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Diverse chemotypes disrupt ion homeostasis in the malaria parasite

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Summary

The antimalarial spiroindolones disrupt *Plasmodium falciparum* Na^+ regulation and induce an alkalinization of the parasite cytosol. It has been proposed that they do so by inhibiting PfATP4, a parasite plasma membrane P-type ATPase postulated to export Na^+ and import H^+ equivalents. Here, we screened the 400 antiplasmodial compounds of the open access 'Malaria Box' for their effects on parasite ion regulation. *Twenty eight* compounds affected parasite Na^+ and pH regulation in a manner consistent with PfATP4 inhibition. Six of these, with chemically diverse structures, were selected for further analysis. All six showed reduced antiplasmodial activity against spiroindolone-resistant parasites carrying mutations in *pfatp4*. We exposed parasites to incrementally increasing concentrations of two of the six compounds and in both cases obtained resistant parasites with mutations in *pfatp4*. The finding that diverse chemotypes have an apparently similar mechanism of action indicates that PfATP4 may be a significant Achilles' heel for the parasite.

Introduction

Nearly half of the world's population live in malaria-endemic regions. With the most virulent of the human malaria parasites, *Plasmodium falciparum*, having acquired resistance to most of the antimalarial drugs that have been deployed, and with increasing reports that resistance to the front-line artemisinin antimalarials may be developing (Wongsrichanalai and Sibley, 2013; Ashley *et al*., 2014), there is an urgent need to identify new antimalarial agents. In the last five years, millions of compounds have been tested in high throughput 'whole-cell assays' to identify inhibitors of the *in vitro* growth of (asexual) blood-stage *P. falciparum* parasites (Plouffe *et al*., 2008; Gamo *et al*., 2010; Guiguemde *et al*., 2010). This approach has uncovered many novel compounds that show potent antiplasmodial activity *in vitro*. One such compound is the spiroindolone NITD609 (also known as KAE609; Rottmann *et al*., 2010; Fig. 1), which in a recently completed Phase II trial was shown to clear parasites rapidly in adults with uncomplicated *P. vivax* or *P. falciparum* malaria (White *et al*., 2014). NITD609 also possesses *in vitro* activity against the sexual gametocyte, the parasite form transmitted to mosquitoes (van Pelt-Koops *et al*., 2012).

The mechanism by which spiroindolones kill the parasite has been investigated. Long-term *in vitro* drug pressure experiments with NITD609 led to the selection of *P. falciparum* parasites with low-level resistance to the drug (Rottmann *et al*., 2010). The resistant parasites had acquired mutations in PfATP4 (PF3D7_1211900), a P-type ATPase on the parasite plasma membrane (Rottmann *et al*., 2010). Although PfATP4 was originally annotated as a Ca^{2+} pump (Krishna *et al*., 2001) it has been pointed out (Spillman *et al*., 2013a) that the protein shows significant sequence similarities to the ENA (*e*xitus *na*trus) Na^+-ATPases which efflux Na^+ from the cells of various lower eukaryotes including protozoa (e.g. Iizumi *et al*., 2006; Lunde *et al*., 2007).

The intraerythrocytic *P. falciparum* parasite exerts a tight control over its internal Na^+ levels, maintaining a cytosolic Na^+ concentration ($[Na^+]_i$) some 10-fold lower than that in the extracellular medium (Lee *et al*., 1988; Mauritz *et al*., 2011; Pillai *et al*., 2013; Spillman *et al*., 2013a). In parasites functionally isolated from their host cells the addition, at nanomolar concentrations, of growth-inhibiting spiroindolones results in an immediate increase in $[Na^+]_i$, consistent with inhibition of a Na^+ efflux mechanism (Spillman *et al*., 2013a). A spiroindolone has also been shown to inhibit Na^+-dependent ATPase activity (i.e. ATP hydrolysis) in parasitized-erythrocyte membrane preparations (Spillman *et al*., 2013a), and parasites with spiroindolone-resistance-conferring mutations in PfATP4 have reduced Na^+ efflux and an increased resting $[Na^+]_i$ (Spillman *et al*., 2013a). Together, the available data are consistent with the hypotheses that PfATP4 serves as a Na^+ efflux pump, that it is a target of the spiroindolone

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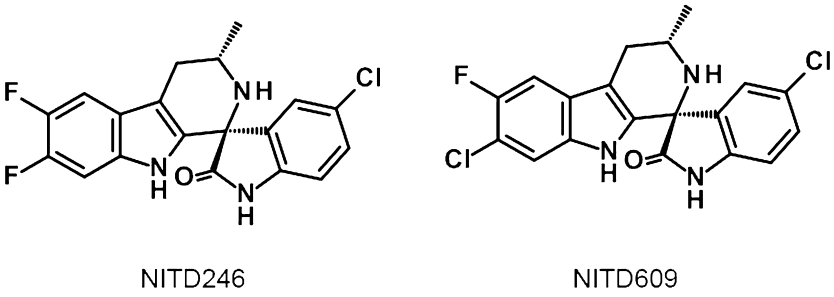


Fig. 1. Chemical structures of the antiplasmodial spiroindolones NITD609 and NITD246. NITD609 is also known as KAE609.

antimalarials, and that resistance-conferring mutations in PfATP4 result in reduced sensitivity of the protein to inhibition by the spiroindolones, as well as some impairment of function.

