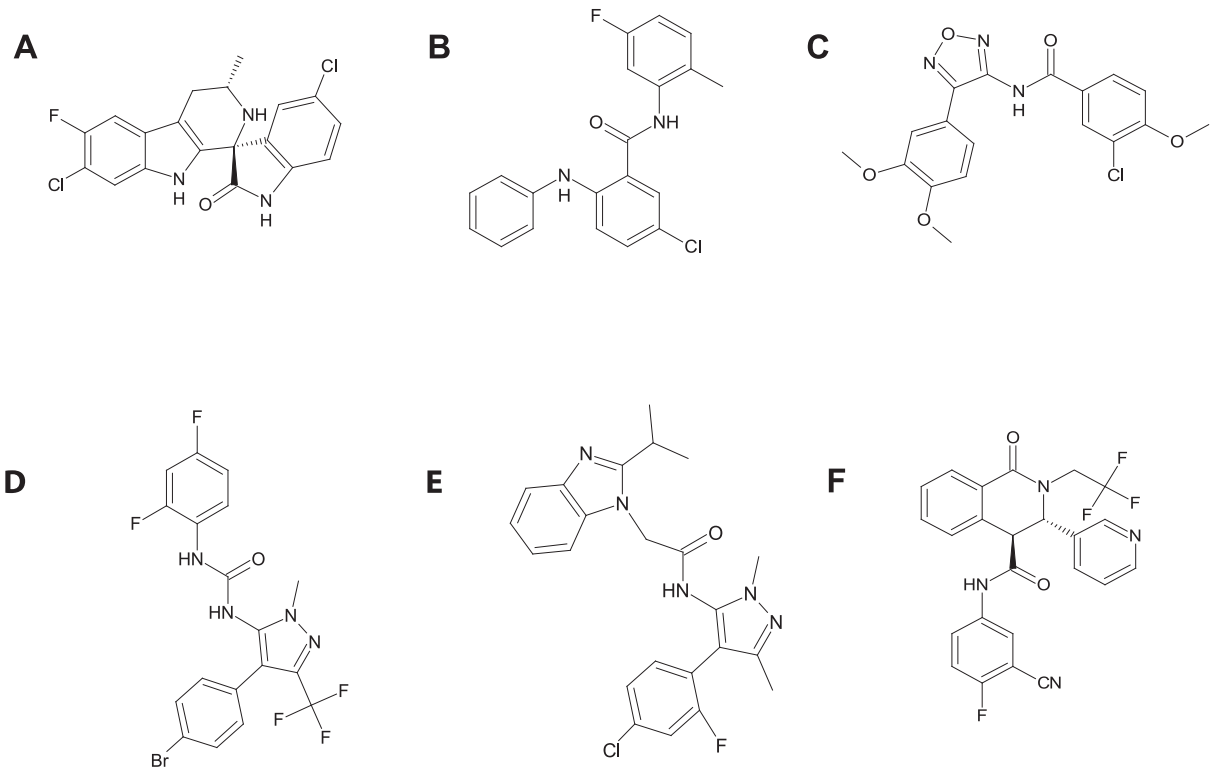


Fig. 2. Representative chemical structures of multiple chemotype classes showing an association with PfATP4. A) KAE609/cipargamin, a spiroindolone, B) MMV007275 and C) MMV0011567, two carboxamides from the MMV Malaria box, D) GNF-Pf4492, an aminopyrazole, E) 21A092, a pyrazoleamide, and F) (+)-SJ733, a dihydroisoquinolone.

compounds that kill asexual blood-stage *P. falciparum* parasites in whole-cell assays (see detailed compilation information in Spangenberg et al., 2013). In a study by Lehane et al. (2014) all 400 compounds were screened for their ability to disrupt parasite Na^+ regulation. Remarkably, 28 of the 400 compounds (i.e. 7%) disrupted Na^+ homeostasis at a test concentration of 1 μM (Lehane et al., 2014). All 28 compounds also disrupted parasite pH regulation in the same manner as the spiroindolones (causing an increase in pH_i, as well as reduced acidification in response to inhibition of the plasma membrane V-type H^+ ATPase). Along with three spiroindolone-like compounds, multiple new potential PfATP4-targeting chemotypes were identified (Lehane et al., 2014). Twenty two of the 28 compounds contained a carboxamide functional group. Six compounds, representing multiple chemotypes (MMV011567, MMV665949, MMV007275, MMV006656, MMV006764, MMV665826), were tested for cross-resistance against the spiroindolone-resistant line, NITD609-R_{Dd2}-clone#2 (the line generated in Rottmann et al., 2010), and for all six of these Malaria Box compounds, the spiroindolone-resistant line showed significant resistance compared to the parental line (Lehane et al., 2014). For two of these compounds (MMV011567 and MMV007275; Fig. 2B, C) resistant parasites were generated by long-term culture of parasites in the presence of sub-lethal concentrations of the compound. For both compounds, the EC_{50} values for inhibition of parasite growth increased approximately 5-fold in the compound-resistant lines (Lehane et al., 2014). Whole genome sequencing was not undertaken, though Sanger sequencing of the *pfatp4* locus revealed that for both compounds there were mutations at single, distinct positions in *pfatp4* (Lehane et al., 2014; Table 1; Fig. 3B). These mutations were different to the mutations identified in the spiroindolone-resistant lines.

In another study, an undisclosed subset of the Malaria Box compounds was screened against parasite lines resistant to another PfATP4 interacting compound, the pyrazole-urea GNF-Pf4492 (Flannery et al., 2014; see Section 3.3 below). For two compounds, MMV666124 and MMV020660 (both identified as causing disruption of ion homeostasis in the Lehane et al. study (2014)), significant cross resistance was observed (Flannery et al., 2014).

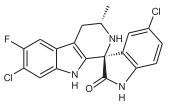
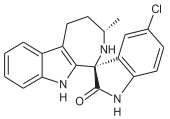
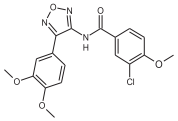
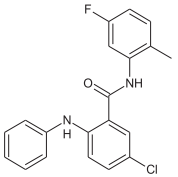
3.3. Pyrazoles

The pyrazole-ureas were first identified in the GNF high throughput screen (the same screen identifying the spiroindolone chemotype; Plouffe et al., 2008). They were identified independently in an *in silico* screen of 300,000 compounds for inhibitors of the *Plasmodium* 'myosin tail interacting protein'/myosin A interaction (Kortagere et al., 2010). This interaction is thought to play a crucial role in invasion of host cells by the parasite (Bosch et al., 2006). However, when the compounds identified as *in silico* hits were exposed to parasites, they were found to kill the parasite throughout the entire life cycle, not just during the invasion process (Kortagere et al., 2010). This is consistent with the pyrazole-urea compounds having one or more additional/alternative targets, or with the aforementioned interaction playing a role additional to that in invasion.

3.3.1. Pyrazole-ureas (aminopyrazoles)

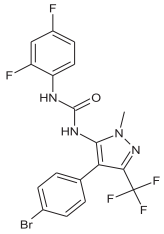
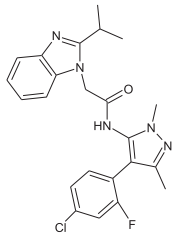
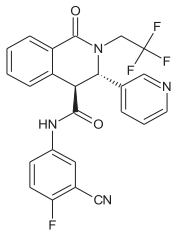
The pyrazole-urea GNF-Pf4492 (Fig. 2D) inhibits the growth of asexual *P. falciparum* blood-stage, parasites with an EC_{50} value of 184 nM (Flannery et al., 2014). Like KAE609, GNF-Pf4492 also inhibits oocyst formation in the mosquito midgut, demonstrating its transmission blocking potential (Flannery et al., 2014). Parasites resistant to GNF-Pf4492 were generated by long-term exposure of parasites to sub-lethal concentrations of the compound, and three independently-generated resistant lines were subjected to whole genome sequencing (Flannery et al., 2014). The EC_{50} values for

Table 1
Summary of antimalarial compounds for which mutations in PfATP4 have been associated with resistance. The parent parasite strain is the Dd2 line, and transgenic parasites all have the Dd2 parent background, unless indicated otherwise. Additional mutations in genes other than PfATP4 are included (in italics, after the relevant PfATP4 mutations). EC_{50} values were determined in asexual *in vitro* culture assays. Further information, particularly with regard to EC_{50} values for multiple compound derivatives and cross resistance EC_{50} values between the different compounds are available from the original publications referred to. NR = not reported.

Compound class	Derivative	EC_{50} against parent parasites (± SEM)	PfATP4 mutations identified after resistance selection, and EC_{50} against mutant parasites (± SEM)		Relevant transgenic parasites generated and EC_{50} against transgenic parasites (± SEM)		Reference
Spiroindolones	KAE609 (NITD609) 	0.44 ± 0.03 nM	I398F, P990R (NITD609-RDd2 clone#1) Also D1247Y	10.9 ± 1.6 nM	Dd2attB wild-type (EF1α promoter, overexpression)	0.92 ± 0.03 nM	(Rottmann et al., 2010)
			T418N, P990R (NITD609-RDd2 clone#2)	3.7 ± 1.0 nM	Dd2attB D1247Y (EF1α promoter, overexpression)	1.57 ± 0.14 nM	
			D1247Y (NITD609-RDd2 clone#3)	3.2 ± 0.6 nM	Dd2attB I398F/P990R (EF1α promoter, overexpression)	1.89 ± 0.06 nM	
					Dd2attB D1247Y (CAM promoter, overexpression)	3.90 ± 0.65 nM	
					Dd2attB I398F/P990R (CAM promoter, overexpression)	4.25 ± 0.54 nM	
	NITD678 	21.9 ± 1.2 nM	G223R (NITD678-R_{Dd2} clone#1)	193 ± 39 nM			
			A184S, P990R (NITD678-R_{Dd2} clone#2) *and I119S (ADP/ATP transporter)* (Bopp et al., 2013)	162 ± 16 nM			
			I203M, I263V (NITD678-R_{Dd2} clone#3)	241 ± 32 nM			
Malaria Box compounds	MMV011567 	0.34 ± 0.05 μM	Q172K (MMV011567-pressured culture 1)	1.63 ± 0.22 μM			(Lehane et al., 2014)
			A353E* (MMV011567-pressured culture 2) *This mutation was originally reported as A353Q (Lehane et al., 2014) but was corrected in a subsequent corrigendum to A353E.	1.89 ± 0.24 μM			
	MMV007275 	0.23 ± 0.02 μM	Q172H (MMV007275-pressured culture 1)	1.10 ± 0.18 μM			

(*continued on next page*)

Table 1 (*continued*)

