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Inhibition of *Plasmodium falciparum* pH regulation by small molecule indole derivatives results in rapid parasite death

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ABSTRACT

The V-type H^+-ATPase is critical during the intraerythrocytic stage of the human malaria parasite *Plasmodium falciparum*. It is responsible for maintaining a near-neutral cytosolic pH (pH 7.3), an acidic digestive vacuole (pH 4.5–5.5) and the generation of an inside-negative plasma membrane potential (~−95 mV). Inhibition of this pump is therefore likely to result in profound physiological disturbances within the parasite and parasite death, as illustrated previously by the antiplasmodial activity of the potent and specific inhibitors of the V-type H^+-ATPase, bafilomycin A_1 and concanamycin A. In this study we examined the antiplasmodial activity of a series of compounds previously designed, on the basis of the active structural constituents of bafilomycin A_1, to inhibit the osteoclast V-type H^+-ATPase. The compounds were tested against up to 4 strains of *P. falciparum* with varying chloroquine sensitivities. Of the 30 novel compounds tested, 9 had sub-micromolar antiplasmodial IC_{50} values, with the most active compound having an IC_{50} of 160 ± 20 nM. The activity of a number of these compounds was investigated in more detail. We show that these inhibitors acidify the parasite cytosol within seconds and that some inhibitors irreversibly kill the parasite within 0.5–4 h. The antiplasmodial activity of the V-type H^+-ATPase inhibitors was strongly correlated with their ability to acidify the parasite cytosol (correlation coefficient 0.98). In combination studies, we show that the inhibitors act indifferently when combined with current antimalarials. Our data support the disruption of parasite pH regulation through inhibition of its V-type H^+-ATPase as an antimalarial approach.

1. Introduction

The V-type H^+-pumping ATPase, which has been characterised in a number of organisms, is made up of 14 different subunits that are organised into 2 domains [1]. The V_0 domain is a 260-kDa complex embedded into the membrane that is responsible for proton translocation from the cytoplasm into either the extracellular space or into the lumen of an intracellular organelle, typically vacuoles. The V_1 domain is a 650-kDa complex responsible for ATP hydrolysis [1,2]. In the human malaria parasite *Plasmodium falciparum*, two V_1 subunits and one V_0 subunit have so far been cloned and localised in the intraerythrocytic stage of the parasite lifecycle [3–5]. Using antibodies to subunits *A* and *B*, the V-type H^+-ATPase has been localised to both the digestive vacuole (DV) and parasite plasma membranes, as well as small clear vesicles of unknown identity [6]. In addition, the V-type H^+-ATPase has also been argued to be present on the infected erythrocyte membrane [7].

The parasite has been shown to regulate its cytosolic pH by pumping protons out of the parasite cytosol across the parasite plasma membrane using a V-type H^+-ATPase [6,8,9]. This V-type H^+-ATPase-mediated regulation of cytosolic pH is important for enzyme function and for the generation of a pH gradient across the plasma membrane and across the membranes of internal organelles. For example, the pH gradient across the parasite plasma membrane is critical for the transport of the essential nutrient pantothenate (vitamin B_5) [10] and H^+ also plays a role in the removal of the waste product lactate [11]. The V-type H^+-ATPase is also used to generate an inside-negative (~−95 mV) membrane potential [12]. This membrane potential is utilised by the parasite to regulate the concentration of ions (e.g., K^+) in the cytosol and the uptake of nutrients, such as phosphate [13] and

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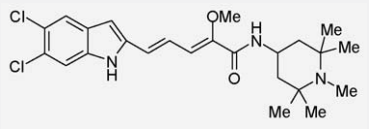
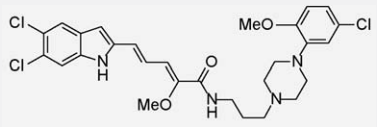
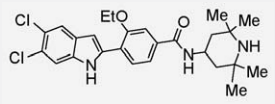
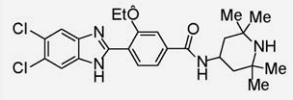
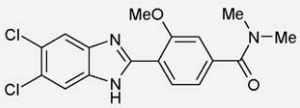
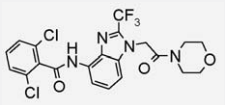
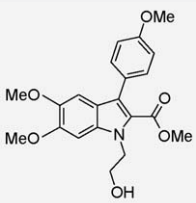
choline [14,15]. Additionally, the *P. falciparum* V-type H^+-ATPase is critical for the acidification of the parasite's DV. The DV is the site of hemoglobin digestion and heme crystallisation [16–18]. Both of these processes are critical for the parasite and both rely on an acidic pH for optimum activity [19,20]. The role played by the V-type H^+-ATPase on the parasite-infected erythrocyte membrane is less clear but it may contribute to infected erythrocyte cytosol pH regulation [7].

The inhibition of the parasite V-type H^+-ATPase is therefore likely to be devastating to the physiological integrity of the parasite and would be expected to result in inhibition of parasite growth. Two potent and specific inhibitors of the V-type H^+-ATPase, concanamycin A and bafilomycin A_1, have been reported to inhibit the growth of *P. falciparum* in vitro in the low micromolar to sub-nanomolar range [21,22], but whether their inhibitory effect was related to an effect on the parasite's V-type H^+-ATPase was not investigated.