In addition to dissipating the Na^+ gradient across the plasma membrane of isolated parasites, the antiplasmodial spiroindolones cause an increase in the parasite's cytosolic pH (pH_i), thereby *increasing* the pH gradient across the parasite plasma membrane (Spillman *et al.*, 2013a). It has been postulated that PfATP4 expels Na^+ from the parasite in exchange for H^+ (Spillman *et al.*, 2013a), thereby imposing a significant acid load on the parasite (Spillman *et al.*, 2013b). The alkalinization seen in response to inhibition of the Na^+ extrusion mechanism has been attributed to the lifting of this acid load (Spillman *et al.*, 2013a). H^+ entering the cell in exchange for Na^+, via the putative Na^+-ATPase, is extruded by a second plasma membrane pump, a V-type H^+-ATPase which is inhibited by concanamycin A (Saliba and Kirk, 1999). Under conditions in which the Na^+-ATPase is operational (and therefore imposing an acid load), inhibition of the V-type H^+-ATPase by concanamycin A results in a marked acidification of the parasite cytosol. If the Na^+-ATPase is inactivated (either by an inhibitor or by removal of extracellular Na^+) then the acidification seen on addition of concanamycin A is reduced (Spillman *et al.*, 2013b).

Together, an increase in $[Na^+]_i$, an increase in pH_i, and a decrease in the extent of cytosolic acidification seen on inhibition of the V-type H^+-ATPase with concanamycin A can be considered a 'signature' of PfATP4 inhibition.

In a bid to further research into novel antiplasmodial compounds identified in the recent large-scale screens, the Medicines for Malaria Venture (MMV) has compiled the open access 'Malaria Box', a collection of 400 structurally diverse antiplasmodial compounds that were selected from the whole-cell drug screen 'hits' and for which the mechanisms of action are unknown (Spangenberg *et al.*, 2013). Here, we sought to determine whether any of the novel chemotypes contained within the Malaria Box disrupt Na^+ regulation in asexual blood-stage *P. falciparum* parasites.

Results

Screening the Malaria Box for inhibitors of parasite Na^+ *regulation*

For the initial screen of the Malaria Box, each of the 400 compounds were tested at a concentration of 1 μM for an effect on parasite $[Na^+]_i$. A 1 μl aliquot of a 1 mM DMSO stock of each compound was added to a 1 ml suspension of trophozoite-stage *P. falciparum* parasites, isolated by saponin-permeabilization of the erythrocyte membrane and loaded with the Na^+-sensitive fluorescent dye SBFI. The suspension was then monitored for any change in the fluorescence ratio. If, on addition of the compound, there was no change in the fluorescence ratio in the ensuing 2 min another compound was added to the same cells. For those compounds for which their addition resulted in an immediate gradual increase in the fluorescence ratio, signifying an increase in $[Na^+]_i$, the trace was continued without further additions until the fluorescence ratio levelled off at a new value (see Fig. 2A), after which time the screen was continued with a new batch of cells.

Some compounds gave rise to an 'optical effect', detectable as an abrupt change in the fluorescence ratio (see Fig. 2B), and interpreted as being due to an intrinsic fluorescence of the compound or perhaps a chemical interaction between the compound and the fluorescent Na^+ indicator. In such cases the cells were exchanged for a new batch of cells before testing additional compounds.

For compounds that were without effect on the fluorescence ratio a maximum of nine such compounds were tested on a single batch of cells before the spiroindolone NITD246 (50 nM; Fig. 1) was added as a positive control to ensure that a 'hit' was still detectable in the cells (see Fig. 2C).

From the initial screen, 24 compounds out of the 400 tested were shown to induce an immediate gradual increase in the fluorescence ratio (Table 1). These hits were all re-tested in subsequent Na^+ experiments, and their effects confirmed.

Fifty five of the 400 compounds gave rise to optical effects in the initial Na^+ assays. For many of these com-

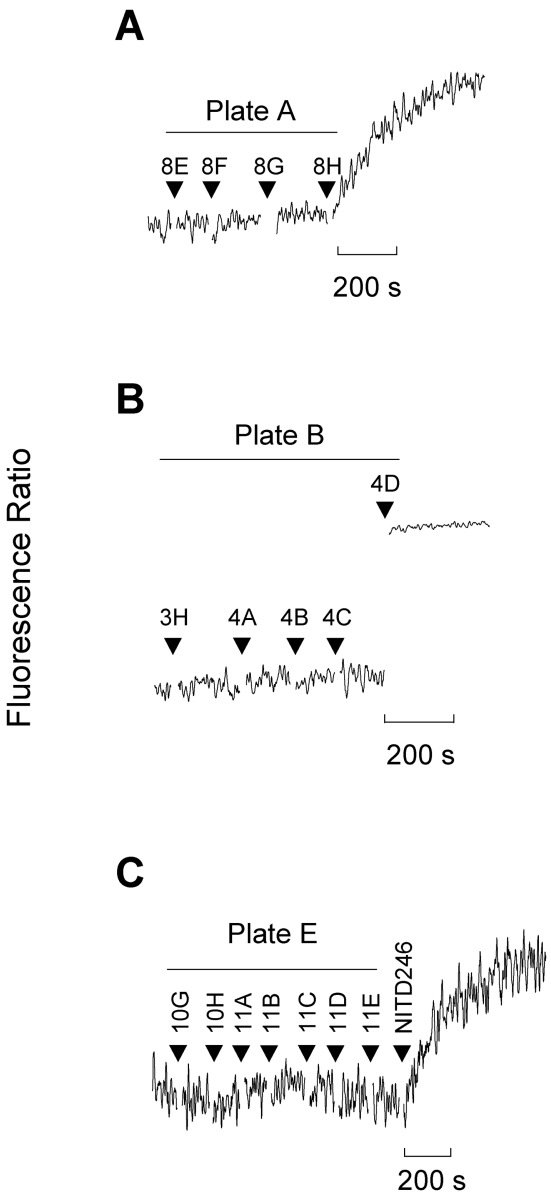


Fig. 2. Representative traces illustrating the strategy used to screen the Malaria Box for inhibitors of parasite Na^+ regulation. Malaria Box compounds (1 μM) were added successively to SBFI-loaded isolated trophozoite-stage parasites until either a hit was detected (A) or a compound caused an optical effect, manifest as an abrupt change in the fluorescence ratio (B). A maximum of nine inactive compounds were tested on a single batch of cells before the spiroindolone NITD246 (50 nM) was added as a positive control to ensure that a hit was still detectable in the cells (C).

