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Discovery of antiplasmodial pyridine carboxamides and thiocarboxamides

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ABSTRACT

Malaria continues to be a significant burden, particularly in Africa, which accounts for 95% of malaria deaths worldwide. Despite advances in malaria treatments, malaria eradication is hampered by insecticide and anti-malarial drug resistance. Consequently, the need to discover new antimalarial lead compounds remains urgent. To help address this need, we evaluated the antiplasmodial activity of twenty-two amides and thioamides with pyridine cores and their non-pyridine analogues. Twelve of these compounds showed *in vitro* anti-proliferative activity against the intraerythrocytic stage of *Plasmodium falciparum*, the most virulent species of *Plasmodium* infecting humans. Thiopicolinamide **13i** was found to possess submicromolar activity (IC_{50} = 142 nM) and was >88-fold less active against a human cell line. The compound was equally effective against chloroquine-sensitive and -resistant parasites and did not inhibit β-hematin formation, pH regulation or *Pf*ATP4. Compound **13i** may therefore possess a novel mechanism of action.

1. Introduction

In humans, malaria is caused by parasites of the genus *Plasmodium* namely *P. falciparum*, *P. vivax*, *P. malariae*, *P. knowlesi*, *P. ovale curtisi* and *P. ovale wallikeri* (Singh et al., 2004; Sutherland et al., 2010). This infectious disease, transmitted by female *Anopheles* mosquitoes, was responsible for 249 million deaths and 608,000 deaths worldwide in 2022 (WHO, 2023). Africa accounted for 94% of the malaria cases in 2022 with pregnant women and children under five years being the most impacted by the disease (WHO, 2023). *P. falciparum*, the predominant parasite in this region, is responsible for most of the deaths from malaria (WHO, 2023). Currently, the cornerstone of antimalarial chemotherapy are artemisinin-based combination therapies (ACTs), in which a derivative of artemisinin (**1**) is paired with a longer-acting antimalarial drug. The spread of parasite resistance to ACTs in the Southeast Asian Western Cambodia region, nonetheless, highlights the continued need to discover and develop antimalarial medicines (Noedl et al., 2008). This is critical in the fight against malaria, which is being undermined both by parasite resistance to antimalarial drugs and mosquito resistance to insecticides (Ranson and Lissenden, 2016). Therefore, new drug leads, preferably with mechanisms of action different from existing drugs, are needed for possible use in combination therapy.

Quinine (**2**), mefloquine (**3**) and chloroquine (**4**) are antimalarials used clinically that contain a quinoline ring (see Fig. 1 for structures). Quinine (**2**), originally isolated from the bark of *Cinchona ledgeriana* and *Cinchona succirubra*, was first shown to possess antimalarial activity and was later modified to create mefloquine (**3**) and chloroquine (**4**) (Cragg and Newman, 2013; Christensen, 2015; Jones et al., 2015). Quinine (**2**), mefloquine (**3**) and chloroquine (**4**) kill *P. falciparum* via inhibition of hemozoin formation (Sullivan et al., 1996).

The formation of hemozoin permits the malaria parasite to avoid the toxicity of the free heme produced as a consequence of the digestion of host cell hemoglobin (Pandey et al., 2003). Despite the effectiveness of chloroquine and its success as an antimalarial drug, it has become largely useless against *P. falciparum* as a result of parasite resistance (Duraisingh and Cowman, 2005).

Methylene blue (**5**), which has a benzothiazine core, also possesses antiplasmodial activity (Vennerstrom et al., 1995; Ehrhardt et al., 2013).