In this study, we tested the antiplasmodial activity of 30 novel indole derivatives of the V-type H^+-ATPase inhibitor bafilomycin A_1. All the small molecule inhibitors of the V-type H^+-ATPase we tested were synthesised previously within the framework of research programmes directed at the identification of novel antiosteoporotic or anticancer therapeutic agents [23–27]. Their activity against the V-type H^+-ATPase present in *P. falciparum* cannot be predicted on the basis of the data previously obtained from mammalian enzymes, due to the fact that very little information is available about the overall features of this enzyme (i.e., the identity, and therefore sequence, of individual subunits and the manner in which they are assembled to form a functional enzyme) in the parasite. Most of these inhibitors had low nanomolar inhibitory activity against mammalian V-type H^+-ATPases such as those isolated from chicken or human osteoclasts, bovine chromaffin granules, chicken adrenals, or mouse macrophages (Table 1). For this reason, we decided to elucidate the

Table 1
Structural classes of the novel small molecule indole derivatives of bafilomycin A_1 tested in this study against the malaria parasite. Representative compounds and their structures are provided for each structural class. Included also is the concentration required to inhibit the activity of the mammalian or chicken V-type H^+-ATPase activity by 50% (IC_{50}).

Class	Name	Compound number	Structure	IC_{50} (μM) V-type H^+-ATPase	Reference
A	Indolepentadiene amides	11383 (SB242784)[a]		0.026[b]	[23,24,28]
		50010		0.02[b]	[30]
B	Indole benzamides	12192		0.017[c]	[25,26]
C	Benzoimidazole benzamides	12194		0.005[c]	[55]
		22434		14[c]	
D	Benzoimidazole acetamides	11384 (FR-177995)[a]		0.46[d]	[32]
E	3-Phenylindoles	14121		0.25[c]	[29]

[a] Previously published codes.
[b] Chicken osteoclast.
[c] Bovine chromaffin granules.
[d] Mouse macrophages.

antiplasmodial activity of a structurally diverse selection of these inhibitors. The structural classes employed and representative compounds of each class are listed in Table 1.

After establishing the antiplasmodial activity, we examined the mechanism through which these derivatives exert their antiplasmodial activity in vitro. We show that these V-type H^+-ATPase inhibitors induce a rapid acidification of the parasite cytosol and that this acidification correlates with the antimalarial activity of the inhibitors. We demonstrate that the inhibitors act rapidly to inhibit parasite growth and when combined with other antimalarials are neither antagonistic nor synergistic. We propose that the inhibition of the V-type H^+-ATPase is responsible for the antiplasmodial activity of the inhibitors, and that this proton pump might be a suitable drug target.

2. Materials and methods

2.1. Reagents

Chloroquine diphosphate, nigericin, artemisinin and quinine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and concanamycin A was purchased from MP Biomedicals (Santa Ana, CA, USA). Albumax II, gentamicin sulphate, HEPES and the acetoxymethyl ester form of the fluorescent pH indicator 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) were purchased from Invitrogen (Carlsbad, CA, USA). [^{3}H]Hypoxanthine was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Bafilomycin A_1 and the novel derivatives were obtained from NiKem Research (Milan, ITA) and were synthesised as described previously [23,24,28–32].

2.2. Parasite culture

Experiments were performed on the chloroquine sensitive (CQ^S) 3D7 and D10 strains and the chloroquine resistant (CQ^R) K1 and 7G8 strains of *P. falciparum* during their intraerythrocytic stage of development. The intraerythrocytic stage is initiated when a merozoite infects the erythrocyte. During the first 16 h post-invasion, the parasite is referred to as being in the ring stage. In the following 24 h, the parasite matures into the metabolically active trophozoite. The parasite then enters the schizont stage (40–48 h post-invasion) during which it divides to produce 20–30 daughter merozoites. At 48 h post-infection, the infected erythrocyte ruptures releasing these merozoites. The released merozoites then infect other erythrocytes to continue the intraerythrocytic lifecycle. The parasites were maintained in human erythrocytes suspended in RPMI-1640 culture medium as described previously [12]. The culture medium was supplemented with sodium bicarbonate (25 mM), gentamicin sulphate (24 μg/mL), glucose (11 mM), HEPES (25 mM), hypoxanthine (200 μM) and Albumax II (6 g/L). The parasites were kept in continuous culture at 37 °C under a gas mixture of 3% CO_2, 1% O_2 and 96% N_2. Cultures were synchronized in the ring stage (~12 h post-invasion) with 10 volumes of 5% (w/v) D-sorbitol [33].

2.3. Antiplasmodial activity assay

The [^{3}H]hypoxanthine incorporation assay was used to measure parasite viability in the presence or absence of the test compounds [34]. All experiments were initiated with the parasites in the late trophozoite stage (36–40 h post-invasion) with the parasitaemia set to 2% and the haematocrit set to 1%. The assay was set up in 96-well microtitre plates as described previously [35]. [^{3}H]Hypoxanthine (final concentration 2 μCi/mL) was added after 24 h and the parasite viability measured at 48 h [35].