pounds it was possible to establish whether or not they were hits in subsequent Na^+ assays. If an increase in $[Na^+]_i$ by a known hit could be detected in the same cells after the addition of a compound that caused an optical effect, the compound that caused the optical effect was deemed a non-hit. This is because a subsequent hit would not have been detectable had the Na^+ gradient already been dissipated. Compounds that gave rise to an optical effect and for which a subsequent hit-induced rise in $[Na^+]_i$ could not be detected were tested in pH experiments (below).

Testing the Malaria Box hits for effects on parasite pH_i

It has been shown previously that compounds such as the spiroindolone NITD246 that inhibit the extrusion of Na^+ from the parasite (thereby *decreasing* the Na^+ gradient across the parasite plasma membrane) cause an *increase* in the transmembrane pH gradient (Spillman *et al.*, 2013a), as well as reducing the extent of acidification that occurs on addition of the V-type H^+-ATPase inhibitor concanamycin A (Spillman *et al.*, 2013b). We therefore tested whether the Malaria Box compounds found to cause an increase in $[Na^+]_i$ in the parasite had an effect on pH_i. Experiments were performed on saponin-isolated trophozoite-stage parasites loaded with the pH-sensitive fluorescent dye BCECF.

As expected, the addition of the solvent DMSO at the concentration used to add the test compounds to BCECF-loaded parasites suspended in saline solution (0.1% v/v) had no effect on pH_i, and on addition of the V-type H^+-ATPase inhibitor concanamycin A the pH_i decreased to well below that of the extracellular solution (pH 7.1; see representative traces in Fig. 3). As shown previously (Spillman *et al.*, 2013a,b), the addition of NITD246 (50 nM) caused an increase in pH_i and a reduction in the magnitude of the acidification seen in response to a subsequent addition of concanamycin A (Fig. 3). All 24 of the Malaria Box hits detected in the initial Na^+ screen, when added at a concentration of 1 μM, produced an increase in pH_i and a reduction in the acidification induced by concanamycin A (see examples in Fig. 3). None of them gave rise to optical effects in the pH assays.

Plate A of the Malaria Box contains the 40 most potent (growth-inhibitory) 'drug-like' compounds and the 40 most potent (growth-inhibitory) 'probe-like' compounds. At a concentration of 1 μM, all of the hits on Plate A of the Malaria Box gave rise to pH_i effects that were similar in magnitude to those induced by 50 nM NITD246 [a maximally effective concentration of this compound (Spillman *et al.*, 2013a)]. For most of the compounds on other plates, the effects at 1 μM were not as pronounced as those caused by 50 nM NITD246. The finding of submaximal effects at 1 μM for these compounds is consistent with the fact that many of the Malaria Box compounds on plates B-E have 50% inhibitory concentrations (IC_{50} values) for parasite growth > 1 μM (http://www.mmv.org/research-development/malaria-box-supporting-information). As shown in Fig. 3, increasing the concentration of one of the Plate B hits (9G, MMV665796, which has an IC_{50} for parasite growth of 1.43 μM;

Table 1. The Malaria Box compounds designated as hits in parasite Na^+ and pH assays, and their effects on parasite ATP levels (after a 30 min incubation with 1 μM of the compound; mean ± SEM derived from 3–4 independent experiments). The 28 compounds identified as hits belong to 17 unique chemotypes. Those (four) chemotypes that include multiple hit compounds are labelled in the Table as Chemotypes I, II, III and IV.

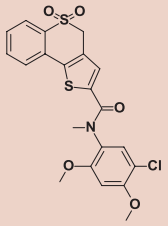
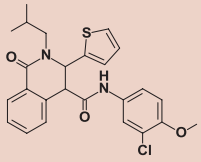
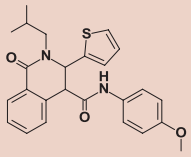
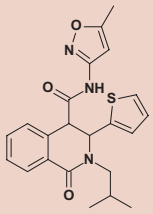
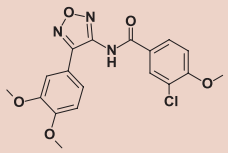
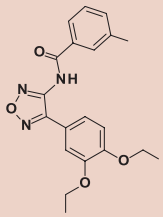
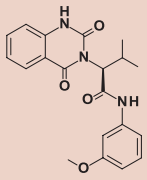
Location in Malaria Box (May 2012 batch)	Compound identifier	Structure	$[ATP]_i$ (% of control)	Optical effect?
Plate A: 9% Hit Rate				
2B	MMV006427		91 ± 5	No
3E	MMV000642 (Chemotype I)		94 ± 4	No
3H	MMV000662 (Chemotype I)		91 ± 0	No
6F	MMV006429 (Chemotype I)		97 ± 3	No
8H	MMV011567 (Chemotype II)		87 ± 4	No
10H	MMV665805 (Chemotype II)		90 ± 1	No
11C	MMV665878		86 ± 1	No