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Its mechanism of action involves both inhibition of *P. falciparum* glutathione reductase and, like the quinolines, inhibition of hemozoin formation (Vennerstrom et al., 1995; Färber et al., 1998). Inhibition of hemozoin formation and glutathione reductase results in increased oxidative stress (Becker et al., 2004; Percário et al., 2012). A nitrogen acyclic core is present in antimalarial drugs such as proguanil (**6**) and halofantrine (**7**) (Greenwood, 1995). Both continue to be used clinically. Proguanil (**6**) is effective for both prophylaxis and treatment while halofantrine (**7**) is used for treatment only (Radloff et al., 1996; McKeage and Scott, 2003). However, the use of both drugs is under threat with the emergence of resistant strains (Høgh et al., 2000). The preparation of new antimalarial agents bearing a quinoline core remains a feasible strategy for the development of new antimalarial compounds for use in combination therapy amidst the widespread quinoline resistance. This is because the process by which the parasite forms hemozoin is presumably unchanged in quinoline-resistant parasites (Wicht et al., 2016). Non-quinoline benzamides have also been shown to inhibit β-hematin (synthetic hemozoin) formation (Wicht et al., 2016). Recently, arterolane (**8**), which contains the trioxolane skeleton and amide functionality (Fig. 1), has been shown to possess excellent activity against the erythrocytic stages of *P. falciparum* and *P. vivax* (Fontaine et al., 2015; Toure et al., 2016). Furthermore, 2-acetylpyridine-4-phenyl-3-thiosemicarbazone (**9**), which bears a pyridine core, has shown antiplasmodial activity (Marella et al., 2015). Wicht et al. (2016) also reported that electron-deficient and pyridyl-containing amides display antiplasmodial activity. Given the prevalence of the quinoline/pyridine and amide/thioamide moieties in antiplasmodial compounds, we hypothesized that the series of pyridine carboxamides and thio derivatives generated in our lab while exploring electronic and steric effects of picolinamides and related systems on thionations using Lawesson's reagent, could be expanded and evaluated as potential antiplasmodial agents. The objective of this study was therefore to synthesize a series of pyridine carboxamides as well as their thiocarboxamides and to assess their antiplasmodial activity against *P. falciparum*.