2.4. Cytosolic pH measurements

The effect of the compounds on the parasite cytosolic pH was determined using the pH-sensitive fluorescent dye, BCECF. Saponin-isolated late trophozoite-stage parasites were loaded with the acetoxymethyl ester of BCECF as described elsewhere [8]. The parasites were then suspended in saline (120 mM NaCl, 5 mM KCl, 25 mM HEPES, 20 mM glucose and 1 mM $MgCl_2$, pH 7.1) in the absence or presence of varying concentrations of the compounds. All fluorometry experiments were performed at a parasite density of 5×10^7 cells/mL in a temperature controlled chamber set to 37 °C. In experiments designed to test the effect of a single concentration of the compounds on the pH of the parasite cytosol, the parasites were placed into a cuvette and loaded into a Perkin-Elmer LS 50B fluorometer (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). Using the "Fast Filter" accessory of the fluorometer, the sample is excited, in succession, at 440 nm and 490 nm and the fluorescence measured at an emission wavelength of 520 nm. In experiments designed to test the effect of multiple concentrations of the compounds on the pH of the parasite cytosol, the parasites were loaded (200 μL/well) into a 96-well microtitre plate and the change in fluorescence, measured at the wavelengths mentioned above, recorded in a FLUOstar Optima microplate reader (BMG Labtech, Durham, NC, USA). The ratio of the fluorescence measured at the dual wavelengths (490 nm/440 nm) provides a qualitative measure of the pH. For both experimental approaches, a pH calibration was performed in each experiment. The BCECF-loaded parasites were suspended in a high K^+ saline (130 mM KCl, 25 mM HEPES, 20 mM glucose and 1 mM $MgCl_2$) at a pH of 6.8, 7.1 and 7.8. The subsequent addition of 15 μM nigericin sets the intracellular pH to the pH of the extracellular solution. The equation derived from the linear regression of the plot between the intracellular fluorescence ratios at the different pH values was used to convert the experimental fluorescence ratio into intracellular pH.

2.5. Onset of action experiments

The contact time between the parasite and the compounds necessary for irreversible inhibition of parasite growth was investigated. At the start of the experiment the trophozoite-stage parasites were split into four samples for each of the time points to be tested (0.5, 1, 4 and 24 h). Then, 500 μL aliquots from each parasite sample were mixed with equal volumes of 10 2-fold serial dilutions of the inhibitor to be tested (the final volume was 1 mL and the parasitaemia and haematocrit were 2% and 1%, respectively). After 30 min at 37 °C the first set of parasites exposed to the 10 inhibitor concentrations was centrifuged (10,000 × *g*, 30 s) and the supernatant removed. The parasites were then washed twice in the same volume (1 mL) of drug-free culture medium. The parasites were subsequently suspended in 1 mL of drug-free medium and 200 μL aliquots were removed from each dilution and plated in triplicate in a 96-well microtitre plate. The procedure was then repeated at the remaining three time points. At 24 h (i.e., immediately after the last time point had been added to the 96-well plate), [^{3}H]hypoxanthine (final concentration 2 μCi/mL) was added to each well and incubated for an additional 24 h. The plates were then incubated overnight at −20 °C to freeze the contents and lyse the cells. After thawing, the nucleic acids were harvested onto glass fibre filters and the incorporated [^{3}H]hypoxanthine was measured in a microplate scintillation counter. In each experiment and for each of the four time points, a drug-free control was included where the parasites were treated in exactly the same way as the drug-treated parasites.

2.6. Drug combination studies

The in vitro antiplasmodial activity of a number of antimalarial compounds was tested in combination with two candidate proton pump inhibitors, 12192 and 50010, using fixed-ratio isobolograms [36]. The assay was set up as described previously [37]. Each assay was initiated with parasites in the trophozoite stage and the parasite viability determined 48 h later using the [^{3}H]hypoxanthine incorporation assay.

The fractional inhibitory concentrations (FICs) were calculated for each drug in a particular combination as follows: FIC (A) = IC_{50} for A in a combination/IC_{50} for A alone; FIC (B) = IC_{50} for B in the combination/IC_{50} for B alone. The isobolograms were constructed by plotting FIC (A) versus FIC (B) for each combination. It is generally accepted that a drug combination is synergistic if the FIC values are lower than 0.5 and antagonistic if they are greater than 2 [38] or perhaps even 4 [39,40]. The nature of the interaction between drugs with an FIC of between 0.5 and 2 (or 4) should be considered as indifferent [40].

2.7. Screening the V-type H^+-ATPase inhibitors in vivo

The activity of the compounds in vivo was determined in a murine malaria model using the standard 4-day suppression test. Briefly, female BALB/c mice (8–9 weeks old) were infected intraperitoneally with 200 μL of 10^7 *Plasmodium vinckei vinckei* parasites suspended in saline. Approximately 2 h later the compounds were administered either intraperitoneally or orally by gavage to groups of randomly selected mice. Compounds 12192, 12193, 12194, 15063, 24275 and 11383 were tested at concentrations ranging from 15 mg/kg/day to 60 mg/kg/day. Treatment with the compounds was continued for the following 3 days. All mice were weighed daily. The day after the last dose was administered, blood smears were made to monitor the parasitaemia. The parasitaemia was monitored daily thereafter and when the parasitaemia exceeded 25%, the mice were euthanised. Approval to carry out the mouse experiments was granted by the Animal Experimentation Ethics Committee of The Australian National University (Protocol No.: F.BMB.31.07).

2.8. Statistics

p-Values were determined using Student's two-tailed *t*-test for paired or unpaired samples, as appropriate.

3. Results

3.1. Antiplasmodial activity

We screened 30 compounds against at least 2, but as many as 4, different strains of *P. falciparum* with a range of sensitivities to chloroquine (Table 2). As controls we also tested

Table 2
The in vitro antiplasmodial activity of the indole derivatives of bafilomycin A_1 against 2 CQ^S (D10, 3D7) and 2 CQ^R (7G8, K1) strains of *P. falciparum*. For comparison the IC_{50} values of the classic proton pump inhibitors concanamycin A and bafilomycin A_1 as well as the quinoline antimalarial chloroquine are provided.