Table 1. *cont.*

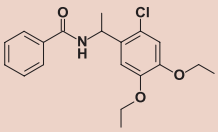
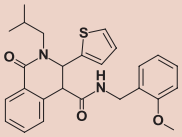
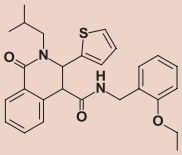
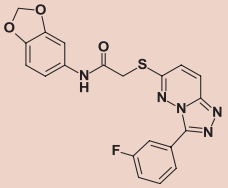
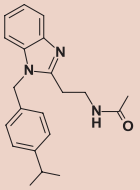
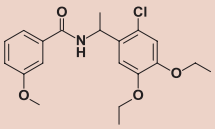
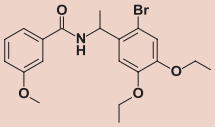
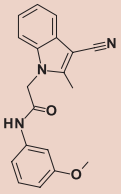
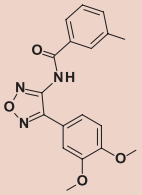
Location in Malaria Box (May 2012 batch)	Compound identifier	Structure	$[ATP]_i$ (% of control)	Optical effect?
Plate B: 11% Hit Rate				
2A	MMV665800 (Chemotype III)		96 ± 4	No
5G	MMV000648 (Chemotype I)		98 ± 1	No
6B	MMV000653 (Chemotype I)		96 ± 6	No
8C	MMV665918		98 ± 4	No
8G	MMV007617		93 ± 3	No
9F	MMV665803 (Chemotype III)		87 ± 2	No
9G	MMV665796 (Chemotype III)		90 ± 4	No
10C	MMV665826		94 ± 4	No
10H	MMV665890 (Chemotype II)		96 ± 3	No

Table 1. *cont.*

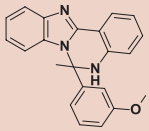
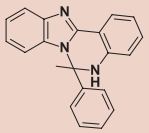
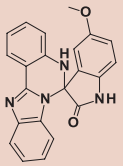
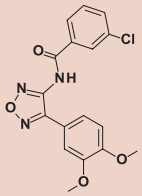
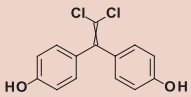
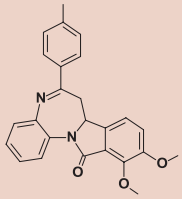
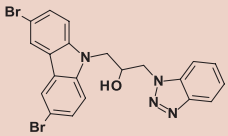
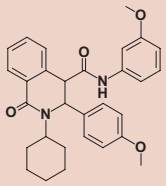
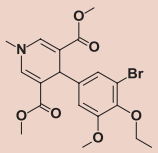
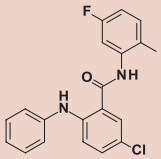
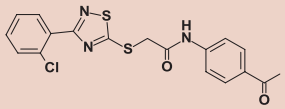
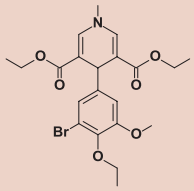
Location in Malaria Box (May 2012 batch)	Compound identifier	Structure	$[ATP]_i$ (% of control)	Optical effect?
Plate C: 5% Hit Rate				
3C	MMV396719 (Chemotype IV)		85 ± 3	Yes (SBFI)
3E	MMV396715 (Chemotype IV)		91 ± 1	Yes (SBFI)
5F	MMV396749 (Chemotype IV)		93 ± 5	No
10E	MMV020660 (Chemotype II)		91 ± 3	No
Plate D: 9% Hit Rate				
2B	MMV665949		96 ± 4	No
2D	MMV000917		93 ± 5	No
3H	MMV666025		94 ± 7	No
8E	MMV008455		95 ± 4	No
8G	MMV006764		94 ± 3	Yes (SBFI, BCECF denominator)

Table 1. *cont.*

Location in Malaria Box (May 2012 batch)	Compound identifier	Structure	$[ATP]_i$ (% of control)	Optical effect?
9G	MMV007275		101 ± 7	No
10B	MMV666124		99 ± 9	No
Plate E: 1% Hit Rate				
3C	MMV006656		90 ± 3	Yes (SBFI, BCECF denominator)

http://www.mmv.org/research-development/malaria-box-supporting-information) from 1 μM to 5 μM increased its effects on pH_i towards those seen for 50 nM NITD246.

One of the hits on Plate D (MMV666025) was a weaker hit than all of the others, with even a 5 μM concentration giving rise to a submaximal effect in the pH assay. Furthermore, although compounds similar to MMV666025 are present in the Malaria Box, they were not picked up as hits in our assays. It is possible that if a higher concentration (i.e. > 1 μM) had been tested in the initial screen (Na^+ assays), some additional compounds may have caused a detectable increase in fluorescence ratio. However in screening at a concentration of 1 μM we were following the recommendation of MMV (http://www.mmv.org/research-development/malaria-box-faqs), with this recommendation being based on the fact that the vast majority of compounds comprising the Malaria Box have IC_{50} values for inhibition of parasite growth of < 2 μM. Thus for any compounds that affected ion regulation only at concentrations higher than 1 μM it is unlikely that disruption of ion regulation was their primary mechanism of parasite killing.