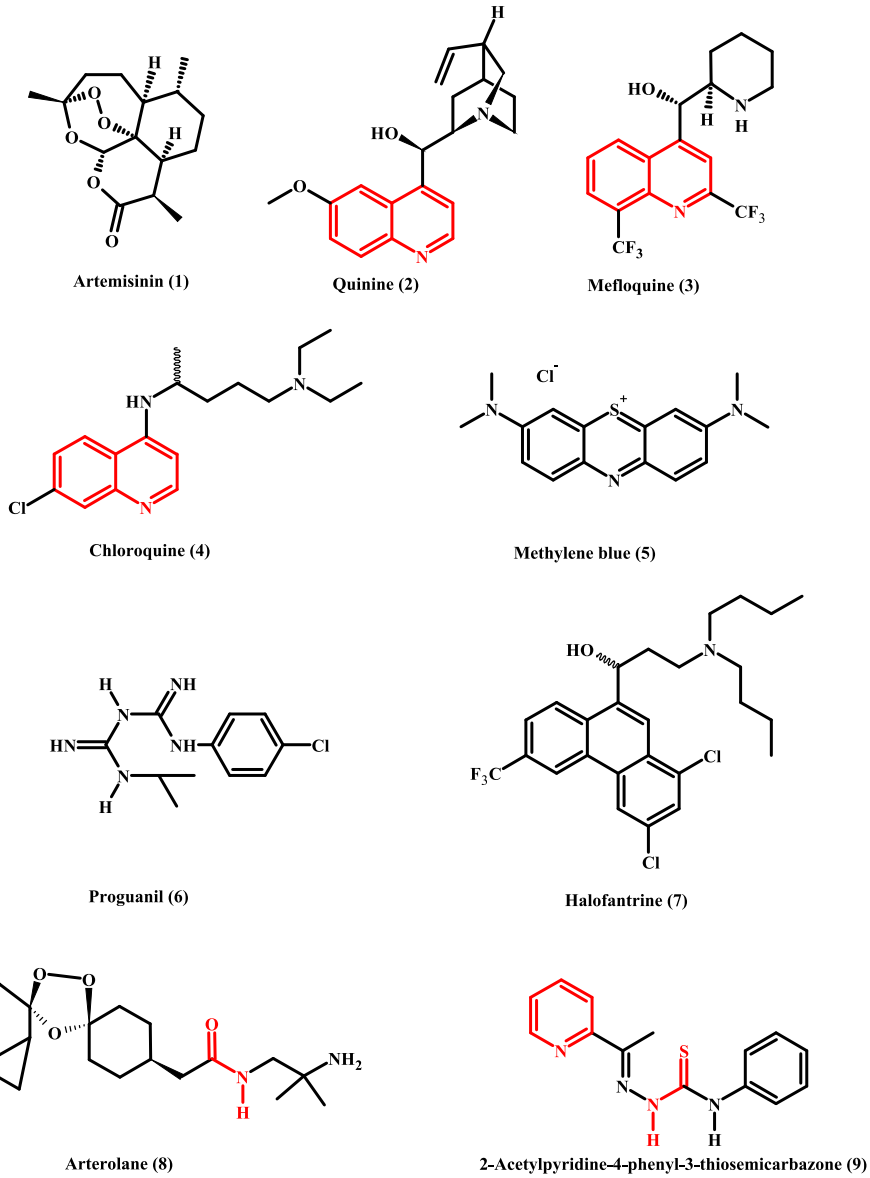


Fig. 1. Antimalarial agents: Artemisinin (**1**), Quinine (**2**), Mefloquine (**3**), Chloroquine (**4**), Methylene blue (**5**), Proguanil (**6**), Halofantrine (**7**), Arterolane (**8**), 2-acetylpyridine-4-phenyl-3-thiosemicarbazone (**9**). Quinoline, amide, thioamide and pyridine moieties are shown in red.

2. Methods

2.1. General information for drug synthesis

Nuclear magnetic resonance spectra were obtained using Bruker Avance 200 and 500 MHz instruments. Unless otherwise stated, the spectra were obtained in $CDCl_3$ and resonances are reported in δ units downfield from tetramethylsilane (TMS), which was used as an internal standard; *J* values are given in Hz. Multiplicities are described as follows: s = singlet, d = doublet, dd = doublet of doublets, ddd = doublet of doublet of doublets, t = triplet, q = quartet, m = multiplet. FT-IR spectra were obtained on a Vector 22 or Bruker Tensor 37 with Pike Technology MIRacle single reflection ATR instruments. Column chromatography was carried out using silica gel as an adsorbent. Elemental analyses were done at the University of the West Indies, Mona on a PE 2400 CHNS/O Analyzer or on a Thermo Flash EA112 CHN Analyzer at MEDAC Ltd., Surrey, United Kingdom. HR-MS analyses were carried out on a Waters LCT Premier (Es-Tof)/Acquiring i-Class instrument at MEDAC Ltd., Surrey, United Kingdom. Melting points (uncorrected) were determined on a Gallenkamp instrument. Microwave reactions were carried out using an Anton Paar Synthos 3000 reaction system. Specific details about the synthesis of compounds **12a-l** and **13a-l** are available in the supporting information.

2.2. General considerations for in vitro assays

All compounds were dissolved in DMSO to a concentration of 200 mM, except for compounds **12a**, **12d**, **12i** and **13c**, which exhibited lower solubility in DMSO. These compounds were dissolved in DMSO to concentrations between 10 and 100 mM. All compounds were subsequently diluted in culture medium (in some cases after a further dilution in DMSO), with the final concentration of DMSO present in assays never exceeding 0.1% (v/v).

2.3. Cell culture

Synchronous intraerythrocytic stage *P. falciparum* parasites (strains 3D7 and Dd2) and human foreskin fibroblast (HFF) cells were maintained *in vitro* as described previously (Jacot et al., 2014; Guan et al., 2021). Briefly, the *P. falciparum* parasites were maintained within O^+ human erythrocytes, suspended in HEPES- and GlutaMAX™-supplemented RPMI 1640 (Thermo Fisher Scientific, order number 72400120) to which 11 mM D-glucose, 200 μM hypoxanthine, 24 mg/L gentamicin and 0.6% (w/v) Albumax II were added. The HFF cells were maintained in DMEM (Sigma-Aldrich, order number D5638) to which 10% (v/v) bovine calf serum, 50 units/mL penicillin, 50 mg/L streptomycin, 10 mg/L gentamicin, 0.2 mM L-glutamine, and 0.25 mg/L amphotericin B was added.