Compound	Class	*Plasmodium falciparum* strains			
		CQ^S		CQ^R	
		3D7	D10	7G8	K1
		IC_{50} values (nM)			
Chloroquine		15.9 ± 2	17.8 ± 3	118 ± 14	138 ± 12
Bafilomycin A_1		19.1 ± 1	19.8 ± 3	12.3 ± 3	17.9 ± 2
Concanamycin A		0.89 ± 0.1	0.76 ± 0.1	0.55 ± 0.1	0.67 ± 0.02
		IC_{50} values (μM)			
11383	A	0.51 ± 0.11	0.52 ± 0.08	0.49 ± 0.03	0.54 ± 0.04
21098	A	0.41 ± 0.02	0.43 ± 0.03	0.45 ± 0.12	0.48 ± 0.07
50008	A	1.08 ± 0.12	NT	NT	0.69 ± 0.03
50009	A	0.58 ± 0.01	NT	NT	0.59 ± 0.01
50010	A	0.33 ± 0.01	NT	NT	0.36 ± 0.03
12192	B	0.24 ± 0.03	0.20 ± 0.01	0.41 ± 0.08	0.39 ± 0.02
12193	B	0.62 ± 0.06	0.70 ± 0.04	0.64 ± 0.13	0.42 ± 0.06
15038	B	0.55 ± 0.09	0.34 ± 0.03	1.4 ± 0.3	1.4 ± 0.4
15114	B	0.55 ± 0.04	1.9 ± 0.4	0.74 ± 0.04	2.8 ± 1.1
15115	B	0.41 ± 0.06	0.39 ± 0.06	0.50 ± 0.02	0.71 ± 0.04
25092	B	4.26 ± 0.48	NT	NT	0.79 ± 0.09
25216	B	6.19 ± 0.30	NT	NT	2.72 ± 0.13
25221	B	2.24 ± 0.20	NT	NT	1.40 ± 0.14
25222	B	5.20 ± 0.02	NT	NT	3.03 ± 0.40
25223	B	0.98 ± 0.03	NT	NT	1.17 ± 0.05
25335	B	2.13 ± 0.34	NT	NT	1.39 ± 0.15
25337	B	>10	NT	NT	>10
12194	C	0.16 ± 0.02	0.21 ± 0.02	0.38 ± 0.10	0.34 ± 0.01
16246	C	0.86 ± 0.12	NT	NT	0.45 ± 0.02
22434	C	5.7 ± 0.8	4.7 ± 0.4	4.8 ± 1.3	3.9 ± 0.7
11384	D	>10	>10	>10	>10
11382	E	>10	>10	4.5 ± 0.3	8.2 ± 1.8
14121	E	>10	6.4 ± 0.5	3.8 ± 0.4	5.6 ± 0.5
14122	E	>10	>10	6.2 ± 0.4	>10
14135	E	>10	>10	>10	>10
14136	E	>10	>10	3.2 ± 0.7	>10
15063	E	>10	5.0 ± 0.6	3.3 ± 0.2	4.2 ± 0.5
15081	E	>10	>10	>10	>10
15083	E	>10	>10	>10	>10
17801	E	>10	>10	3.9 ± 0.2	5.1 ± 1.0

The IC_{50} values for chloroquine, concanamycin A and bafilomycin A_1 are presented as nM concentrations while the derivatives are shown as μM concentrations. All IC_{50} values represent the mean ± S.E.M. of at least 3 independent experiments each performed in triplicate. >10 indicates that the IC_{50} value exceeded the highest concentration tested (10 μM). NT indicates that the derivatives were not tested in these strains.

the antiplasmodial activity of bafilomycin A_1 and concanamycin A against these strains. As expected, both bafilomycin A_1 and concanamycin A were potent inhibitors of parasite growth, although the antiplasmodial activity of bafilomycin A_1 was somewhat better than what has been reported previously [22]. As reported previously [21,41] concanamycin A was highly active and had a sub-nanomolar IC_{50} against all the strains tested. A few (5) of the analogues did not inhibit parasite growth by more than 50% at the highest concentration tested (10 μM). Another 5 had IC_{50} values between 1 and 10 μM and 9 had IC_{50} values below 1 μM. The remaining 11 compounds had variable IC_{50} values among the strains tested thereby making it difficult to categorise them accurately within the concentration ranges described above. Most analogues were equally effective against both CQ^S and CQ^R strains (e.g., 11383, 21098, Table 2; $p \geq 0.38$) but some were more active (2–5-fold) against CQ^R parasites (e.g., 25092, 25216, Table 2; $p \leq 0.01$). Furthermore, some inhibitors appeared to be more effective (~2-fold) against CQ^S parasites (e.g., 12192, 12194, Table 2), though this difference was only statistically significant when the antiplasmodial activity of these compounds against the CQ^S strains was compared with their activity against the CQ^R K1 strain ($p \leq 0.01$). Among the most active analogues were compounds 12192 and 50010 with IC_{50} values in the range of 250–400 nM.

3.2. Effect on cytosolic pH

We next investigated whether these inhibitors were able to affect the parasite's ability to maintain a stable cytosolic pH. Saponin-permeabilised *P. falciparum*-infected erythrocytes were loaded with the fluorescent pH indicator BCECF and the fluorescence monitored in a fluorometer. Fig. 1A shows the effect of 12192 (20 μM) on the resting pH of the parasite cytosol; a 0.5–0.6 pH unit reduction in the cytosolic pH within 20 min. A similar effect was observed with concanamycin A (50 nM) and a number of the other analogues (data not shown). A solvent control (DMSO; 0.1%) was also included and had no effect (data not shown).