We also used the pH assays to test the compounds that gave rise to optical effects in Na^+ assays. The majority of these compounds were revealed in the pH assays to be

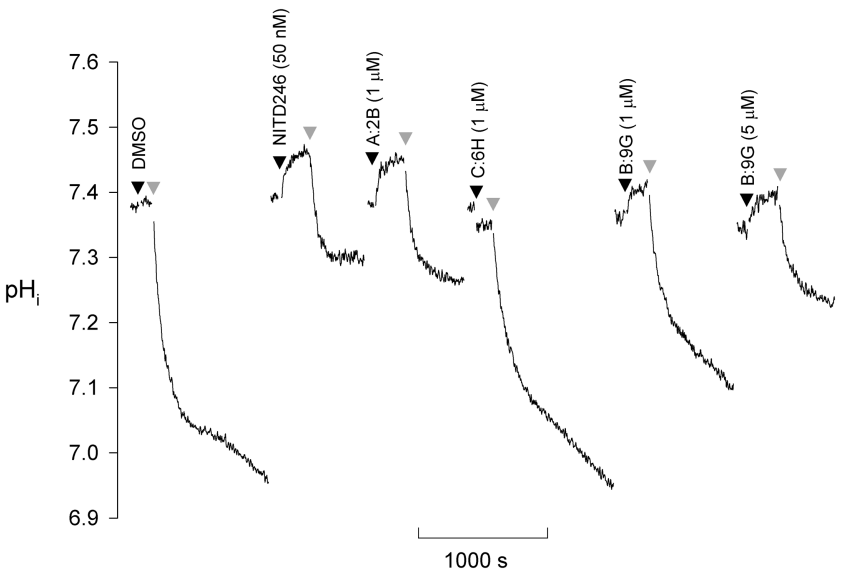


Fig. 3. Representative traces showing the effects of various compounds on pH_i in isolated trophozoite-stage parasites. DMSO did not increase pH_i, and on addition of concanamycin A (100 nM; grey arrow) pH_i decreased to below the pH of the extracellular solution. NITD246 (50 nM) increased pH_i and reduced the magnitude of the concanamycin A-induced acidification, as did the Plate A hit 2B (MMV006427; 1 μM). A non-hit from Plate C (6H) had little effect on pH_i or concanamycin A-induced acidification. A Plate B hit (9G; MMV665796) had an effect intermediate between that of DMSO and NITD246 at 1 μM, and a larger effect (similar to that of NITD246) at 5 μM.

non-hits, despite some of them giving rise to optical effects in these assays. A compound was considered a non-hit if it had no effect on the pH-sensitive BCECF fluorescence ratio (i.e. the ratio of the fluorescence measured at 520 nm using the two excitation wavelengths; 490 nm/440 nm), or on the (pH-sensitive) fluorescence measured at 520 nm following excitation at 490 nm (i.e. the numerator in the fluorescence ratio), or if it had an abrupt (optical) impact on one or other of these parameters but the subsequent addition of NITD246 or one of the confirmed Malaria Box hits nevertheless caused a detectable increase in the fluorescence ratio (indicative of a cytosolic alkalinization), showing that the compound had not eliminated the acid load associated with Na^+ extrusion. There was one compound in the Malaria Box (Plate D, 10D, MMV009127) for which a determination of whether it was a hit or non-hit could not be achieved with either Na^+ or pH assays, due to a strong optical effect that resulted in a loss of ion-sensitive fluorescence for both of the fluorescent indicators.

Four compounds that caused optical effects in Na^+ experiments were promoted to hit status after the pH experiments (Table 1). These were 3C and 3E on Plate C (neither gave rise to optical effects in the pH experiments and both displayed the pre- and post-concanamycin A characteristics of a hit), and 8G on Plate D and 3C on Plate E (both interfered with the pH-insensitive denominator in the BCECF fluorescence ratio in the pH experiments, but gave rise to gradual 'hit-like' increases in the pH-sensitive numerator).

In summary, based on the combination of Na^+ and pH experiments, 28 out of 400 compounds, equating to 7% of the Malaria Box compounds, showed evidence of having spiroindolone-like effects on parasite Na^+ and pH regulation, consistent with inhibition of PfATP4.

Testing the effects of the Malaria Box hits on parasite ATP levels

The possibility that the observed disruption of Na^+ and pH regulation might be an indirect effect resulting from depletion of parasite ATP was investigated by testing whether any of the hits (at 1 μM) had an effect on parasite ATP levels after a 30 min incubation. Parasites suspended in a glucose-free saline solution were used as a positive control for ATP depletion.

The results, expressed as the intracellular ATP concentration relative to that in control parasites incubated for 30 min in the presence of the same concentration of DMSO as was used to deliver the inhibitors (0.1% v/v), are shown in Table 1. For all of the hits, the ATP concentration was within 85–101% of control levels after the 30 min exposure. Note that 30 min is much longer than the time required for these compounds to begin disrupting $[Na^+]_i$ and pH_i. There was no significant difference in the intracellular ATP concentration between the control parasites and parasites treated with any of the compounds ($P > 0.05$; one-way ANOVA with post hoc Tukey test).

Table 2. Growth inhibition of the spiroindolone-resistant line NITD609-R^{Dd2}-clone#2 and its Dd2 parental line (both from Rottmann *et al.*, 2010) by six Malaria Box compounds, the spiroindolone NITD609, and the antimalarial drugs artemisinin and chloroquine.

	IC_{50} (μM)	
Compound	Dd2 parent	NITD609-R^{Dd2}-clone#2
MMV011567	0.33 ± 0.03	1.20 ± 0.20
MMV665949	1.12 ± 0.13	13.2 ± 1.9
MMV007275	0.22 ± 0.02	1.12 ± 0.10
MMV006656	0.15 ± 0.01	1.14 ± 0.07
MMV006764	0.45 ± 0.05	3.9 ± 0.4
MMV665826	0.28 ± 0.04	1.24 ± 0.15
NITD609	0.00062 ± 0.00002	0.0111 ± 0.0002
Artemisinin	0.011 ± 0.001	0.012 ± 0.002
Chloroquine	0.12 ± 0.02	0.11 ± 0.02

The data are from 3 independent experiments and are shown as mean ± SEM.