Compound class	Derivative	EC_{50} against parent parasites (± SEM)	PfATP4 mutations identified after resistance selection, and EC_{50} against mutant parasites (± SEM)		Relevant transgenic parasites generated and EC_{50} against transgenic parasites (± SEM)		Reference
Pyrazoles	GNF-Pf4492 (a pyrazole-urea) 	184.1 nM	A211T (GNF-Pf4492R-1) I203L, P990R (GNF-Pf4492R-2) A187V (GNF-Pf4492R-3) *and I301N (ADP/ATP transporter)*	1170 nM 811.0 nM 631.0 nM	Dd2attB wild-type (CAM promoter, overexpression) Dd2attB A211T (CAM promoter, overexpression)	~1.5 fold shift (EC_{50} NR) ~4.4 fold shift (EC_{50} NR)	(Flannery et al., 2014)
	PA21A092 (a pyrazole-amide) 	12.9 ± 2.0 nM	V178I (Dd2-R-1, Dd2-R21-2 and Dd2-R21-3) *and T392A (PfCDPK5), D133Y (DNA polymerase alpha; PF3D7_0411900), N142K (DEAH box ATP-dependent RNA helicase; PF3D7_0630900), K465E (thioredoxin-like associated protein 2; PF3D7_1034300)*	133 ± 8 nM	Dd2::PfCDPK5^{T392A} (endogenous promoter, integration) Dd2attB PfATP4wild-type (CAM promoter, overexpression) Dd2attB PfATP4^{V178I} (CAM promoter, overexpression) Dd2::PfCDPK5^{T392A} (endogenous promoter, integration) + Dd2attB PfATP4wild-type (CAM promoter, overexpression from an expisome) Dd2::PfCDPK5^{T392A} (endogenous promoter, integration) + Dd2attB PfATP4^{V178I} (CAM promoter, overexpression from an episome)	23 ± 3 nM 22 ± 9 nM 34 ± 3 nM 31 ± 4 nM 176 ± 8 nM	(Vaidya et al., 2014) Note: resistant parasites raised against C2-1 compound
Dihydroiso-quinolones	(+)-SJ733 	20 nM (Dd2 parent) 30 nM (3D7 parent)	V415D (SJ81-733) 3D7 L350H (SJ82-733) 3D7 P412T (SJ83-733) 3D7 G358S (DD2-SJ16-D2) Dd2 L928F (DD2-SJ16-B7) Dd2	EC_{50} NR EC_{50} NR 15 μM 5 fold (EC_{50} NR) 3 fold (EC_{50} NR)	*P. berghei* expressing PfATP4 at the PbATP4 locus		(Jimenez-Diaz et al., 2014)
	SJ311 (structure undisclosed)	20 nM (3D7 parent)	P437S (*P. berghei*) L350H (SJ72-311) 3D7	2 fold (EC_{50} NR) EC_{50} NR			
	SJ247 (structure undisclosed)	50 nM (Dd2)	P966S (UC7-247) W2 parent	330 nM			
	SJ279 (structure undisclosed)	40 nM (Dd2)	P966T (UC8-279) W2 parent	90 nM			
MMV772	Details/structures undisclosed	EC_{50} NR (3D7 parent) EC_{50} NR (W2 parent)	F917L (3D7-C9-ATP4F917L) P412L (W2-F6-ATP4P412L)	100 fold (EC_{50} NR) 10 fold (EC_{50} NR)			Unpublished (http://www.mmv.org/newsroom/news/three-new-roads-leading-common-pathway; Report dated 9 December, 2014; Last accessed May, 2015) and (Jimenez-Diaz et al., 2014)

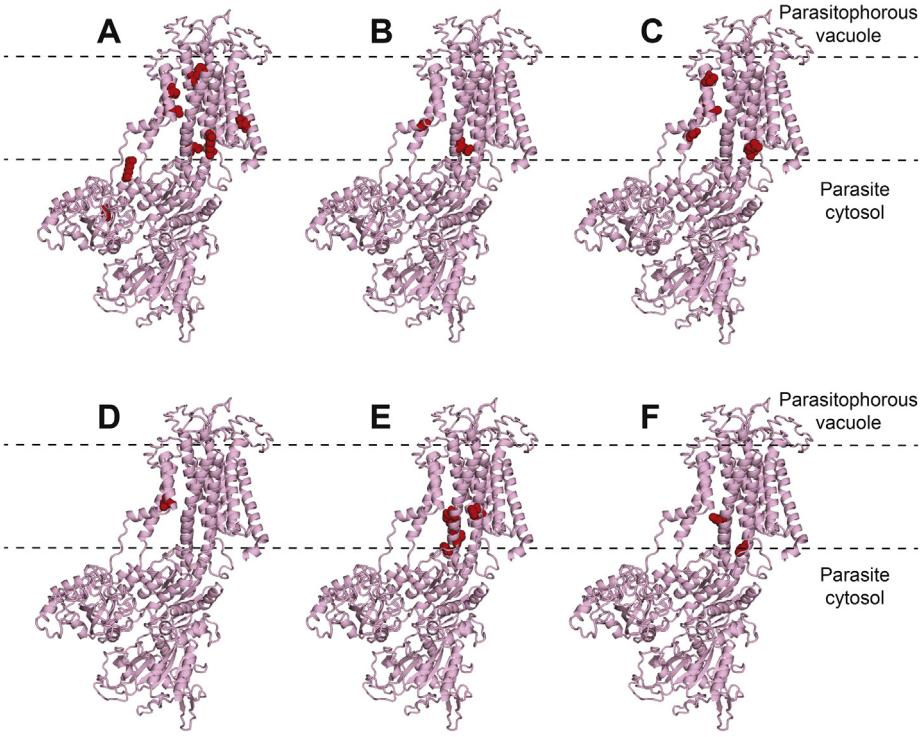


Fig. 3. PfATP4 homology model showing mutations observed in parasites resistant to each of the different chemotypes for which the mechanism of action has been linked to PfATP4. The homology model of PfATP4 (using the full length sequence as input) was generated using the I-TASSER server (Zhang, 2008; Roy et al., 2010), and rendered with PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC. The top structural analogues used were 2DQS (rabbit Ca^{2+}-ATPase), 2KDP (pig Na^+/K^+-ATPase) and 2ZXE (spiny dogfish Na^+/K^+-ATPase). The amino acids represented in red are the resistance-associated mutant residues, with the atoms in these amino acids depicted as red spheres. The predicted approximate transmembrane region of the protein is delineated by the dashed lines. Table 1 provides a full list of the mutations. Resistance-associated mutations occur predominantly in the transmembrane helices for: A) the spiroindolones KAE609 and NITD678; B) two carboxamides from the MMV Malaria box, MMV007275 and MMV0011567 (one of the two alternative mutations reported at position 172 – Q172K – is shown); C) an aminopyrazole, GNF-Pf4492; D) a pyrazoleamide, C2-1; E) the dihydroisoquinolones (+)-SJ733, SJ311, SJ247 and SJ279 (one of the two alternative mutations reported at position 966 – P966S – is shown); and F) MMV772.

inhibition of parasite growth increased 3.4- to 6.4-fold in the three compound-resistant lines relative to the parent line (Flannery et al., 2014). All three had mutations in *pfatp4* (Flannery et al., 2014; Table 1; Fig. 3C). One of these mutations, P990R, had been reported in spiroindolone-resistant parasites (Rottmann et al., 2010). The others were previously undescribed. One of the resistant lines also had a mutation in the final (C-terminal) amino acid of the putative ADP/ATP transporter (PF3D7_1037300). Although the ADP/ATP transporter was initially localised to the inner mitochondrial membrane (Hatin et al., 1992), there is functional evidence for its presence on the parasite plasma membrane (Kanaani and Ginsburg, 1989; Choi and Mikkelsen, 1990; Saliba et al., 2003). What role, if any, either a mitochondrial or plasma membrane ADP/ATP exchanger might play in the resistance phenotype was not investigated. Mutations in the calcium-dependent protein kinase 5 (*pfcdpk5*; PF3D7_1337800) were reported in the pyrazole-urea resistant parasites, although these mutations were also present in the parent line and their functional significance was not investigated (Flannery et al., 2014).

The role of the PfATP4 mutations identified in this study were investigated using multiple transgenic parasite lines. Parasite lines overexpressing wild-type PfATP4 (generated in Rottmann et al., 2010; Flannery et al., 2014) showed a modest, approximately 1.5 fold, decrease in the sensitivity to GNF-Pf4492 relative to the relevant $Dd2^{attB}$ parent line (Flannery et al., 2014). When the mutant PfATP4 was overexpressed, there was a greater decrease in sensitivity (3.6–4.4 fold compared to the $Dd2^{attB}$ parent) (Flannery et al., 2014). Two of the three GNF-Pf4492 resistant parasite lines were cross resistant to the spiroindolones tested (KAE609, KAE678 and NITD246); however the other was found to be hypersensitive to the spiroindolones. The mechanism of this hypersensitivity was not investigated (Flannery et al., 2014).

Addition of GNF-Pf4492 to saponin-isolated parasites resulted in an increase in $[Na^+]_{cyt}$ (Flannery et al., 2014), although the time-frame of Na^+ influx was somewhat slower than that observed in other studies (Spillman et al., 2013a; Jimenez-Diaz et al., 2014; Lehane et al., 2014; Vaidya et al., 2014). Like the spiroindolone-resistant parasite lines (Spillman et al., 2013a), the GNF-Pf4492 resistant lines showed reduced sensitivity to the disruption of Na^+ regulation and an increased resting $[Na^+]_{cyt}$ (in 2/3 lines) (Flannery et al., 2014). These data are consistent with the involvement of PfATP4 in parasite Na^+ regulation.

3.3.2. *Pyrazoleamides*

From a different pyrazole-urea lead (one in which the bromide group of GNF-Pf4492 is replaced with a methoxy group), several pyrazoleamide derivatives were synthesised, with the aim of developing compounds more potent than the pyrazole-ureas at killing parasites (Vaidya et al., 2014). A number of these were found to kill asexual stage *P. falciparum* laboratory strains with EC_{50} values <10 nM, and to kill *P. falciparum* and *P. vivax* field isolates with EC_{50} values <18 nM (Vaidya et al., 2014). The lead compound, PA21A092 (Fig. 2E), like the spiroindolone KAE609, also inhibited gametocyte development, with reduced conversion of gametocytes to gametes (Vaidya et al., 2014). In an *in vivo* mouse model of malaria, using *P. falciparum* parasites in NOD/SCID/IL2Rγ^{null} mice, clearance of parasites following PA21A092 treatment (one dose per day for four consecutive days, commencing on day-3 post-infection) was as rapid as that seen with artesunate (Vaidya et al., 2014). The ED_{90} (effective dose for reduction of parasitaemia by 90%) for PA21A092

was 2.5 mg/kg (Vaidya et al., 2014).