3.3. Correlation between cytosolic pH abrogation and antiplasmodial activity

Although a number of the analogues clearly possessed antiplasmodial activity (Table 2) and were able to inhibit the parasite's ability to maintain a near-neutral cytosolic pH (Fig. 1A), the disruption of pH homeostasis by the compounds is not necessarily related to their antiplasmodial activity. To test this we generated concentration–response curves for the effect of the compounds on cytosolic pH disruption. The maximum pH decrease observed following a 60 min exposure to the various concentrations tested was plotted against the concentration used (Fig. 1B). A 60 min exposure was chosen to reduce the possibility of non-specific effects on parasite health. Similar curves to that shown in Fig. 1B were generated for several of the analogues and the classic V-type H^+-ATPase inhibitors, concanamycin A and bafilomycin A_1. The IC_{50} values for cytosolic pH abrogation were plotted against the IC_{50} values for parasite growth inhibition (Fig. 1C). There is a strong correlation ($R^2 = 0.978$) between the ability of the inhibitors to disrupt parasite cytosolic pH and inhibit parasite growth, consistent with the compounds killing the parasites by interfering with the parasite's ability to regulate its pH.

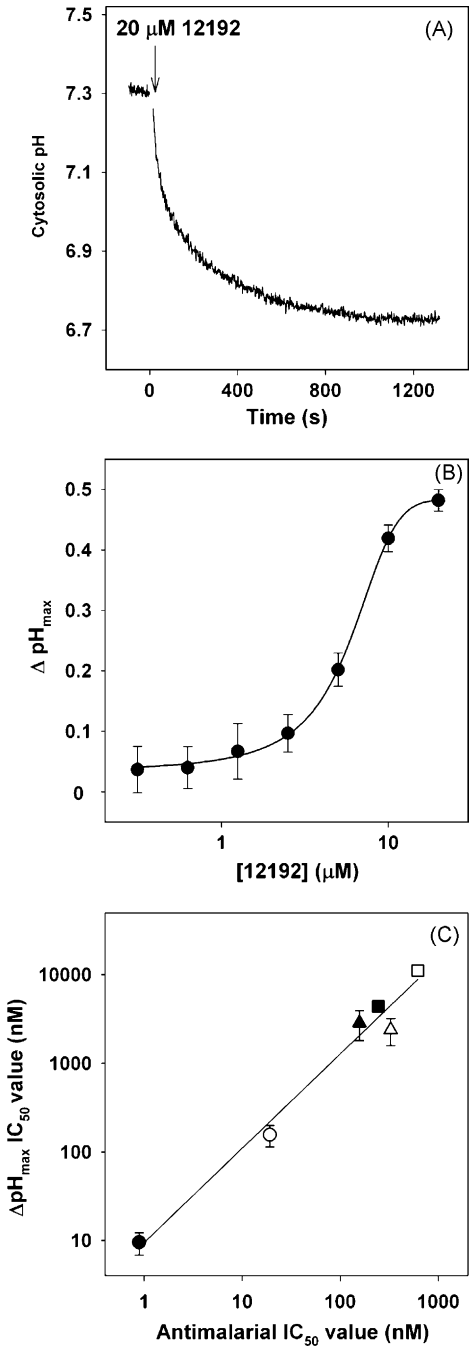


Fig. 1. Correlation between the antiplasmodial activity of and cytosolic pH disruption by the V-type H^+-ATPase inhibitors. (A) Effect of 12192 (20 μM) on the resting cytosolic pH of the 3D7 *P. falciparum* parasites. (B) The effect of different concentrations of 12192 on cytosolic pH expressed as the maximum change in cytosolic pH. IC_{50} values determined from curves such as this one for a number of different H^+-pump inhibitors are plotted in (C) as a function of their antiplasmodial IC_{50}. (C) The linear correlation between antimalarial activity and cytosolic pH change (correlation coefficient; $R^2 = 0.978$). The inhibitors are concanamycin A (filled circle), bafilomycin A_1 (empty circle), 12192 (filled square), 12193 (empty square), 12194 (filled triangle) and 50010 (empty triangle). Values are averaged from at least 3 independent experiments, ± S.E.M. X and Y error bars are included in (C) and, where not visible, are smaller than the symbols.

3.4. Onset of action

To establish the contact time required to inhibit the parasite proliferation to the same extent as that seen in the 48 h assay (Table 2), the parasites were exposed to a range of inhibitor concentrations for various time periods. Fig. 2 shows that high concentrations of compound 12192 (2–4 μM) kill 100% of the parasites after only a 30 min exposure. The antimalarial potency of the 12192 increased with a longer exposure to the parasites as demonstrated by the curves shifting to the left (Fig. 2). The IC_{50} value was determined for each period of exposure and compared