Testing for cross-resistance between six chemically diverse Malaria Box hits and the spiroindolones

Six of the Malaria Box hits (those for which additional quantities could be sourced from MMV or from Princeton BioMolecular Research) were tested for antiplasmodial activity against a spiroindolone-resistant line generated by Rottmann *et al.* (2010) (NITD609-R^{Dd2}-clone#2) and its matched Dd2 parental line. The six hits tested (MMV011567, MMV665949, MMV007275, MMV006656, MMV006764 and MMV665826) all belong to distinct chemotypes (Table 1). The spiroindolone-resistant line was significantly less susceptible than the parental line to each of the Malaria Box compounds tested ($P \leq 0.03$; two-tailed paired t-tests; Table 2). The fold difference in the IC_{50} values between the two lines ranged from 3.6 ± 0.3 (for MMV011567; mean ± SEM) to 8.9 ± 1.1 (MMV006764). There was no difference in the susceptibility of the two lines to either chloroquine or artemisinin ($P \geq 0.1$).

Generating parasites resistant to the Malaria Box hits MMV011567 and MMV007275

For each of two chemically distinct Malaria Box hits, MMV011567 and MMV007275, parasite cultures were exposed to increasing concentrations of the compound for a prolonged period (142–150 days) to generate resistant parasites. In the case of MMV011567 results were obtained for two independent duplicate cultures; in the

Table 3. *pfatp4* mutations and growth inhibition by MMV011567, MMV007275 and the spiroindolone NITD609 in parasites generated by prolonged exposure to increasing concentrations of MMV011567 or MMV007275.

	Mutation in PfATP4 (relative to Dd2 parent)	MMV011567 IC_{50} value (μM)	MMV007275 IC_{50} value (μM)	NITD609 IC_{50} value (μM)
Dd2 parent	–	0.34 ± 0.05	0.23 ± 0.02	0.00092 ± 0.00016
MMV011567-pressured culture 1	Q172K	1.63 ± 0.22	nd	0.0028 ± 0.0002
MMV011567-pressured culture 2	A353Q	1.89 ± 0.24	nd	0.0035 ± 0.0004
MMV007275-pressured culture	Q172H	nd	1.10 ± 0.18	0.0037 ± 0.0006

The data are from 3 independent growth inhibition experiments and are shown as mean ± SEM. nd, no data.

case of MMV007275 results were obtained for a single culture. The experiment was performed with a Dd2 line that had recently been cloned by limiting dilution, and 'drug pressure' (i.e. culturing in the presence of the compound of interest) commenced with the addition of the appropriate compound at its 50% growth inhibitory concentration (IC_{50}) against the Dd2 clone (214 nM for MMV011567 and 93 nM for MMV007275). Drug pressure was increased in a stepwise manner: when parasites reached a parasitaemia of ~ 5% and the drug IC_{50} had increased, the drug concentration was increased to the new IC_{50}.

Table 3 shows IC_{50} values for the effects of MMV011567, MMV007275 and NITD609 on the growth of the different parasites, as measured at the end of the drug pressure period. The IC_{50} values for the effect of MMV011567 on parasites from both of the two MMV011567-pressured cultures were ~ 5-fold higher than those of the parental parasites ($P \leq 0.03$; two-tailed paired t-tests). Approximately 5-fold higher IC_{50} values relative to parental parasites were also observed for MMV007275 in parasites from the MMV007275-pressured culture ($P = 0.03$). Parasites from all three of the drug-pressured cultures were also found to be significantly less susceptible to NITD609 than the parental parasites ($P \leq 0.03$).

Genomic DNA was extracted from each of the drug-pressured cultures at the end of the drug pressure period and from the parental Dd2 culture. The entire *pfatp4* gene was then sequenced. The parental Dd2 parasites had two polymorphisms in *pfatp4* relative to the 3D7 strain (PlasmoDB ID PF3D7_1211900). These were C669A (synonymous) and G3383A (which codes for a G1128R mutation). Parasites from each of the drug-pressured cultures were found to have one additional mutation in *pfatp4* (Table 3). Parasites from one of the MMV011567-pressured cultures had a C514A mutation in *pfatp4*, corresponding to a Q172K mutation in the protein. Parasites from the other MMV011567-pressured culture were found to have a C1058A mutation in *pfatp4*, corresponding to an A353Q mutation in the protein. Parasites from the MMV007275-pressured culture had an A516T mutation in *pfatp4*, corresponding to a Q172H mutation in the protein. The three mutations identified here have not been reported previously. The NITD609-R^{Dd2}-clone#2 parasites described by Rottmann *et al*. (2010) have two mutations in PfATP4: T418N and P990R. I398F, D1247Y, G223R, A184S, I203M and I263V have also been identified in PfATP4 in other spiroindolone-resistant lines, with each line having a maximum of two mutations (Rottmann *et al*., 2010).

Discussion

Recent whole-cell assays screening some 4 million compounds from the chemical libraries of St Jude Children's Research Hospital, the Genomics Institute of the Novartis Research Foundation and GlaxoSmithKline have yielded some 20 000 inhibitors of the growth *in vitro* of blood-stage *P. falciparum* parasites (Plouffe *et al*., 2008; Gamo *et al*., 2010; Guiguemde *et al*., 2010). In compiling the 400-compound Malaria Box, MMV evaluated the (~ 5000) commercially available hits with the aim of maximizing the collection's structural diversity (Spangenberg *et al*., 2013).

The present study revealed that, when added at a concentration of 1 μM, 28 of the 400 compounds comprising the Malaria Box caused an immediate disruption of malaria parasite ion homeostasis. The nature of the disruption was, in each case, the same as that reported previously for the spiroindolones (Spillman *et al*., 2013a,b): on addition of the compounds there was, within seconds, an increase in both $[Na^+]_i$ and pH_i, reflecting a *decrease* in the $[Na^+]$ gradient and an *increase* in the pH gradient across the parasite plasma membrane, with the subsequent inhibition of the parasite's plasma membrane V-type H^+-ATPase giving rise to a lesser acidification than was seen under control conditions.