2.4. In vitro antiplasmodial assays

In vitro antiplasmodial activity against *P. falciparum* was assessed over 96 h using the malaria SYBR Green I-based fluorescence assay described by Smilkstein et al. (2004) with previously described modifications (Spry et al., 2013). Assays were initiated with parasites in the ring stage at a parasitemia and hematocrit of 0.5 and 1%. Infected erythrocytes incubated with 0.25 μM chloroquine served as zero proliferation controls. Following the 96 h incubation, assay plates were frozen and stored at −80 °C. Subsequent to thawing, the contents of each well were mixed, and a 100 μL aliquot transferred to a second plate containing 100 μL/well SYBR Safe DNA gel stain in 20 mM Tris, pH 7.5, containing 5 mM ethylenediaminetetraacetic acid (EDTA), 0.008% (w/v) saponin and 0.08% (v/v) Triton X-100. The fluorescence in each well was measured as previously described (Spry et al., 2013). Following subtraction of the average fluorescence measured in the zero proliferation control wells, fluorescence in the test-compound-containing wells was expressed as a percentage of the average background-corrected fluorescence measured in the no-inhibitor control wells. To obtain IC_{50} values, the processed data were fitted with the equation $y = \text{Bottom} + (\text{Top - Bottom})/(1+(IC_{50}/x)^{\text{Hill Slope}})$ using GraphPad Prism 9. Where incomplete inhibition was observed at the highest concentration, the 'bottom' was constrained to zero. IC_{50} values were compared following $\log_{10}$ transformation, by performing a one-way ANOVA with Tukey's multiple comparisons test.

2.5. In vitro HFF cell proliferation assays

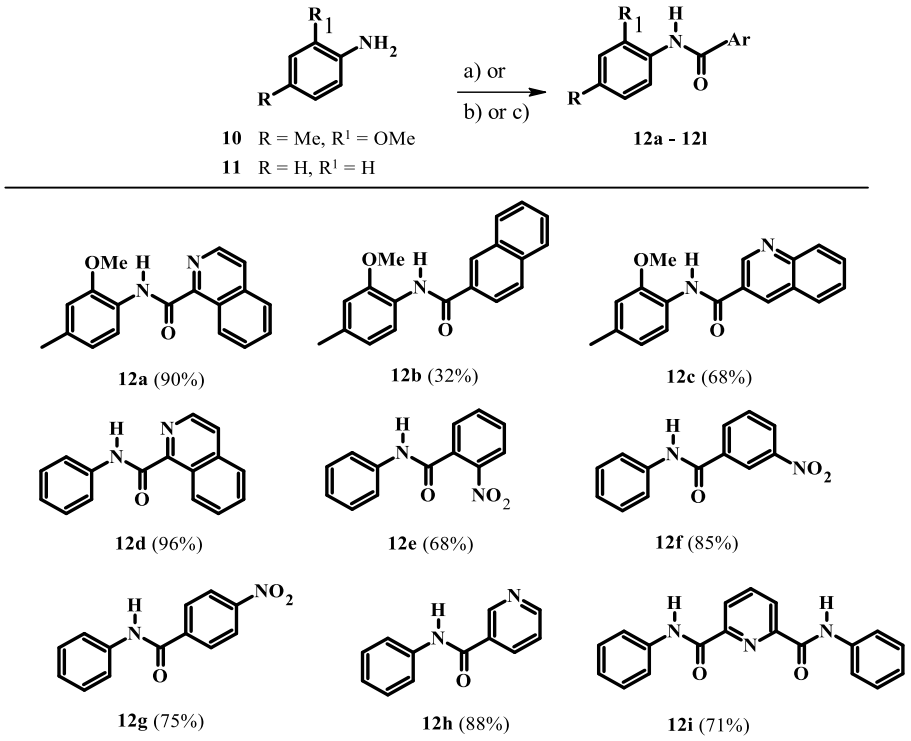
Activity against HFF cells was assessed over a period of four days using a SYBR Safe-based assay, essentially as previously reported (Hoegl et al., 2014; Spry et al., 2020). Briefly, assays were performed in 96-well plates, initiated with HFF cells seeded at 1.5–2.4 × 10^5 cells/mL. HFF cells incubated with the protein synthesis inhibitor cycloheximide (at 10 μM) served as zero proliferation controls. Plates were incubated at 37 °C in a humidified 5% CO_2 incubator. After 4 days, an aliquot of the supernatant (150 μL) was carefully removed from each well and discarded, prior to storing the plates at −80 °C. After thawing, the SYBR Safe lysis solution also used for the antiplasmodial assays (150 μL) was added to each well and mixed via pipetting to ensure the HFF cells were detached from the plate and lysed. The plates were then processed as described for the antiplasmodial assay.