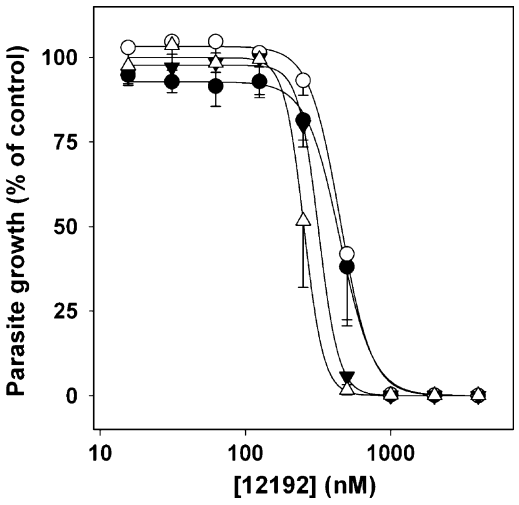


Fig. 2. Effect of compound 12192 exposure time on parasite viability. Parasites were exposed to various concentrations of compound 12192 for 0.5 h (filled circles), 1 h (empty circles), 4 h (filled triangles) or 24 h (empty triangles). The parasites were then washed twice and resuspended in drug-free growth media. Parasite growth was measured at 48 h using the [^{3}H]hypoxanthine incorporation assay. The curves represent the average of 3 independent experiments. Error bars show S.E.M. For clarity, only negative error bars have been included.

with the IC_{50} value obtained after a 48 h exposure to the drug (Table 3). From the data presented in Table 3 it is evident that the inhibitor 12192 is both potent and rapid-acting. After an only 30 min exposure, 12192 had an IC_{50} value that was only slightly higher (2-fold; $p = 0.03$) than the IC_{50} value found after a 48 h exposure. After a 4 h exposure, 12192 had an IC_{50} value that was statistically indistinguishable ($p = 0.09$) from that seen after a 48 h exposure. The full antiplasmodial effect of 50010 manifested slower, with a 24 h period required to exert an effect comparable to that seen after 48 h. Again there was no significant difference in the IC_{50} values for compound 50010 at 24 h and 48 h ($p = 0.89$). Surprisingly, after a full 24 h exposure to the highly potent inhibitor bafilomycin A_1, the IC_{50} value was still twice as high as the IC_{50} value after a 48 h exposure. Similar to compound 12192, the antimalarial chloroquine exerted its maximal antiproliferative effect within 4 h, with no significant difference observed between the IC_{50} values obtained after a 4 h or 48 h exposure ($p = 0.14$).

3.5. Interaction with antimalarial drugs

We used fixed-ratio isobolograms to examine the in vitro antiplasmodial interaction of two of the proton pump inhibitors, 12192 and 50010, with the standard antimalarials chloroquine, quinine and artemisinin (Fig. 3). The interaction of both 12192 and 50010 with each of the 3 antimalarials was similar. When the inhibitors were combined with the two quinoline-based antimalarials, chloroquine and quinine (Fig. 3A and B), all the FIC values fell within the range 1.0–1.5. Although the trend was towards antagonism, none of the FIC values exceeded a value of 2.0 which is regarded as a cut-off for antagonism [40]. When combined with artemisinin (Fig. 3C), 12192 and 50010 FIC values fell within a narrower range of between 1.0 and 1.3. Again these FIC values represent an indifferent interaction between the inhibitors and the standard antimalarials.

3.6. In vivo activity

In addition to the in vitro screens against *P. falciparum*, we have also screened several of the compounds in vivo against *P. vinckei vinckei* in a murine model of malaria. Six inhibitors were selected (12192, 12193, 12194, 15063, 24275 and 11383) and were examined in the standard 4-day suppression test [35] at single daily doses ranging from 15 mg/kg/day to 60 mg/kg/day, administered either orally or intraperitoneally on 4 consecutive days. None of the inhibitors reduced the parasitaemia of the mice when compared to drug-free control mice (data not shown). Furthermore, some of the compounds exhibited toxicity towards the mice (weight loss, reduced activity, rough coat).

4. Discussion

4.1. The V-type H^+-ATPase is essential for the intraerythrocytic stage of *P. falciparum*

The V-type H^+-ATPase is crucial in regulating the pH of the *P. falciparum* parasite cytosol [8], the digestive vacuole [42] and may contribute to pH regulation in the infected red blood cell cytosol [7]. In addition, it is responsible for generating the membrane potential that plays a role in the uptake of essential nutrients by the parasite [12–15]. Using V-type H^+-ATPase inhibitor candidates based on the active core structure of bafilomycin A_1, we have identified 9 compounds that kill malaria parasites at sub-micromolar concentrations and provide data consistent with them doing so through the disruption of the parasite's intracellular pH.

The malaria parasite was initially reported [43], and again recently [44], to regulate its cytosolic pH by a Na^+/H^+ exchanger. Suggestions have also been made that two V-type H^+-pyrophosphatases (one K^+-dependent, the other K^+-independent) may also play a role in parasite pH regulation [42,45–47]. The suggestion that a Na^+/H^+ exchanger plays a role in cytosolic pH regulation [43,44] has been refuted in the past [6,8] and more recently by Spillman et al. [9], and although a pyrophosphate-dependent DV-acidifying mechanism has been demonstrated [42], whether the H^+-pyrophosphatases play any role in cytosolic pH regulation is still unclear. If, as we postulate, the compounds tested in this study are targeting the *P. falciparum* V-type H^+-ATPase, then the data presented in this study, in particular the strong correlation between the antiplasmodial activity of the H^+-pump inhibitors and their effect on the cytosolic

Table 3
The contact time required for the V-type H^+-ATPase inhibitors to inhibit *P. falciparum* parasite proliferation in vitro.