To investigate the mechanism of pyrazoleamide killing, *P. falciparum* lines resistant to the pyrazoleamides were generated by exposure to a high concentration (3× EC_{50}) of one of the pyrazoleamide derivatives, compound C2-1 (Vaidya et al., 2014). Three independent resistant lines were generated, showing an approximately 20-fold increase in the EC_{50} value for the inhibition of parasite growth relative to the parent line. All three showed cross-resistance to other pyrazoleamides, consistent with a common mechanism of action for the compounds of this class. Whole genome sequencing revealed that all three resistant parasite lines harboured a mutation in *pfatp4* (Fig. 3D), as well as a mutation in each of four other genes including the gene encoding PfCDPK5 (Vaidya et al., 2014).

Introduction of the T392A mutation (alone) into the endogenous PfCDPK5 protein resulted in a slight decrease in the sensitivity of the parasite to the pyrazoleamides (Vaidya et al., 2014). When the PfATP4 V178I mutant (alone), was overexpressed (and compared to a line in which the wild-type PfATP4 was overexpressed), this resulted in a low level of pyrazoleamide resistance (Vaidya et al., 2014). However, when the PfCDPK5 T392A and PfATP4 V178I mutations were combined (with the mutation in the *pfcdpk5* gene integrated into the endogenous locus and the mutant *pfatp4* gene overexpressed episomally) a much higher degree of resistance was achieved, consistent with both PfATP4 and PfCDPK5 playing a role in pyrazoleamide resistance (Vaidya et al., 2014). Notably, a pyrazoleamide resistant parasite line (R2-1) generated by prolonged exposure to pyrazoleamide compound C2-1 did not display significant cross resistance to KAE609. Similarly, of the transgenic lines only the combination of the PfCDPK5 T392A and PfATP4 V178I mutations resulted in significant cross resistance to KAE609 (Vaidya et al., 2014).

As is seen for the spiroindolones, addition of the pyrazoleamide PA21A050 to saponin-isolated parasites resulted in a rapid increase of $[Na^+]_{cyt}$, an increase in cytosolic pH, and no disruption of resting cytosolic $[Ca^{2+}]$ (Vaidya et al., 2014). Concomitant with an increase in $[Na^+]_{cyt}$, the pyrazoleamide treated parasites underwent swelling, consistent with the osmotic uptake of water (Vaidya et al., 2014). When parasites were grown in low $[Na^+]$ media, the parent parasites and resistant parasites generated by prolonged exposure to a pyrazoleamide both showed reduced sensitivity to both pyrazoleamides and KAE609, consistent with Na^+ playing a role in the mechanism of action of both compound classes (Vaidya et al., 2014).

PfCDPK5 is essential for merozoite release in the final stages of schizogony (Dvorin et al., 2010), a process that is thought to involve schizont swelling (Gruring et al., 2011). Vaidya et al. (2014) postulated that PfCDPK5 may regulate PfATP4 activity, perhaps serving to inhibit its function and thus allowing an influx of Na^+ and water in order to induce schizont swelling. It was proposed that the pyrazoleamides (and perhaps the spiroindolones as well) might trigger prematurely some of the events associated with the egress of parasites from their host erythrocyte (Vaidya et al., 2014) (see Section 4 and Fig. 4).

3.4. Dihydroisoquinolones

The antimalarial dihydroisoquinolones were identified in a high-throughput phenotypic screen of approximately 300,000 compounds conducted at St Jude Children's Research Hospital (Guiguemde et al., 2010). The optimised dihydroisoquinolone (+)-SJ733 (Fig. 2F) kills asexual parasites with EC_{50} values of 10–60 nM in *in vitro* growth assays. (+)-SJ733 was faster than artesunate at clearing *P. falciparum* parasites in the NOD/SCID/IL2Rγ^{null} mice model (one dose per day for four consecutive days, commencing on day three post infection; ED_{90} of 1.9 mg/kg) and blocked transmission of the mouse malaria parasite *P. berghei* from infected mice to mosquitos (Jimenez-Diaz et al., 2014). On the basis of its promising safety profile, oral availability and preclinical pharmacokinetic properties, (+)-SJ733 was endorsed for clinical development by MMV in 2013 (http://www.mmv.org/research-development/project-portfolio/sj733; Accessed May, 2015).

To assess the mechanism of action of (+)-SJ733, drug-resistant parasite strains were selected, both *in vitro* in *P. falciparum* cultures (with (+)-SJ733), and *in vivo* in *P. berghei* (with the dihydroisoquinolone SJ311). In the dihydroisoquinolone-resistant parasite lines, the EC_{50} values for parasite killing increased from approximately 2- to 750-fold (Jimenez-Diaz et al., 2014). Whole genome sequencing was performed for six independent *P. falciparum* selections, and in all cases mutations in *pfatp4* were observed (Jimenez-Diaz et al., 2014; Fig. 3E). Sanger sequencing at the *pfatp4* locus was performed for an additional two independent *P. falciparum* selections and the *P. berghei* selection, and again mutations were found in *pfatp4* and *pbatp4*, respectively (Jimenez-Diaz et al., 2014). The (+)-SJ733-resistant parasite lines were cross-

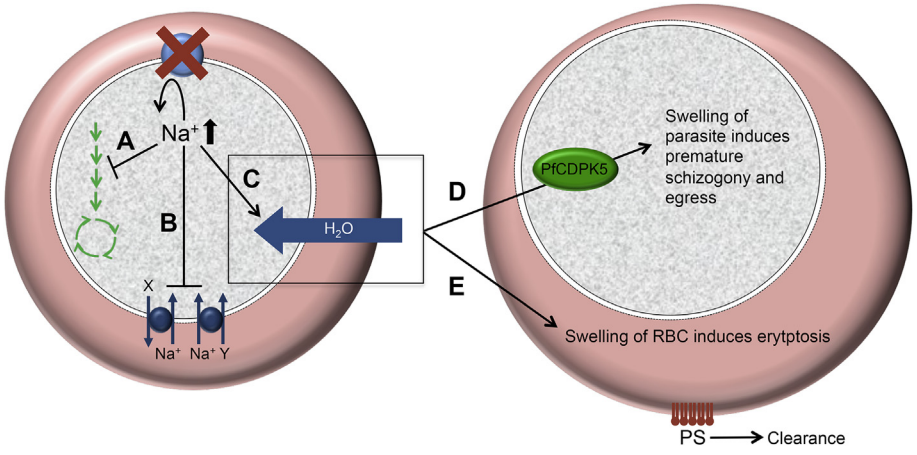


Fig. 4. Potential mechanisms of parasite killing following disruption of Na^+ regulation by PfATP4-associated antimalarials. PfATP4 inhibition results in a net influx of Na^+ into the parasite. This may cause: A) disruption of enzyme function, B) disruption of physiological processes that are dependent on the Na^+ electrochemical gradient, and/or C) parasite and host cell swelling/lysis due to the osmotic load associated with the influx of Na^+. It has been proposed that parasite and/or host cell swelling might be associated with: D) induction of premature schizogony and egress via a mechanism involving the kinase PfCDPK5 and/or E) activation of eryptosis (erythrocyte suicide pathway) in the host cell via a mechanism that entails the display on the erythrocyte surface of phosphatidylserine (PS), cell rounding and enhanced clearance *in vivo*.

resistant to other dihydroisoquinolone derivatives, and were all cross-resistant to the spiroindolones KAE246 and NITD138 (Jimenez-Diaz et al., 2014). For two mutant strains (drug-selected lines with PfATP4 mutations L350H and P966T) their fitness, compared to wild-type parasites, was tested in competition assays. Wild-type and mutant parasites were mixed in equal proportions (i.e. a wild-type:mutant parasite ratio of 1) and the mixed cultures were grown in the absence of drug pressure. After 20–30 days >90% of the parasites were wild type (a wild-type:mutant parasite ratio >10), indicating that the mutant parasites had a significantly slower growth rate (i.e. fitness) relative to their wild-type counterparts (Jimenez-Diaz et al., 2014). The mutant parasites (with PfATP4 mutation L350H) were shown to have a higher resting $[Na^+]_{cyt}$ and it was suggested that this may contribute to the observed fitness cost (Jimenez-Diaz et al., 2014).

As for the other PfATP4-associated antimalarials, addition of (+)-SJ733 to isolated parasites caused a rapid increase in $[Na^+]_{cyt}$ and a rapid alkalinisation of cytosolic pH, consistent with inhibition of PfATP4 activity (Jimenez-Diaz et al., 2014). A (+)-SJ733-resistant parasite (L350H) showed reduced sensitivity to Na^+ disruption by (+)-SJ733 (Jimenez-Diaz et al., 2014).

In multiple *in vivo* mouse malaria models, (+)-SJ733 treatment resulted in a rapid clearance of parasites (estimated to be four times faster than *in vitro* killing) (Jimenez-Diaz et al., 2014). Treatment with (+)-SJ733, or KAE246, led to a rapid exposure of phosphatidylserine on the outer plasma membrane leaflet (Jimenez-Diaz et al., 2014). This is typical of 'eryptotic' or senescent erythrocytes and serves as a signal for the erythrocytes to be removed by erythrophagocytosis or splenic clearance (Lang et al., 2010, 2012). As was reported for the pyrazoleamides (Vaidya et al., 2014), infected erythrocytes were shown to undergo swelling after (+)-SJ733 or KAE246 treatment, with the infected erythrocytes changing from a typical biconcave disc shape to a more spherical shape (Jimenez-Diaz et al., 2014). The authors proposed that osmotic swelling of the parasite leads to premature eryptosis and hence to changes in the infected erythrocyte that facilitate rapid clearance of these cells *in vivo* (Jimenez-Diaz et al., 2014) (see Section 4 and Fig. 4).

3.5. GlaxoSmithKline compounds (MMV772)

Recently, MMV has revealed the development, by GlaxoSmithKline, of another novel chemical class that shows an association with PfATP4 (http://www.mmv.org/newsroom/news/three-new-roads-leading-common-pathway; Report dated 9 December, 2014; Last accessed May, 2015). As is the case for the lead pyrazoleamide (21A092) and the lead dihydroisoquinolone (SJ733), the lead compound of this chemotype (MMV772) has been "recommended for full development" by the MMV External Scientific Advisory Committee. Although the structure of these compounds has not been revealed, it is known that MMV772-selected parasites develop mutations in PfATP4 (Jimenez-Diaz et al., 2014) (Table 1) that confer 10- to 100-fold resistance to MMV772, as well as cross resistance to the dihydroisoquinolones (Jimenez-Diaz et al., 2014).

4. The nature of the association of compounds with PfATP4

The convergence on PfATP4 of so many distinct chemotypes raises a number of key questions. How do mutations in PfATP4 confer resistance? Is PfATP4 the direct target of these compounds? How might inhibition of PfATP4 lead to parasite killing?