Within these 28 hits there are some compounds with similar structures. Nevertheless the 28 compounds represent 17 unique chemotypes (Table 1). One of these, comprising MMV396719, MMV396715 and MMV396749 on Plate C, is similar in structure to the spiroindolones (Rottmann *et al*., 2010); however, the compounds comprising the other 16 chemotypes are all quite different in structure from the spiroindolones.

It has been reported that the spiroindolone NITD609 has a detrimental effect on the sexual gametocyte stage of *P. falciparum* (van Pelt-Koops *et al.*, 2012). The Malaria Box compounds have recently been screened for anti-gametocyte activity (Duffy and Avery, 2013; Lucantoni *et al.*, 2013; Bowman *et al.*, 2014). For all but one of the Plate A compounds identified as hits in our study it was reported that a concentration of 5 μM reduced the survival of early gametocytes [by ≥ 91% in the study by Duffy and Avery (2013) and by ≥ 70% in the study by Lucantoni *et al.* (2013)]. The one exception, MMV011567, is the least potent of the Plate A hits at killing asexual stage parasites, with an IC_{50} value > 3.7-fold higher than those for the other compounds (http://www.mmv.org/research-development/malaria-box-supporting-information). Thus, the lower level of activity against early gametocytes for this compound [a 32% decrease in survival rate at 5 μM in the study by Duffy and Avery (2013) and an 11% decrease in survival rate at 5 μM in the study by Lucantoni *et al.* (2013)], and for the hits on the other plates (which contain less potent compounds) may reflect a sensitivity issue. The activity of the hits against late-stage gametocytes tended to be lower than their activity against early-stage gametocytes. The average level of growth inhibition at 5 μM for our hits was 32 ± 27% (mean ± SD) in the study by Duffy and Avery (2013) and 67 ± 25% in the study by Bowman *et al.* (2014). When considering only the Plate A hits, the level of growth inhibition was 48 ± 17% in the study by Duffy and Avery (2013) and 76 ± 4% in the study by Bowman *et al.* (2014).

The observation that all of the hits identified here in the initial $[Na^+]_i$ assays caused an *increase* in the transmembrane pH gradient rules out the possibility that the increase in $[Na^+]_i$ was due to a general disruption of the integrity of the parasite plasma membrane. The finding that none of the hits caused a marked depletion of parasite ATP levels on the timescale of the experiment rules out the possibility of the disruption being a secondary consequence of de-energization of the cell. Instead, the data are consistent with the hypothesis that the compounds identified here bring about a more specific inhibition of parasite Na^+ extrusion, postulated previously to involve the exchange of Na^+ for H^+ equivalents, and to be mediated by PfATP4 (Spillman *et al.*, 2013a,b).

There are a number of alternative mechanisms by which the compounds might inhibit Na^+ extrusion. One possibility is that the Na^+ extrusion mechanism is dependent for its activity on a number of other cellular components, and that inhibition of Na^+ extrusion may be achieved through the interaction of pharmacological agents with any of these. The parasite's putative plasma membrane Na^+ pump, PfATP4, is closely related to the plasma membrane Ca^{2+}-ATPases (Krishna *et al.*, 2001; Rottmann *et al.*, 2010; Spillman *et al.*, 2013a). Recent work on plasma membrane Ca^{2+}-ATPases has revealed that they are regulated by a diverse range of scaffolding, signalling and regulatory proteins (Strehler, 2013), raising the possibility that the same may be true of PfATP4. Phosphorylation sites have been identified in PfATP4 (Treeck *et al.*, 2011), consistent with kinases being involved in its regulation. Thus, it is possible that the inhibition of Na^+ extrusion by the diversity of compounds identified here reflects, in at least some cases, an indirect effect.

An alternative possibility is, however, that the inhibition is due in each case to a direct interaction between the compounds identified here and PfATP4. Our finding that each of the six chemically diverse Malaria Box compounds that were identified as hits in the ion regulation assays and then tested in growth assays had reduced antiplasmodial activity against *pfatp4* mutant NITD609-resistant parasites compared to the matched parental parasites is consistent with this scenario. Furthermore, our finding of *pfatp4* mutations in parasites exposed to increasing concentrations of either MMV011567 or MMV007275 is consistent with at least these two compounds targeting PfATP4 itself.

Our study therefore raises the question of why 7% of a group of compounds selected initially on the basis of their ability to inhibit the growth of *P. falciparum* parasites in *in vitro* whole-cell assays, and subsequently with a view to providing the broadest cross-section of structural diversity (Spangenberg *et al.*, 2013), might interact with the one protein. For a protein to serve as an effective target in *in vitro* whole-cell assays such as those used in the initial high throughput screens it is necessary that it be: (i) essential (i.e. necessary for parasite survival), (ii) inhibitable (i.e. able to bind to a pharmacological agent in such a way as to prevent the function of the protein), and (iii) accessible (i.e. able to be accessed by pharmacological agents introduced into the extracellular medium). Perhaps PfATP4 is one of relatively few malaria parasite proteins that, at least in asexual blood-stage parasites grown under *in vitro* conditions, and perhaps in gametocytes as well, meets these requirements. As such, it may represent a significant Achilles' heel for the parasite.