Parasite growth inhibitor	IC_{50} values (nM)				
	Contact time				
	0.5 h	1 h	4 h	24 h	48 h
12192	453 ± 68 (1.8)	452 ± 74 (1.8)	317 ± 8 (1.3)	269 ± 56 (1.1)	245 ± 28 (1.0)
50010	5254 ± 802 (16)	2919 ± 303 (8.9)	576 ± 22 (1.8)	336 ± 61 (1.0)	327 ± 14 (1.0)
Bafilomycin A_1	>200 (>10)	>200 (>10)	92 ± 24 (4.8)	38 ± 4 (2.0)	19 ± 1 (1.0)
Chloroquine	155 ± 38 (9.7)	39 ± 6 (2.4)	22 ± 4 (1.4)	12 ± 2 (0.8)	16 ± 2 (1.0)

The IC_{50} values are expressed as nM concentrations and are the mean ± S.E.M of at least 3 experiments each performed in triplicate. >200 indicates the IC_{50} value exceeded the highest concentration tested (200 nM). The values reported in parentheses show the fold-increase in the IC_{50} values following an exposure to the compounds for the indicated times when compared to those obtained following a 48 h incubation in the presence of the inhibitors.

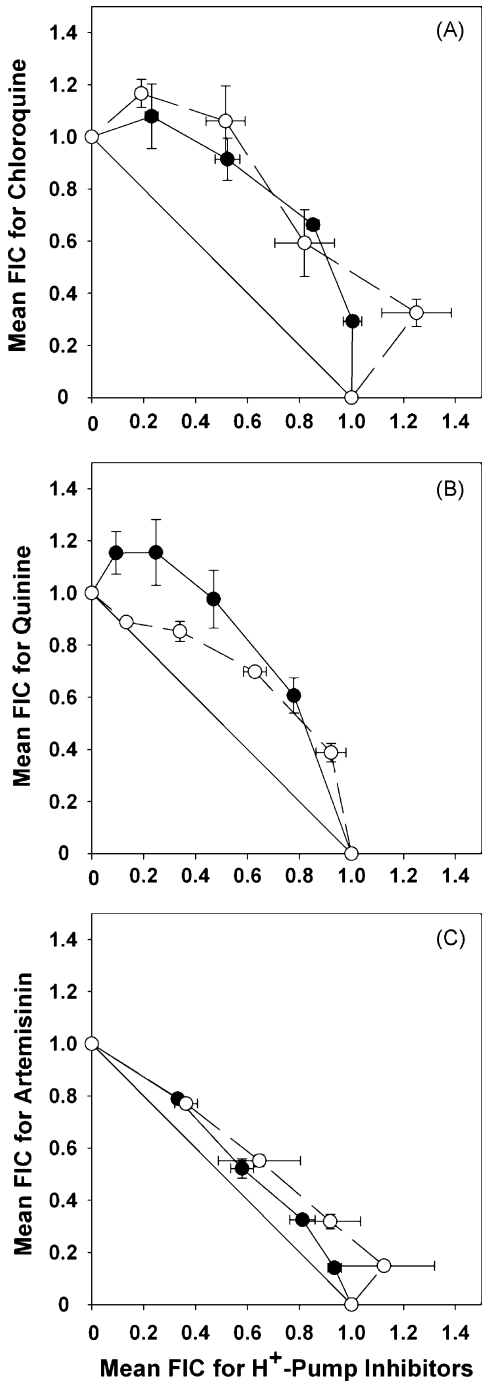


Fig. 3. Isobolograms showing the interaction of 2 of the inhibitors 50010 (empty circles) or 12192 (filled circles) with the standard antimalarials chloroquine (A), quinine (B) or artemisinin (C) performed on the CQ^S 3D7 strain. Axes represent the mean fractional inhibitory concentration (FIC) for the compounds tested. The straight line represents a hypothetical indifferent interaction. FIC values need to be below 0.5 or above 2–4 in order to represent synergism or antagonism, respectively [39,40]. The data are averaged from 3 independent experiments. Error bars represent S.E.M., and where not visible, are smaller than the symbols.

pH, are consistent with these alternate pH-regulatory mechanisms (if they do play a role in cytosolic pH regulation) being insufficient to compensate for the V-type H^+-ATPase activity and do not allow the parasite to survive if the proton pump is inhibited. We would therefore suggest that, at least in vitro, the V-type H^+-ATPase is the main cytosolic pH-regulating mechanism in *P. falciparum* and that strategies designed to block its activity would make for promising antimalarial approaches.

From the antiplasmodial activity reported in Table 2 it is evident that inhibitors belonging to the first three structural classes, i.e., indolepentadiene amides (Class A), indole benzamides (Class B) and benzoimidazole benzamides (Class C), have, in general, a similar inhibitory potency against all the CQ^S and CQ^R parasite strains tested. Compound 11384, the only benzoimidazole acetamides (Class D, Table 1), was prepared as a representative of the V-type H^+-ATPase inhibitors designed and characterised by Astellas Pharma [32], and showed no antimalarial activity at the highest concentration tested even though it was a sub-micromolar inhibitor of macrophage V-type H^+-ATPase [32]. Surprisingly, 3-phenylindoles belonging to class E showed an inhibitory preference for some strains of the parasite, in particular those resistant to chloroquine. Whether this effect is due to a greater affinity of these compounds for the V-type H^+-ATPase present in CQ^R parasites or is due to a difference between these strains that is unrelated to the proton pump remains to be investigated.