One well established mechanism by which mutations in transporters confer drug-resistance involves the mutant transporter mediating the transport of the drug, away from its site of action. P-type cation ATPases transport inorganic cations (e.g. Na^+, K^+, H^+, Ca^{2+}, Zn^{2+}) across membranes or catalyse the translocation of phospholipids across membrane leaflets (flippases) (Yatime et al., 2009; Palmgren and Nissen, 2011). Although P-type ATPases are themselves attractive pharmacological targets (Yatime et al., 2009), there is not, to our knowledge, any known example of an ion translocating P-type ATPase transporting a drug. P-type ATPases do not share homology with known multidrug efflux transporters (e.g. members of the ATP-binding cassette (ABC) transporter family which can transport a multitude of metabolites and drugs). Thus, there is no prior evidence to support the hypothesis that PfATP4 might efflux the various compounds of interest from the parasite.

Another possible explanation for the diversity of chemotypes that has been associated with PfATP4 is that PfATP4 has a multiplicity of interacting partners that are themselves the pharmacological targets of the various compounds of interest. P-type ATPases are known to interact with a number of cytosolic and membrane bound proteins, and it is feasible that the activity of PfATP4 is modulated by a range of other proteins and that compounds interacting with these proteins might thereby affect PfATP4 function. The plasma-membrane P-type Ca^{2+}-ATPase interacts with a large number of proteins, including calmodulin, calcineurin A, ankyrin, actin, and 14-3-3ε proteins (Strehler, 2013). Other examples of proteins interacting with P-type ATPases include the interaction of sarcolipin with the SERCA-type $Ca2^+$-ATPases and 14-3-3 proteins with plant H^+-ATPases (Bublitz et al., 2011). These interacting partners may regulate transporter activation or function, perhaps through allosteric regulation, by altering trafficking or membrane localisation, or by regulating downstream signalling (Strehler, 2013).

Small molecule inhibition of the interacting partner may result in a downregulation/inactivation of PfATP4 activity, leading to the Na^+ influx observed experimentally. Mutations in p*fatp4* may compensate for altered interactions with regulating partners. PfATP4 may be regulated by kinases/phosphatases; six phosphorylation sites have been identified (S28, S69, S86, S103, S113, Y116) (Aurrecoechea et al., 2009; Treeck et al., 2011). Mutations in the kinase PfCDPK5 have been associated with aminopyrazole resistance (Section 3.3.2) and it is proposed that PfCDPK5 may be involved in PfATP4 regulation (Vaidya et al., 2014), although phosphorylation of PfATP4 by PfCDPK5 is yet to be demonstrated experimentally.

Compensatory mutations in *pfatp4* may also arise if the Na^+-pumping capacity of PfATP4 is disrupted by the various compounds in a non-specific manner, for example if the compounds were to act as ionophores. In the case of *P. falciparum*, the various small molecules reviewed in Section 3 do not seem to function as protonophores. Protonophores act to decrease or eliminate the transmembrane H^+ gradient, whereas the spiroindolones (NITD246) (Spillman et al., 2013a), the PfATP4-active Malaria Box Compounds (Lehane et al., 2014), the pyrazoleamides (21A050) (Vaidya et al., 2014) and the dihydroisoquinolones ((+)-SJ733) (Jimenez-Diaz et al., 2014) have all been shown to induce an *increase* in the transmembrane H^+ gradient across the plasma membrane of isolated parasites. It is also unlikely that the compounds act as Na^+ ionophores, as the maximal Na^+ influx rate observed on addition to isolated parasites of the spiroindolones (NITD246,139,247; approximately 0.1 mM/s) is not significantly different from that observed in response to treatment of the parasites with the general P-type ATPase inhibitor orthovanadate (Spillman et al., 2013a). This is consistent with Na^+ gradient disruption being due to P-type ATPase inhibition, and not to an ionophoretic effect. A similar maximal Na^+ influx rate, of approximately 0.08 mM/s was also measured for the pyrazoleamide 21A050 (Vaidya et al., 2014).

The simplest explanation that would account for the data currently available is that PfATP4 functions as a Na^+ efflux pump, that the PfATP4-associated compounds bind directly to PfATP4, and that binding results in inhibition of one or more steps in the

alternating access catalytic cycle of the protein, leading to a halt in cation translocation (i.e. Na^+ efflux). In this model, the time-dependent increase in parasite Na^+ observed experimentally following the addition of ATP4-associated compounds reflects the influx of Na^+ via (unknown) endogenous pathways. P-type ATPases are long-established as drug targets with well-characterised inhibitor binding pockets (Yatime et al., 2009; Bublitz et al., 2011). The resistance-associated mutations in PfATP4 are postulated to reduce the affinity of the interaction of the inhibitors with their binding site(s) on the protein. The reduced sensitivity of the PfATP4 mutant parasites to the disruption of Na^+ regulation (Spillman et al., 2013a; Jimenez-Diaz et al., 2014), is consistent with this hypothesis. The majority (though not all) of the resistance-associated mutations are in a transmembrane region of the protein (which is thereby identified as a candidate inhibitor binding site; see Fig. 3). For at least one of the PfATP4-associated compounds, (+)-SJ7733, *in silico* docking studies have revealed how the compound might interact with this site (Jimenez-Diaz et al., 2014). Nevertheless the direct interaction of the small molecule inhibitors with PfATP4 has not been demonstrated directly (see Section 7). Understanding how the inhibitors interact with PfATP4, in both the wild-type and mutant proteins, may help to explain why the fold-change in sensitivity to a given compound varies between resistant parasites carrying different mutations in PfATP4, and why for any given resistance-conferring mutation(s) the fold-decrease in sensitivity varies between the different chemotypes.

The disruption of Na^+ (and pH) homeostasis occurs immediately following the exposure of parasites to the PfATP4-associated compound. The question of the precise mechanism(s) by which a disruption of parasite ion homeostasis leads to parasite death, and clearance of parasitised erythrocytes from the circulation, remains to be elucidated. As summarised in Fig. 4, there are several possibilities: (A) a high $[Na^+]_{cyt}$ may be detrimental to enzyme function; (B) disruption of the Na^+ electrochemical gradient might perturb important physiological mechanisms that are reliant on this gradient; and/or (C) the net influx of Na^+ that occurs on inhibition of the Na^+ efflux pump may cause the parasites and perhaps, thereby, the infected cell as a whole to swell to such an extent as to threaten their integrity and/or trigger detrimental swelling-associated processes in the parasite and/or erythrocyte. These three possibilities are not mutually exclusive. All warrant further investigation.

In plants, direct Na^+ toxicity (and hence the need to maintain $[Na^+]_{cyt}$ at low levels) has been linked to the ability of Na^+ to interfere with the regulation of various metabolic pathways (including glycolysis) by competing with K^+ (Munns and Tester, 2008). Increased $[Na^+]_{cyt}$ also inhibits cellular protein synthesis (Carrasco and Smith, 1976), as has been reported to occur with the spiroindolones and pyrazole-urea compounds (Rottmann et al., 2010; Flannery et al., 2014).

The parasite uses the inwardly directed Na^+ electrochemical gradient to energise the uptake of at least one essential nutrient, inorganic phosphate (Saliba et al., 2006), though whether this plays a critical physiological role *in vitro* and, more importantly, *in vivo*, is unclear. There are other potential Na^+-coupled transporters annotated in the parasite genome, including amino acid symporters (in the neurotransmitter:Na^+ symporter family), a sugar transporter (in the glycoside–pentoside–hexuronide:cation symporter family), a multi-antimicrobial extrusion family transporter and major facilitator superfamily transporters (Martin et al., 2005). However, the localisations and physiological roles of these proteins have not been investigated, and nor has their dependence on Na^+ been demonstrated.

Both the pyrazoleamides and dihydroisoquinolones (Jimenez-Diaz et al., 2014; Vaidya et al., 2014) have been reported (on the basis of light microscopy measurements) to induce swelling of asexual blood-stage parasites, which is postulated to occur as a result of an osmotic imbalance resulting from the net influx of Na^+ (Fig. 4C). Asexual-stage parasitised erythrocytes treated with the spiroindolone KAE609 become rounded, and have an increased rigidity (measured using micropipette aspiration) compared to uninfected erythrocytes (Zhang et al., in press). Gametocytes treated with KAE609 were also reported to became swollen and rounded (van Pelt-Koops et al., 2012), again consistent with a disruption of osmotic stability. Many cells have the capacity to regulate their volume in response to osmotic perturbation. The ability of the parasite to do so is unknown.

The swelling of parasites following the addition of PfATP4-associated compounds has been proposed to have a number of different consequences (Fig. 4). It has been suggested that swelling might trigger the premature lysis of the host erythrocyte by activating a merozoite release pathway, normally activated during the last moments of schizogony (Vaidya et al., 2014). This hypothesis is based upon the report that mutations in PfCDPK5 (a regulator of schizont rupture (Dvorin et al., 2010)) caused an increase in the parasite resistance to aminopyrazoles (Vaidya et al., 2014). It has also been proposed that parasite swelling triggers eryptosis (an endogenous erythrocyte suicide pathway) in the infected erythrocyte, leading to exposure of phosphatidylserine at the erythrocyte surface, to an increase in erythrocyte rigidity and to the infected cell becoming more spherical (Jimenez-Diaz et al., 2014). Signs of eryptosis were also observed in a second study; however the authors reported there to be no exposure of phosphatidylserine at the erythrocyte surface (Zhang et al., in press). The end result of eryptosis is the rapid clearance of infected erythrocytes from the host organism. Some parasite death through lysis (inside an intact erythrocyte) has also been observed (Jimenez-Diaz et al., 2014), perhaps occurring as a result of osmotic water uptake. Understanding the precise mechanism, and downstream consequences, of parasite swelling requires further investigation.

5. ATP4 in other *Plasmodium* species and other Apicomplexa

There are syntenic PfATP4 orthologues in the human malaria parasites *P. vivax* and *Plasmodium knowlesi*, in the non-human primate malaria parasites *Plasmodium cynomolgi* and *Plasmodium reichenowi*, and in the rodent malaria parasites *P. berghei*, *Plasmodium chabaudi* and *Plasmodium yoelii* (PlasmoDB (v24.0); (Aurrecoechea et al., 2009)). *P. berghei*, *Plasmodium vinckei* and *P. chabaudi* are less sensitive to (+)-SJ733 than is *P. falciparum* (in both *ex vivo* and *in vivo* experiments) (Jimenez-Diaz et al., 2014), and it has been suggested that the differences in compound sensitivity between the species may be due to variations in a loop region close to the predicted (+)-SJ733 binding site (Jimenez-Diaz et al., 2014). There have not yet been any site directed mutagenesis studies to investigate the residues involved; however, in transgenic *P. berghei* parasites in which the entire *pbatp4* locus was replaced with the *pfatp4* locus, the sensitivity to (+)-SJ733 was restored almost to that observed in *P. falciparum* parasites (Jimenez-Diaz et al., 2014).