Experimental procedures

Compounds

The Malaria Box was provided by MMV. Each compound was diluted from 10 mM to 1 mM in DMSO and aliquoted into multiple plates. Details of the compounds are available via ChEMBL-NTD (https://www.ebi.ac.uk/chemblntd). The spiroindolone NITD246 (Rottmann *et al.*, 2010), kindly provided by the Novartis Institute for Tropical Diseases (Singapore) through a Material Transfer Agreement, was used as a positive control. NITD609 and additional quantities of the

Malaria Box compounds MMV011567, MMV007275 and MMV665949 were supplied by MMV. Additional quantities of the Malaria Box compounds MMV006656, MMV006764 and MMV665826 were purchased from Princeton BioMolecular Research.

Parasite culture and isolation

Unless stated otherwise, all experiments were performed with the chloroquine-resistant Dd2 strain of *P. falciparum*, which is of Thai origin. The spiroindolone-resistant line NITD609-R^{Dd2}-clone#2 generated by Rottmann *et al.* (2010) and its matched parental line were generously provided by Professor Elizabeth Winzeler. Cultures were maintained as described previously (Trager and Jensen, 1976), with some modifications (Allen and Kirk, 2010), and were synchronized by sorbitol treatment (Lambros and Vanderberg, 1979). Parasites were functionally isolated from their host erythrocytes by exposing the (~ 4% haematocrit) cultures briefly to saponin (0.05% w/v, of which ≥ 10% was the active agent sapogenin) (Saliba *et al.*, 1998), and then washing the cells several times in bicarbonate-free RPMI1640 supplemented with 20 mM glucose, 0.2 mM hypoxanthine, 25 mM HEPES, and 25 mg l^{-1} gentamicin sulphate (pH 7.10). The isolated parasites were maintained in this medium at 37°C and at a density of 1×10^7–3×10^7 parasites ml^{-1} until immediately before their use in either Na^+ or pH experiments. For experiments in which parasite ATP levels were measured, the isolated parasites were washed and maintained in 'saline solution' (125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM glucose, 25 mM HEPES; pH 7.10) instead of bicarbonate-free RPMI.

Monitoring changes in parasite [Na^+]

Saponin-isolated trophozoite-stage parasites were loaded with the Na^+-sensitive dye SBFI (Molecular Probes, Life Technologies), as described previously (Spillman *et al.*, 2013a). Suspensions of SBFI-loaded, saponin-isolated trophozoites (1×10^7–3×10^7 cells ml^{-1}) in saline solution were excited at 340 nm and 380 nm successively, and the fluorescence measured at 515 nm, using a PerkinElmer LS 50B Fluorescence Spectrometer with a dual excitation Fast Filter accessory. The ratio of the two measurements (340 nm/380 nm) was used as an indicator of parasite $[Na^+]_i$. For the purpose of this study the relationship between fluorescence ratio and $[Na^+]_i$ was not calibrated. The signal-to-noise ratios obtained in the Na^+ assays varied between experiments, as can be seen from the representative traces shown in Fig. 2.

Measuring parasite pH

pH_i was measured at 37°C in saponin-isolated trophozoite-stage parasites using the ratiometric pH-sensitive fluorescent dye BCECF (Molecular Probes, Life Technologies), as described previously (Saliba and Kirk, 1999). Suspensions of BCECF-loaded, saponin-isolated trophozoites (1×10^7–3×10^7 cells ml^{-1}) in saline solution were excited at 440 and 490 nm successively, and the fluorescence measured at 520 nm. The fluorescence measured on excitation at 490 nm is pH-sensitive whereas that measured on excitation at 440 nm is pH-insensitive. The ratio of the two measurements (490 nm/440 nm) provides an effective measure of cytosolic pH. The relationship between this fluorescence ratio and the cytosolic pH was calibrated as described previously (Saliba and Kirk, 1999). The signal-to-noise ratios obtained in pH experiments were typically better than those obtained in Na^+ experiments (~ 10 for the initial alkalinization; cf. Figs 2 and 3).

Measuring parasite ATP levels

ATP concentrations in isolated trophozoite-stage parasites suspended at 37°C at a density of 4×10^7–10×10^7 cells ml^{-1} in saline solution were measured using firefly lantern extract, as described elsewhere (van Schalkwyk *et al.*, 2008).

Parasite proliferation assays

The effect of compounds on parasite proliferation was assessed using a fluorescent DNA-intercalating dye (Smilkstein *et al.*, 2004), essentially as described previously (Spry *et al.*, 2013). The initial parasitaemia (consisting of predominantly ring-stage parasites) and haematocrit were both 1%, and assays were terminated after 72 h.

Generating and characterizing resistant parasites

A clone of the chloroquine-resistant Dd2 strain of *P. falciparum* was obtained by limiting dilution, essentially as described previously by Adjalley *et al.* (2010). For each of two Malaria Box compounds of interest (MMV011567 and MMV007275), flasks containing 1.8×10^8 parasites were exposed to increasing concentrations of the compound for ~ 5 months. The starting concentrations of the compounds were their IC_{50} values against the clonal Dd2 parasites. For each compound the concentration was increased to the newly determined IC_{50} value once parasite proliferation was restored and a parasitaemia of ~ 5%, as well as a higher IC_{50} value, were reached. The cultures were provided with fresh medium and blood and diluted to lower the parasitaemia as required.

Genomic DNA was isolated from saponin-isolated parasites by phenol/chloroform extraction with prior Proteinase K treatment (Gross-Bellard *et al.*, 1973). The entire *pfatp4* gene was PCR-amplified using KOD Hot Start DNA polymerase (Novagen) using identical primers to those described previously (Rottmann *et al.*, 2010). The entire *pfatp4* gene was sequenced at the Australian Genome Research Facility using the primers used to amplify the gene, the internal sequencing primers described by Rottmann *et al.* (2010), and three additional internal primers (TATCTCCGTCTTCTACATTATTG, ATGACAGCAATTAATGCAGTTAC and CATGTAGTATATC TGCAACTTTAAC).

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