4.2. V-type H^+-ATPase inhibition results in rapid parasite death

The progression of a malaria infection occurs very rapidly within its human host and most deaths from severe malaria will occur within 24 h of starting treatment [48]. It is therefore of utmost importance that treatment be administered as soon as possible after the symptoms are identified. It is also critical that the drug treatment acts rapidly to clear the parasites before neurological symptoms develop [48]. A previous study comparing the onset of the antiplasmodial effect of chloroquine, quinine, artemisinin and sodium artelinate [49] found that chloroquine had the most rapid onset of action [49]. We compared the onset of action of the V-type H^+-ATPase inhibitors with chloroquine (Table 3). The time of contact necessary to obtain almost maximal antimalarial activity (as measured using IC_{50} values) for 12192 was approximately 4 h. However, it should be noted that at higher concentrations (2–4 μM) a complete parasite kill (i.e., 100% inhibition of parasite proliferation) can be attained within 30 min (Fig. 2). Surprisingly, there was variability in the contact time necessary for maximal parasite killing by the V-type H^+-ATPase inhibitors (Table 3). Bafilomycin A_1 (100 nM) was shown previously to decrease the pH of the parasite cytosol by 0.5–0.6 units within 30 min [8], similar to the effect observed for 12192 (20 μM) in Fig. 1A. Yet the data in Table 3 suggests that the inhibitory effect of bafilomycin A_1 is reversible since complete parasite killing was not observed after washing the drug off at 30 min and 1 h, even when the drug was present at 200 μM. Compound 50010 took 24 h to exert its maximal antimalarial effect, although again it should be noted that a 100% parasite kill was observed at 30 min (as with 12192) at higher concentrations (data not shown). This evidence is consistent with the different inhibitors interacting with the V-type H^+-ATPase with different affinities and that some of the compounds (like 12192) are perhaps more tightly bound than the other candidates tested. The rapid onset of activity of compound 12192 is similar to that observed for chloroquine which was shown previously to act more rapidly than artemisinin and quinine [49].

4.3. Standard antimalarials combined with V-type H^+-ATPase inhibitors act indifferently

With resistance having been reported to almost all currently used antimalarials, combination therapies have been adopted to delay the onset of resistance to new antimalarial drugs used to treat malaria [50,51]. Disruption of the cytosolic and digestive vacuole pH might be expected to alter the uptake of certain

antimalarials and thereby reduce their potency. This is especially relevant to certain quinoline-based compounds (e.g., chloroquine) that accumulate in the parasite's acidic digestive vacuole in part because of the weak base effect. An altered pH might also be expected to disrupt the function of many enzymes, which in turn could disrupt the activity of antimalarials that target enzymes (e.g., artemisinin which has been proposed to target the *P. falciparum* SERCA; [52]). However, we show a lack of antagonism and an indifferent in vitro interaction of the 3 antimalarials tested in combination with 2 (12192 and 50010) of the V-type H^+-ATPase inhibitors (Fig. 3). This indifferent interaction is similar to the results reported previously for a combination of concanamycin A with pyronaridine, a novel antimalarial that accumulates and acts in a manner similar to chloroquine [21]. Whether these results can be extended to in vivo models of malaria remains to be tested.

4.4. Lack of effect of V-type ATPase inhibitors in vivo in a mouse malaria model

The reason for the lack of in vivo antimalarial activity is unclear. One possibility is that the pharmacokinetic properties of the compounds and the administration regime used (i.e., single daily doses) are inadequate to maintain the compounds within the therapeutic range for a time sufficiently long to kill the parasites. Administering the inhibitors in more frequent, yet smaller, doses might overcome this problem. It might also overcome some of the toxicity issues, as the smaller doses might prevent toxic plasma concentrations of the inhibitors. Another possibility is that murine malaria is insensitive to these novel V-type H^+-ATPase inhibitors or possibly that these parasites have alternative pH-regulating mechanisms (e.g., the Na^+/H^+ exchanger and/or the H^+-pyrophosphatases mentioned above) that are able to compensate for the inhibition of the V-type H^+-ATPase. Additional studies will need to be performed to establish the reason for the lack of effect in this mouse malaria model.

4.5. Concluding remarks

We report on the activity of a series of novel V-type H^+-ATPase inhibitors. Although inhibition of targets other than the V-type H^+-ATPase (e.g., those involved in ATP generation) cannot be excluded, we propose that the nanomolar antimalarial activity of some of these inhibitors, coupled with their rapid effect at inhibiting both pH regulation and parasite proliferation, warrant further investigation of the V-type H^+-ATPase as an antimalarial drug target.

In addition to the macrolide antibiotics (e.g., bafilomycins and concanamycins), there are two other classes of compounds that have been shown to inhibit V-type H^+-ATPases. Benzolactone enamides (e.g., salicylihalamide and lobatamide A) inhibit mammalian V-type H^+-ATPases at nanomolar concentrations but have very little effect at inhibiting the fungal V-type H^+-ATPase [53]. The chondropsins are polyketide-derived macrolide lactams isolated from marine sponges. These compounds, although less active than the other classes at inhibiting the proton pump, are more selective against the fungal V-type H^+-ATPase than against the mammalian equivalent [54]. This varying selectivity of V-type H^+-ATPase inhibitors raises the possibility that compounds may be identified which selectively inhibit plasmodial proton pumps in preference to the host counterpart. Given the heavy reliance of the parasite on its V-type H^+-ATPase to maintain both the cytosolic and digestive vacuole pH of the intaerythrocytic stage of its growth (at least in vitro), the identification of new, potent and selective inhibitors of the plasmodial proton pump that are active in vivo should provide a novel therapeutic approach in the treatment of malaria.

Conflict of interest

PM, SG and CF were/are employees of NiKem Research, the company that used to own the intellectual property of the novel compounds tested in this study. NiKem Research also provided some financial support for this project. Rights relating to some of these novel compounds now belong to a third party that has not been involved in this study.

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