Understanding the interaction of the various antimalarial agents with *P. vivax* PvATP4 will be important, given the burden of *P. vivax* worldwide (Baird, 2013). Initial evidence suggests that PfATP4 and PvATP4 are similarly sensitive to spiroindolones (NITD609/cipargamin), both *ex vivo* (Rottmann et al., 2010) and *in vivo* (White et al., 2014). By contrast a pyrazoleamide (PA21A092) was significantly more active (*ex vivo*) against *P. vivax* than against *P. falciparum* (Vaidya et al., 2014).

Na^+-ATPases are found in many pathogenic fungi and parasites (Rodriguez-Navarro and Benito, 2010), raising the possibility that the new PfATP4-associated antimalarial agents could be more general anti-parasitic/anti-fungal drugs. The growth inhibitory

effects and the ion-regulation disrupting effects of the compounds should be tested in other Apicomplexa and fungi. *Toxoplasma gondii*, a protozoan parasite, has a readily identifiable PfATP4 homologue (TgATP4, with 51% sequence identity to PfATP4 (Zhou et al., 2014)), and a preliminary report suggests that KAE609 kills *Toxoplasma* tachyzoites both *in vitro*, with an EC_{50} of approximately 1 μM, and in an *in vivo* mouse model (Zhou et al., 2014). Knockdown of TgATP4 led to inhibition of tachyzoite replication, consistent with TgATP4 function playing an important role in the *T. gondii* tachyzoite stage (Zhou et al., 2014). The identification of novel broad anti-parasitic/anti-fungal drugs would be a significant breakthrough in the current age of multi-drug resistant pathogens. Additionally, as the development of KAE609 and other PfATP4-associated compounds continue it may be possible to repurpose the drugs to treat other infections.

6. Drug development and clinical trials

The translation of 'bench' experimental findings to 'bedside' clinical success presents a significant challenge. Target Product Profiles for antimalarial drug development have been established (Burrows et al., 2013; Leroy et al., 2014), with fast clearance and transmission blocking ability being the key characteristics of an ideal SERCaP (Single-Exposure Radical Cure and Prophylaxis) drug. Initial characterisation of several of the PfATP4–related compounds has revealed promising clearance times as well as gametocyte blocking abilities, and this has underpinned the decision to proceed with the development of multiple different chemotypes (http://www.mmv.org/newsroom/news/three-new-roads-leading-common-pathway; Report dated 9 December, 2014; Last accessed May, 2015).

The most advanced of the PfATP4-associated compounds (KAE609/cipargamin) underwent Phase I clinical trials in 2010 and 2011 (Leong et al., 2014). Cipargamin was well tolerated, with mild to moderate gastrointestinal and genitourinary adverse events recorded (Leong et al., 2014). Data from this study, and data from pharmacokinetic–pharmacodynamic preclinical investigations led to the proposal that a daily dose of 30 mg/70 kg human would be efficacious (Lakshminarayana et al., 2014; Leong et al., 2014). Data on the pharmacokinetic–pharmacodynamic properties of various spiroindolone derivatives is being used to optimise dose and dosing regimens, to minimise the risk of sub-optimal doses resulting in spiroindolone resistance (Lakshminarayana et al., 2014).

In a Phase II trial, performed in Thailand in 2012, patients infected with *P. falciparum* or *P. vivax* malaria were treated with 30 mg cipargamin for three sequential days (White et al., 2014). For both parasite species, the median parasite clearance half-life was less than one hour, with median parasite clearance times of approximately 12 h. This is quicker than the current fastest acting antimalarials, the artemisinins (White et al., 2014). The rapid antimalarial activity of cipargamin bodes well for continued development of this compound class, with further Phase II confirmatory and combination clinical studies the next step in the process.

Other PfATP4-associated compounds are also beginning Phase I trials with the dihydroisoquinolone (+)-SJ733 scheduled for first-in-human trials in 2015 (http://www.mmv.org/newsroom/news/three-new-roads-leading-common-pathway; Report dated 9 December, 2014; Last accessed May, 2015).

7. Future directions

Although results obtained to date are consistent with PfATP4 functioning as a Na^+-efflux ATPase, this awaits confirmation by expression of the protein in a heterologous system. Successful heterologous expression of PfATP4 has been reported in *X. laevis* oocytes (Krishna et al., 2001), and the oocyte system has been shown to provide a useful method for the characterisation of other *Plasmodium* transporters; e.g. PfENT1 (Equilibrative Nucleoside Transporter 1; Carter et al., 2000; Parker et al., 2000), PfHT (Hexose Transporter; Woodrow et al., 1999, Woodrow et al., 2000), PfAQP (aquaglyceroporin; Hansen et al., 2002), PfCHA (Ca^{2+}/H^+ exchanger; Rotmann et al., 2010), PfMDR1 (P-glycoprotein homologue 1; Sanchez et al., 2008), PfFT1 and 2 (Folate Transporter; Salcedo-Sora et al., 2011), PfENT4 (Equilibrative Nucleoside Transporter 4; Frame et al., 2012), PfPiT (inorganic phosphate transporter; Saliba et al., 2006), PfCRT (Chloroquine Resistance Transporter; Martin et al., 2009b) and PfFNT (Formate-Nitrite Transporter; Marchetti et al., 2015). If PfATP4 is, as has been postulated, the parasite Na^+-ATPase, then expression of PfATP4 in *Xenopus* oocytes may give rise to an increase in Na^+-ATPase activity in the membrane fraction (under conditions in which the endogenous Na^+-ATPase activity of the oocyte is inhibited by the cardiac glycoside ouabain).

An alternative to expression in *X. laevis* is expression in the yeast, *Saccharomyces cerevisiae*, which has also been used to characterise a number of malaria parasite transport proteins (e.g. the *Plasmodium* Ca^{2+}/H^+ exchanger (Salcedo-Sora et al., 2012; Guttery et al., 2013), the Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} pump PfATP6 (Pulcini et al., 2013), PfCRT (Baro et al., 2011), PfMDR1 (Amoah et al., 2007) and PfFNT (Wu et al., 2015)). A mutant Na^+-sensitive strain of *S. cerevisiae* (B31) has been used previously to study ENA-type Na^+-ATPases (Lunde et al., 2007) and this may well serve as a suitable strain for the functional expression of PfATP4. Expression of the *Physcomitrella patens* ENA-type Na^+-ATPase in B31 yeast conferred resistance to a high extracellular [Na^+] in complementation assays (Lunde et al., 2007). B31 yeast expressing PfATP4 could be assayed for Na^+-dependent ATPase activity and/or used in complementation assays in which the yeast are grown under high-[Na^+] conditions (as in (Lunde et al., 2007)) to assess whether PfATP4 confers an increased Na^+ tolerance on the mutant B31 strain. There has been one reported case of an ENA-type Na^+-ATPase (from *Trypanosoma*, TcENA) failing to express in the B31 yeast system (Iizumi et al., 2006); it remains to be seen whether expression of PfATP4 in this system is feasible.

The development of such heterologous expression systems for PfATP4 would allow an investigation of the nature of the interaction of the different PfATP4-associated compound classes with PfATP4 and an analysis of the functional significance of the various resistance-associated mutations that have been reported to date. It will also pave the way for the development of high throughput assays that could be used for testing and refining antimalarial compounds with this mechanism of action.

8. Final perspectives

Historically, parasites have developed resistance to all of the antimalarial drugs used in the field, including the 'wonder-drug' chloroquine (Wellems and Plowe, 2001). The current frontline antimalarial, artemisinin, is used throughout the developing world; however control of the parasite in Southeast Asia is now under significant threat, with evidence from multiple sites that artemisinin is taking longer to clear parasite infections than was previously the case (Dondorp et al., 2009, 2011; Ashley et al., 2014).

Maintaining a constant arsenal of new antimalarials is critical to our fight against the disease. PfATP4 has been identified as a target for antimalarial drug development, and the fact that multiple high throughput screens with asexual blood-stage parasites have converged upon this protein is consistent with PfATP4 being essential, inhibitable, and pharmacologically accessible. The data are also consistent with PfATP4 being essential for the survival of sexual-stage gametocytes. As noted above, parasites showing some

degree of resistance to many of the different classes of PfATP4-associated antimalarials have been generated in laboratory (*in vitro*) studies. Resistance was slower to develop *in vivo* (Jimenez-Diaz et al., 2014), perhaps because resistance-associated mutations result in a fitness cost. Parasites are cleared rapidly in mouse *in vivo* models as well as in preliminary human trials; this may help to counter the development of resistance in the clinical setting. The ability to monitor PfATP4 resistance in the field, and to distinguish between genetic drift and relevant mutations, will undoubtedly aid in making decisions relating to drug control.

Although a lack of understanding the mechanism of action of a particular drug candidate does not preclude drug development, insights into PfATP4 function, and into the nature of the interaction of the PfATP4-associated drugs with the protein will provide a rational basis for the design and refinement of compounds of this class, as well as facilitating an understanding of mechanisms of resistance.

Acknowledgements

This work was supported by an Australian National Health and Medical Research Council (NHMRC) Overseas Biomedical Fellowship 1072217 (to N.J.S.), and by NHMRC Project Grants 585473 and 1042272 (to K.K.). We thank Dr. C.J. Wanty for assistance with figure preparation and Dr. A.M. Lehane for comments on the manuscript.

References

Allen, R.J., Kirk, K., 2004. The membrane potential of the intraerythrocytic malaria parasite *Plasmodium falciparum*. J. Biol. Chem. 279, 11264–11272.

Amoah, L.E., Lekostaj, J.K., Roepe, P.D., 2007. Heterologous expression and ATPase activity of mutant versus wild type PfMDR1 protein. Biochemistry 46, 6060–6073.

Ashley, E.A., Dhorda, M., Fairhurst, R.M., Amaratunga, C., Lim, P., Suon, S., Sreng, S., Anderson, J.M., Mao, S., Sam, B., Sopha, C., Chuor, C.M., Nguon, C., Sovannaroth, S., Pukrittayakamee, S., Jittamala, P., Chotivanich, K., Chutasmit, K., Suchatsoonthorn, C., Runcharoen, R., Hien, T.T., Thuy-Nhien, N.T., Thanh, N.V., Phu, N.H., Htut, Y., Han, K.T., Aye, K.H., Mokuolu, O.A., Olaosebikan, R.R., Folaranmi, O.O., Mayxay, M., Khanthavong, M., Hongvanthong, B., Newton, P.N., Onyamboko, M.A., Fanello, C.I., Tshefu, A.K., Mishra, N., Valecha, N., Phyo, A.P., Nosten, F., Yi, P., Tripura, R., Borrmann, S., Bashraheil, M., Peshu, J., Faiz, M.A., Ghose, A., Hossain, M.A., Samad, R., Rahman, M.R., Hasan, M.M., Islam, A., Miotto, O., Amato, R., MacInnis, B., Stalker, J., Kwiatkowski, D.P., Bozdech, Z., Jeeyapant, A., Cheah, P.Y., Sakulthaew, T., Chalk, J., Intharabut, B., Silamut, K., Lee, S.J., Vihokhern, B., Kunasol, C., Imwong, M., Tarning, J., Taylor, W.J., Yeung, S., Woodrow, C.J., Flegg, J.A., Das, D., Smith, J., Venkatesan, M., Plowe, C.V., Stepniewska, K., Guerin, P.J., Dondorp, A.M., Day, N.P., White, N.J., Tracking Resistance to Artemisinin C, 2014. Spread of artemisinin resistance in *Plasmodium falciparum* malaria. N. Engl. J. Med. 371, 411–423.

Atamna, H., Ginsburg, H., 1997. The malaria parasite supplies glutathione to its host cell- investigation of glutathione transport and metabolism in human erythrocytes infected with *Plasmodium falciparum*. Eur. J. Biochem. 250, 670–679.

Aurrecoechea, C., Brestelli, J., Brunk, B.P., Dommer, J., Fischer, S., Gajria, B., Gao, X., Gingle, A., Grant, G., Harb, O.S., Heiges, M., Innamorato, F., Iodice, J., Kissinger, J.C., Kraemer, E., Li, W., Miller, J.A., Nayak, V., Pennington, C., Pinney, D.F., Roos, D.S., Ross, C., Stoeckert Jr., C.J., Treatman, C., Wang, H., 2009. PlasmoDB: a functional genomic database for malaria parasites. Nucleic Acids Res. 37, D539–D543.

Baird, J.K., 2013. Malaria caused by *Plasmodium vivax*: recurrent, difficult to treat, disabling, and threatening to life–the infectious bite preempts these hazards. Pathog. Glob. Health 107, 475–479.

Baro, N.K., Pooput, C., Roepe, P.D., 2011. Analysis of chloroquine resistance transporter (CRT) isoforms and orthologues in *S. cerevisiae* yeast. Biochemistry 50, 6701–6710.

Bopp, S.E., Manary, M.J., Bright, A.T., Johnston, G.L., Dharia, N.V., Luna, F.L., McCormack, S., Plouffe, D., McNamara, C.W., Walker, J.R., Fidock, D.A., Denchi, E.L., Winzeler, E.A., 2013. Mitotic evolution of *Plasmodium falciparum* shows a stable core genome but recombination in antigen families. PLoS Genet. 9, e1003293.

Bosch, J., Turley, S., Daly, T.M., Bogh, S.M., Villasmil, M.L., Roach, C., Zhou, N., Morrisey, J.M., Vaidya, A.B., Bergman, L.W., Hol, W.G., 2006. Structure of the MTIP-MyoA complex, a key component of the malaria parasite invasion motor. Proc. Natl. Acad. Sci. U. S. A. 103, 4852–4857.

Brand, V.B., Sandu, C.D., Duranton, C., Tanneur, V., Lang, K.S., Huber, S.M., Lang, F., 2003. Dependence of *Plasmodium falciparum in vitro* growth on the cation permeability of the human host erythrocyte. Cell. Physiol. Biochem. 13, 347–356.

Bublitz, M., Morth, J.P., Nissen, P., 2011. P-type ATPases at a glance. J. Cell Sci. 124, 2515–2519.

Burrows, J.N., van Huijsduijnen, R.H., Mohrle, J.J., Oeuvray, C., Wells, T.N., 2013. Designing the next generation of medicines for malaria control and eradication. Malar. J. 12, 187.

Carrasco, L., Smith, A.E., 1976. Sodium ions and the shut-off of host cell protein synthesis by picornaviruses. Nature 264, 807–809.

Carter, N.S., Ben Mamoun, C., Liu, W., Silva, E.O., Landfear, S.M., Goldberg, D.E., Ullman, B., 2000. Isolation and functional characterization of the PfNT1 nucleoside transporter gene from *Plasmodium falciparum*. J. Biol. Chem. 275, 10683–10691.

Caruso-Neves, C., Einicker-Lamas, M., Chagas, C., Oliveira, M.M., Vieyra, A., Lopes, A.G., 1999. Ouabain-insensitive Na^+-ATPase activity in *Trypanosoma cruzi* epimastigotes. Z. Naturforsch. C 54, 100–104.

Chatterjee, A.K., Yeung, B.K., 2012. Back to the future: lessons learned in modern target-based and whole-cell lead optimization of antimalarials. Curr. Top. Med. Chem. 12, 473–483.

Choi, I., Mikkelsen, R.B., 1990. *Plasmodium falciparum*: ATP/ADP transport across the parasitophorous vacuolar and plasma membranes. Exp. Parasitol. 71, 452–462.

Coteron, J.M., Marco, M., Esquivias, J., Deng, X., White, K.L., White, J., Koltun, M., El Mazouni, F., Kokkonda, S., Katneni, K., Bhamidipati, R., Shackleford, D.M., Angulo-Barturen, I., Ferrer, S.B., Jimenez-Diaz, M.B., Gamo, F.J., Goldsmith, E.J., Charman, W.N., Bathurst, I., Floyd, D., Matthews, D., Burrows, J.N., Rathod, P.K., Charman, S.A., Phillips, M.A., 2011. Structure-guided lead optimization of triazolopyrimidine-ring substituents identifies potent *Plasmodium falciparum* dihydroorotate dehydrogenase inhibitors with clinical candidate potential. J. Med. Chem. 54, 5540–5561.

de Almeida-Amaral, E.E., Caruso-Neves, C., Pires, V.M., Meyer-Fernandes, J.R., 2008. *Leishmania amazonensis*: characterization of an ouabain-insensitive Na^+-ATPase activity. Exp. Parasitol. 118, 165–171.

De Souza, A.M., Batista, E.J., Pinheiro, A.A., Carvalhaes, M., Lopes, A.G., De Souza, W., Caruso-Neves, C., 2007. *Entamoeba histolytica*: ouabain-insensitive Na^+-ATPase activity. Exp. Parasitol. 117, 195–200.

Desai, S.A., Rosenberg, R.L., 1997. Pore size of the malaria parasite's nutrient channel. Proc. Natl. Acad. Sci. U. S. A. 94, 2045–2049.

Desai, S.A., Krogstad, D.J., McCleskey, E.W., 1993. A nutrient-permeable channel on the intraerythrocytic malaria parasite. Nature 362, 643–646.

Dondorp, A.M., Nosten, F., Yi, P., Das, D., Phyo, A.P., Tarning, J., Lwin, K.M., Ariey, F., Hanpithakpong, W., Lee, S.J., Ringwald, P., Silamut, K., Imwong, M., Chotivanich, K., Lim, P., Herdman, T., An, S.S., Yeung, S., Singhasivanon, P., Day, N.P., Lindegardh, N., Socheat, D., White, N.J., 2009. Artemisinin resistance in *Plasmodium falciparum* malaria. N. Engl. J. Med. 361, 455–467.

Dondorp, A.M., Fairhurst, R.M., Slutsker, L., Macarthur, J.R., Breman, J.G., Guerin, P.J., Wellems, T.E., Ringwald, P., Newman, R.D., Plowe, C.V., 2011. The threat of artemisinin-resistant malaria. N. Engl. J. Med. 365, 1073–1075.

Dvorin, J.D., Martyn, D.C., Patel, S.D., Grimley, J.S., Collins, C.R., Hopp, C.S., Bright, A.T., Westenberger, S., Winzeler, E., Blackman, M.J., Baker, D.A., Wandless, T.J., Duraisingh, M.T., 2010. A plant-like kinase in *Plasmodium falciparum* regulates parasite egress from erythrocytes. Science 328, 910–912.

Dyer, M., Jackson, M., McWhinney, C., Zhao, G., Mikkelsen, R., 1996. Analysis of a cation-transporting ATPase of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 78, 1–12.

English, M.C., Waruiru, C., Lightowler, C., Murphy, S.A., Kirigha, G., Marsh, K., 1996. Hyponatraemia and dehydration in severe malaria. Arch. Dis. Child. 74, 201–205.

Flannery, E.L., McNamara, C.W., Kim, S.W., Kato, T.S., Li, F., Teng, C.H., Gagaring, K., Manary, M.J., Barboa, R., Meister, S., Kuhen, K., Vinetz, J.M., Chatterjee, A.K., Winzeler, E.A., 2014. Mutations in the P-type cation-transporter ATPase 4, PfATP4, mediate resistance to both aminopyrazole and spiroindolone antimalarials. ACS Chem. Biol. 10, 413–420.

Frame, I.J., Merino, E.F., Schramm, V.L., Cassera, M.B., Akabas, M.H., 2012. Malaria parasite type 4 equilibrative nucleoside transporters (ENT4) are purine transporters with distinct substrate specificity. Biochem. J. 446, 179–190.

Gamo, F.J., Sanz, L.M., Vidal, J., de Cozar, C., Alvarez, E., Lavandera, J.L., Vanderwall, D.E., Green, D.V., Kumar, V., Hasan, S., Brown, J.R., Peishoff, C.E., Cardon, L.R., Garcia-Bustos, J.F., 2010. Thousands of chemical starting points for antimalarial lead identification. Nature 465, 305–310.

Gardner, K.B., Sinha, I., Bustamante, L.Y., Day, N.P., White, N.J., Woodrow, C.J., 2011. Protein-based signatures of functional evolution in *Plasmodium falciparum*. BMC Evol. Biol. 11, 257.

Ginsburg, H., Kutner, S., Krugliak, M., Cabantchik, Z.I., 1985. Characterization of permeation pathways appearing in the host membrane of *Plasmodium falciparum* infected red blood cells. Mol. Biochem. Parasitol. 14, 313–322.

Ginsburg, H., Handeli, S., Friedman, S., Gorodetsky, R., Krugliak, M., 1986. Effects of red blood cell potassium and hypertonicity on the growth of *Plasmodium falciparum* in culture. Z. Parasitenkd. 72, 185–199.

Ginsburg, H., 2002. Abundant proton pumping in *Plasmodium falciparum*, but why? Trends Parasitol. 18, 483–486.

Gruring, C., Heiber, A., Kruse, F., Ungefehr, J., Gilberger, T.W., Spielmann, T., 2011. Development and host cell modifications of *Plasmodium falciparum* blood stages in four dimensions. Nat. Commun. 2, 165.

Guiguemde, W.A., Shelat, A.A., Bouck, D., Duffy, S., Crowther, G.J., Davis, P.H., Smithson, D.C., Connelly, M., Clark, J., Zhu, F., Jimenez-Diaz, M.B., Martinez, M.S., Wilson, E.B., Tripathi, A.K., Gut, J., Sharlow, E.R., Bathurst, I., El Mazouni, F.,

Fowble, J.W., Forquer, I., McGinley, P.L., Castro, S., Angulo-Barturen, I., Ferrer, S., Rosenthal, P.J., Derisi, J.L., Sullivan, D.J., Lazo, J.S., Roos, D.S., Riscoe, M.K., Phillips, M.A., Rathod, P.K., Van Voorhis, W.C., Avery, V.M., Guy, R.K., 2010. Chemical genetics of *Plasmodium falciparum*. Nature 465, 311–315.

Guttery, D.S., Pittman, J.K., Frenal, K., Poulin, B., McFarlane, L.R., Slavic, K., Wheatley, S.P., Soldati-Favre, D., Krishna, S., Tewari, R., Staines, H.M., 2013. The *Plasmodium berghei* Ca^{2+}/H^{+} exchanger, PbCAX, is essential for tolerance to environmental Ca^{2+} during sexual development. PLoS Pathog. 9, e1003191.

Hansen, M., Kun, J.F., Schultz, J.E., Beitz, E., 2002. A single, bi-functional aquaglyceroporin in blood-stage *Plasmodium falciparum* malaria parasites. J. Biol. Chem. 277, 4874–4882.

Hanson, J., Hossain, A., Charunwatthana, P., Hassan, M.U., Davis, T.M., Lam, S.W., Chubb, S.A., Maude, R.J., Yunus, E.B., Haque, G., White, N.J., Day, N.P., Dondorp, A.M., 2009. Hyponatremia in severe malaria: evidence for an appropriate anti-diuretic hormone response to hypovolemia. Am. J. Trop. Med. Hyg. 80, 141–145.

Hatin, I., Jambou, R., Ginsburg, H., Jaureguiberry, G., 1992. Single or multiple localization of ADP/ATP transporter in human malarial *Plasmodium falciparum*. Biochem. Pharmacol. 43, 71–75.

Hayashi, M., Yamada, H., Mitamura, T., Horii, T., Yamamoto, A., Moriyama, Y., 2000. Vacuolar H^{+}-ATPase localized in plasma membranes of malaria parasite cells, *Plasmodium falciparum*, is involved in regional acidification of parasitized erythrocytes. J. Biol. Chem. 275, 34353–34358.

Iizumi, K., Mikami, Y., Hashimoto, M., Nara, T., Hara, Y., Aoki, T., 2006. Molecular cloning and characterization of ouabain-insensitive Na^{+}-ATPase in the parasitic protist, *Trypanosoma cruzi*. Biochim. Biophys. Acta 1758, 738–746.

Jimenez-Diaz, M.B., Ebert, D., Salinas, Y., Pradhan, A., Lehane, A.M., Myrand-Lapierre, M.E., O'Loughlin, K.G., Shackleford, D.M., Justino de Almeida, M., Carrillo, A.K., Clark, J.A., Dennis, A.S., Diep, J., Deng, X., Duffy, S., Endsley, A.N., Fedewa, G., Guiguemde, W.A., Gomez, M.G., Holbrook, G., Horst, J., Kim, C.C., Liu, J., Lee, M.C., Matheny, A., Martinez, M.S., Miller, G., Rodriguez-Alejandre, A., Sanz, L., Sigal, M., Spillman, N.J., Stein, P.D., Wang, Z., Zhu, F., Waterson, D., Knapp, S., Shelat, A., Avery, V.M., Fidock, D.A., Gamo, F.J., Charman, S.A., Mirsalis, J.C., Ma, H., Ferrer, S., Kirk, K., Angulo-Barturen, I., Kyle, D.E., DeRisi, J.L., Floyd, D.M., Guy, R.K., 2014. (+)-SJ733, a clinical candidate for malaria that acts through ATP4 to induce rapid host-mediated clearance of *Plasmodium*. Proc. Natl. Acad. Sci. U. S. A. 111, E5455–E5462.

Kanaani, J., Ginsburg, H., 1989. Metabolic interconnection between the human malarial parasite *Plasmodium falciparum* and its host erythrocyte. Regulation of ATP levels by means of an adenylate translocator and adenylate kinase. J. Biol. Chem. 264, 3194–3199.

Kirk, K., Lehane, A.M., 2014. Membrane transport in the malaria parasite and its host erythrocyte. Biochem. J. 457, 1–18.

Kirk, K., Horner, H.A., Elford, B.C., Ellory, J.C., Newbold, C.I., 1994. Transport of diverse substrates into malaria-infected erythrocytes via a pathway showing functional characteristics of a chloride channel. J. Biol. Chem. 269, 3339–3347.

Kortagere, S., Welsh, W.J., Morrisey, J.M., Daly, T., Ejigiri, I., Sinnis, P., Vaidya, A.B., Bergman, L.W., 2010. Structure-based design of novel small-molecule inhibitors of *Plasmodium falciparum*. J. Chem. Inf. Model. 50, 840–849.

Krishna, S., Woodrow, C., Webb, R., Penny, J., Takeyasu, K., Kimura, M., East, J.M., 2001. Expression and functional characterization of a *Plasmodium falciparum* Ca^{2+}-ATPase (PfATP4) belonging to a subclass unique to apicomplexan organisms. J. Biol. Chem. 276, 10782–10787.

Lakshminarayana, S.B., Freymond, C., Fischli, C., Yu, J., Weber, S., Goh, A., Yeung, B.K., Ho, P.C., Dartois, V., Diagana, T.T., Rottmann, M., Blasco, F., 2014. Pharmacokinetics-pharmacodynamics analysis of spiroindolone analogs and KAE609 in a murine malaria model. Antimicrob. Agents Chemother. 59, 1200–1210.

Lang, F., Gulbins, E., Lang, P.A., Zappulla, D., Foller, M., 2010. Ceramide in suicidal death of erythrocytes. Cell. Physiol. Biochem. 26, 21–28.

Lang, E., Qadri, S.M., Lang, F., 2012. Killing me softly – suicidal erythrocyte death. Int. J. Biochem. Cell Biol. 44, 1236–1243.

Lee, P., Ye, Z., Van Dyke, K., Kirk, R.G., 1988. X-ray microanalysis of *Plasmodium falciparum* and infected red blood cells: effects of qinghaosu and chloroquine on potassium, sodium, and phosphorus composition. Am. J. Trop. Med. Hyg. 39, 157–165.

Lehane, A.M., Ridgway, M.C., Baker, E., Kirk, K., 2014. Diverse chemotypes disrupt ion homeostasis in the malaria parasite. Mol. Microbiol. 94, 327–339.

Leong, F.J., Li, R., Jain, J.P., Lefevre, G., Magnusson, B., Diagana, T.T., Pertel, P., 2014. A first-in-human randomized, double-blind, placebo-controlled, single- and multiple-ascending oral dose study of novel antimalarial Spiroindolone KAE609 (Cipargamin) to assess its safety, tolerability, and pharmacokinetics in healthy adult volunteers. Antimicrob. Agents Chemother. 58, 6209–6214.

Leroy, D., Campo, B., Ding, X.C., Burrows, J.N., Cherbuin, S., 2014. Defining the biology component of the drug discovery strategy for malaria eradication. Trends Parasitol. 30, 478–490.

Lew, V.L., Tiffert, T., Ginsburg, H., 2003. Excess hemoglobin digestion and the osmotic stability of *Plasmodium falciparum*-infected red blood cells. Blood 101, 4189–4194.

Lunde, C., Drew, D.P., Jacobs, A.K., Tester, M., 2007. Exclusion of Na^{+} via sodium ATPase (PpENA1) ensures normal growth of *Physcomitrella patens* under moderate salt stress. Plant Physiol. 144, 1786–1796.

Marchetti, R.V., Lehane, A.M., Shafik, S.H., Winterberg, M., Martin, R.E., Kirk, K., 2015. A lactate and formate transporter in the intraerythrocytic malaria parasite, *Plasmodium falciparum*. Nat. Commun. 6, 6721.

Martin, R.E., Henry, R.I., Abbey, J.L., Clements, J.D., Kirk, K., 2005. The 'permeome' of the malaria parasite: an overview of the membrane transport proteins of *Plasmodium falciparum*. Genome Biol. 6, R26.

Martin, R.E., Ginsburg, H., Kirk, K., 2009a. Membrane transport proteins of the malaria parasite. Mol. Microbiol. 74, 519–528.

Martin, R.E., Marchetti, R.V., Cowan, A.I., Howitt, S.M., Broer, S., Kirk, K., 2009b. Chloroquine transport via the malaria parasite's chloroquine resistance transporter. Science 325, 1680–1682.

Mauritz, J.M., Esposito, A., Ginsburg, H., Kaminski, C.F., Tiffert, T., Lew, V.L., 2009. The homeostasis of *Plasmodium falciparum*-infected red blood cells. PLoS Comput. Biol. 5, e1000339.

Mauritz, J.M., Seear, R., Esposito, A., Kaminski, C.F., Skepper, J.N., Warley, A., Lew, V.L., Tiffert, T., 2011. X-ray microanalysis investigation of the changes in Na, K, and hemoglobin concentration in *Plasmodium falciparum*-infected red blood cells. Biophys. J. 100, 1438–1445.

Munns, R., Tester, M., 2008. Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. 59, 651–681.

Niggli, V., Sigel, E., 2008. Anticipating antiport in P-type ATPases. Trends Biochem. Sci. 33, 156–160.

Palmgren, M.G., Nissen, P., 2011. P-type ATPases. Annu. Rev. Biophys. 40, 243–266.

Parker, M.D., Hyde, R.J., Yao, S.Y., McRobert, L., Cass, C.E., Young, J.D., McConkey, G.A., Baldwin, S.A., 2000. Identification of a nucleoside/nucleobase transporter from *Plasmodium falciparum*, a novel target for anti-malarial chemotherapy. Biochem. J. 349, 67–75.

Pillai, A.D., Addo, R., Sharma, P., Nguitragool, W., Srinivasan, P., Desai, S.A., 2013. Malaria parasites tolerate a broad range of ionic environments and do not require host cation remodelling. Mol. Microbiol. 88, 20–34.

Plouffe, D., Brinker, A., McNamara, C., Henson, K., Kato, N., Kuhen, K., Nagle, A., Adrian, F., Matzen, J.T., Anderson, P., Nam, T.G., Gray, N.S., Chatterjee, A., Janes, J., Yan, S.F., Trager, R., Caldwell, J.S., Schultz, P.G., Zhou, Y., Winzeler, E.A., 2008. *In silico* activity profiling reveals the mechanism of action of antimalarials discovered in a high-throughput screen. Proc. Natl. Acad. Sci. U. S. A. 105, 9059–9064.

Pulcini, S., Staines, H.M., Pittman, J.K., Slavic, K., Doerig, C., Halbert, J., Tewari, R., Shah, F., Avery, M.A., Haynes, R.K., Krishna, S., 2013. Expression in yeast links field polymorphisms in PfATP6 to in vitro artemisinin resistance and identifies new inhibitor classes. J. Infect. Dis. 208, 468–478.

Rodriguez-Navarro, A., Benito, B., 2010. Sodium or potassium efflux ATPase a fungal, bryophyte, and protozoal ATPase. Biochim. Biophys. Acta 1798, 1841–1853.

Rotmann, A., Sanchez, C., Guiguemde, A., Rohrbach, P., Dave, A., Bakouh, N., Planelles, G., Lanzer, M., 2010. PfCHA is a mitochondrial divalent cation/H^{+} antiporter in *Plasmodium falciparum*. Mol. Microbiol. 76, 1591–1606.

Rottmann, M., McNamara, C., Yeung, B.K., Lee, M.C., Zou, B., Russell, B., Seitz, P., Plouffe, D.M., Dharia, N.V., Tan, J., Cohen, S.B., Spencer, K.R., Gonzalez-Paez, G.E., Lakshminarayana, S.B., Goh, A., Suwanarusk, R., Jegla, T., Schmitt, E.K., Beck, H.P., Brun, R., Nosten, F., Renia, L., Dartois, V., Keller, T.H., Fidock, D.A., Winzeler, E.A., Diagana, T.T., 2010. Spiroindolones, a potent compound class for the treatment of malaria. Science 329, 1175–1180.

Roy, A., Kucukural, A., Zhang, Y., 2010. I-TASSER: a unified platform for automated protein structure and function prediction. Nat. Protoc. 5, 725–738.

Salcedo-Sora, J.E., Ochong, E., Beveridge, S., Johnson, D., Nzila, A., Biagini, G.A., Stocks, P.A., O'Neill, P.M., Krishna, S., Bray, P.G., Ward, S.A., 2011. The molecular basis of folate salvage in *Plasmodium falciparum*: characterization of two folate transporters. J. Biol. Chem. 286, 44659–44668.

Salcedo-Sora, J.E., Ward, S.A., Biagini, G.A., 2012. A yeast expression system for functional and pharmacological studies of the malaria parasite Ca^{2+}/H^{+} antiporter. Malar. J. 11, 254.

Saliba, K.J., Kirk, K., 1999. pH regulation in the intracellular malaria parasite, *Plasmodium falciparum*: H^{+} extrusion via a V-type H^{+}-ATPase. J. Biol. Chem. 274, 33213–33219.

Saliba, K.J., Allen, R.J., Zissis, S., Bray, P.G., Ward, S.A., Kirk, K., 2003. Acidification of the malaria parasite's digestive vacuole by a H^{+}-ATPase and a H^{+}-pyrophosphatase. J. Biol. Chem. 278, 5605–5612.

Saliba, K.J., Martin, R.E., Broer, A., Henry, R.I., McCarthy, C.S., Downie, M.J., Allen, R.J., Mullin, K.A., McFadden, G.I., Broer, S., Kirk, K., 2006. Sodium-dependent uptake of inorganic phosphate by the intracellular malaria parasite. Nature 443, 582–585.

Sanchez, C.P., Rotmann, A., Stein, W.D., Lanzer, M., 2008. Polymorphisms within PfMDR1 alter the substrate specificity for anti-malarial drugs in *Plasmodium falciparum*. Mol. Microbiol. 70, 786–798.

Spangenberg, T., Burrows, J.N., Kowalczyk, P., McDonald, S., Wells, T.N., Willis, P., 2013. The open access malaria box: a drug discovery catalyst for neglected diseases. PLoS One 8, e62906.

Spillman, N.J., Allen, R.J.W., McNamara, C.W., Yeung, B.K.S., Winzeler, E.A., Diagana, T.T., Kirk, K., 2013a. Na^{+} regulation in the malaria parasite *Plasmodium falciparum* involves the cation ATPase PfATP4 and is a target of the spiroindolone antimalarials. Cell Host Microbe 13, 227–237.

Spillman, N.J., Allen, R.J., Kirk, K., 2013b. Na^{+} extrusion imposes an acid load on the intraerythrocytic malaria parasite. Mol. Biochem. Parasitol. 189, 1–4.

Staines, H.M., Ellory, J.C., Kirk, K., 2001. Perturbation of the pump-leak balance for Na^{+} and K^{+} in malaria-infected erythrocytes. Am. J. Physiol. Cell Physiol. 280, C1576–C1587.

Stiles, J.K., Kucerova, Z., Sarfo, B., Meade, C.A., Thompson, W., Shah, P., Xue, L., Meade, J.C., 2003. Identification of surface-membrane P-type ATPases resembling fungal K^{+}- and Na^{+}-ATPases, in *Trypanosoma brucei, Trypanosoma cruzi* and *Leishmania donovani*. Ann. Trop. Med. Parasitol. 97, 351–366.

Strehler, E.E., 2013. Plasma membrane calcium ATPases as novel candidates for therapeutic agent development. J. Pharm. Pharm. Sci. Pub. Can. Soc. Pharm. Sci. Soc. Can. Sci. Pharm. 16, 190–206.

Treeck, M., Sanders, J.L., Elias, J.E., Boothroyd, J.C., 2011. The phosphoproteomes of *Plasmodium falciparum* and *Toxoplasma gondii* reveal unusual adaptations within and beyond the parasites' boundaries. Cell Host Microbe 10, 410–419.

Trottein, F., Cowman, A.F., 1995. Molecular cloning and sequence of two novel P-type adenosinetriphosphatases from *Plasmodium falciparum*. Eur. J. Biochem. 227, 214–225.

Trottein, F., Thompson, J., Cowman, A.F., 1995. Cloning of a new cation ATPase from *Plasmodium falciparum*: conservation of critical amino acids involved in calcium binding in mammalian organellar Ca^{2+}-ATPases. Gene 158, 133–137.

Vaidya, A.B., Morrisey, J.M., Zhang, Z., Das, S., Daly, T.M., Otto, T.D., Spillman, N.J., Wyvratt, M., Siegl, P., Marfurt, J., Wirjanata, G., Sebayang, B.F., Price, R.N., Chatterjee, A., Nagle, A., Stasiak, M., Charman, S.A., Angulo-Barturen, I., Ferrer, S., Belen Jimenez-Diaz, M., Martinez, M.S., Gamo, F.J., Avery, V.M., Ruecker, A., Delves, M., Kirk, K., Berriman, M., Kortagere, S., Burrows, J., Fan, E., Bergman, L.W., 2014. Pyrazoleamide compounds are potent antimalarials that target Na^+ homeostasis in intraerythrocytic *Plasmodium falciparum*. Nat. Commun. 5, 5521.

van Pelt-Koops, J.C., Pett, H.E., Graumans, W., van der Vegte-Bolmer, M., van Gemert, G.J., Rottmann, M., Yeung, B.K., Diagana, T.T., Sauerwein, R.W., 2012. The spiroindolone drug candidate NITD609 potently inhibits gametocytogenesis and blocks *Plasmodium falciparum* transmission to anopheles mosquito vector. Antimicrob. Agents Chemother. 56, 3544–3548.

van Wolfswinkel, M.E., Hesselink, D.A., Zietse, R., Hoorn, E.J., van Genderen, P.J., 2010. Hyponatraemia in imported malaria is common and associated with disease severity. Malar. J. 9, 140.

Wellems, T.E., Plowe, C.V., 2001. Chloroquine-resistant malaria. J. Infect. Dis. 184, 770–776.

White, N.J., Pukrittayakamee, S., Phyo, A.P., Rueangweerayut, R., Nosten, F., Jittamala, P., Jeeyapant, A., Jain, J.P., Lefevre, G., Li, R., Magnusson, B., Diagana, T.T., Leong, F.J., 2014. Spiroindolone KAE609 for *falciparum* and *vivax* malaria. N. Engl. J. Med. 371, 403–410.

Woodrow, C.J., Penny, J.I., Krishna, S., 1999. Intraerythrocytic *Plasmodium falciparum* expresses a high affinity facilitative hexose transporter. J. Biol. Chem. 274, 7272–7277.

Woodrow, C.J., Burchmore, R.J., Krishna, S., 2000. Hexose permeation pathways in *Plasmodium falciparum*-infected erythrocytes. Proc. Natl. Acad. Sci. U. S. A. 97, 9931–9936.

World Malaria Report, 2014. WHO Press, Geneva. Available from: http://www.who.int/malaria/publications/world_malaria_report_2014/en/ [last accessed May, 2015].

Wu, B., Rambow, J., Bock, S., Holm-Bertelsen, J., Wiechert, M., Soares, A.B., Spielmann, T., Beitz, E., 2015. Identity of a *Plasmodium* lactate/H(+) symporter structurally unrelated to human transporters. Nat. Commun. 6, 6284.

Wunsch, S., Sanchez, C.P., Gekle, M., Grosse-Wortmann, L., Wiesner, J., Lanzer, M., 1998. Differential stimulation of the Na^+/H^+ exchanger determines chloroquine uptake in *Plasmodium falciparum*. J. Cell Biol. 140, 335–345.

Yatime, L., Buch-Pedersen, M.J., Musgaard, M., Morth, J.P., Lund Winther, A.M., Pedersen, B.P., Olesen, C., Andersen, J.P., Vilsen, B., Schiott, B., Palmgren, M.G., Moller, J.V., Nissen, P., Fedosova, N., 2009. P-type ATPases as drug targets: tools for medicine and science. Biochim. Biophys. Acta 1787, 207–220.

Yeung, B.K., Zou, B., Rottmann, M., Lakshminarayana, S.B., Ang, S.H., Leong, S.Y., Tan, J., Wong, J., Keller-Maerki, S., Fischli, C., Goh, A., Schmitt, E.K., Krastel, P., Francotte, E., Kuhen, K., Plouffe, D., Henson, K., Wagner, T., Winzeler, E.A., Petersen, F., Brun, R., Dartois, V., Diagana, T.T., Keller, T.H., 2010. Spirotetrahydro beta-carbolines (spiroindolones): a new class of potent and orally efficacious compounds for the treatment of malaria. J. Med. Chem. 53, 5155–5164.

Zhang, Y., 2008. I-TASSER server for protein 3D structure prediction. BMC Bioinform. 9, 40.

Zhang, R., Suwanarusk, R., Malleret, B., Cooke, B.M., Nosten, F., Ling, L.Y., Dao, M., Lim, C.T., Renia, L., Tan, K.S.W., Russell, B., 2015. A basis for rapid clearance of circulating ring-stage malaria parasites by the spiroindolone KAE609. J. Infect. Dis. in press.

Zhou, Y., Fomovska, A., Muench, S., Lai, B.S., Mui, E., McLeod, R., 2014. Spiroindolone that inhibits PfATPase4 is a potent, cidal inhibitor of *Toxoplasma gondii* tachyzoites *in vitro* and *in vivo*. Antimicrob. Agents Chemother. 58, 1789–1792.