2.6. Detergent mediated β-hematin assays

Activity against β-hematin formation was assessed using an assay reported previously (de Sousa et al., 2020). All reagents and buffers were purchased from Sigma-Aldrich (rebranded to Merck). Briefly, compound stocks were made up in either DMSO or MilliQ™ water to a final concentration of 20 mM. A working solution of 70% water/20% NP-40 substitute (305.5 μM)/10% DMSO v/v was prepared, of which 100 μL was added to columns 1 to 11 of a 96-well plate (Greiner). To column 12, 140 μL water, 40 μL NP-40 substitute (305.5 μM) and 20 μL of test compound stock (2 mM final concentration) were added. A 1:2 serial dilution was performed sequentially across the plate from column 12 to column 2, leaving column 1 as a blank. A 0.22 mM working solution of Fe(III)PPIX (bovine haemin, Sigma) was prepared by adding 178.8 μL of a 25 mM Fe(III)PPIX stock in DMSO to 20 mL of a 2 M acetate buffer (pH 4.75–4.9). Of this, 100 μL was added to each well, the plate sealed and incubated for 5 h at 37 °C. Following incubation, 32 μL of a pyridine working solution (20% water/20% acetone/10% 2 M HEPES buffer (pH 7.4)/50% pyridine v/v) was added to each well followed by 60 μL of acetone. The contents of the wells were resuspended and the absorbance of the resultant bis-pyridyl-heme complex measured at 405 nm using a ThermoScientific MultiskanGo plate reader. The data were analysed using GraphPad Prism 9.

2.7. Cytosolic Na^+ and pH measurements

Measurements of $[Na^+]_{cyt}$ and pH_{cyt} were performed with trophozoite-stage 3D7 parasites that were isolated from their host erythrocytes via brief exposure to saponin (as described in Qiu et al., 2022) and loaded with the fluorescent dye SBFI ($[Na^+]_{cyt}$ measurements) or BCECF (pH_{cyt} measurements). $[Na^+]_{cyt}$ and pH_{cyt} measurements were carried out in 96-well plates as described in Dennis et al. (2018) and Qiu et al. (2022), respectively. In both cases, the compounds of interest were added to parasites suspended at 37 °C in a saline consisting of 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM glucose and 25 mM HEPES (pH 7.1).

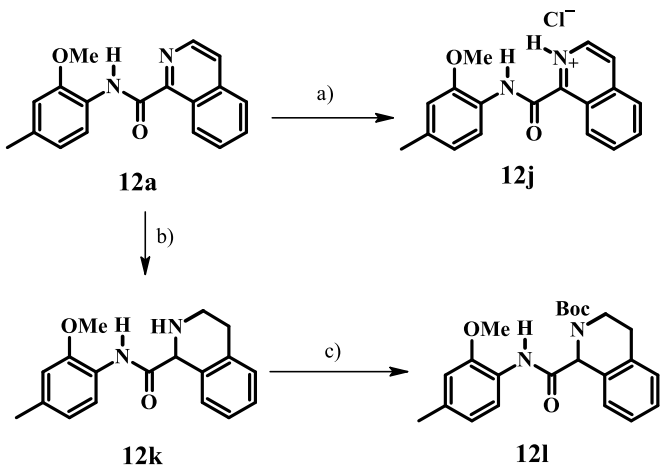


Scheme 1. a) EDCI (HOBt), DCM/DMF, rt, 1–6 days; b) $(COCl)_2$, ArCOOH, DMF, 0 °C then amine, DCM, Et_3N, rt, 16–18 h; c) Et_3N or pyr, DCM/PhMe, ArCOCl, 0 °C then amine rt, 18 h.

3. Results and discussion

3.1. Synthesis

In our study, we chose to synthesize twelve amides (**12a-12l**), and ten thioamides (**13a-13l**) including some with pyridine or quinoline rings. Amides **12a-12l** were prepared in 32–96% yield from the coupling of 2-methoxy-4-methylaniline (**10**) and aniline (**11**) with various acids or acid chlorides (Scheme 1). Salt **12j** was prepared in 81% yield from the addition of concentrated hydrochloric acid in tetrahydrofuran (THF) to a cold solution of amide **12a** in THF (Scheme 2). On the other hand, amide **12l** was prepared in 44% yield over two steps from the reduction of the isoquinoline ring of **12a** using 3 mol. equiv. of sodium cyanoborohydride followed by protection using Boc anhydride (Scheme 2).

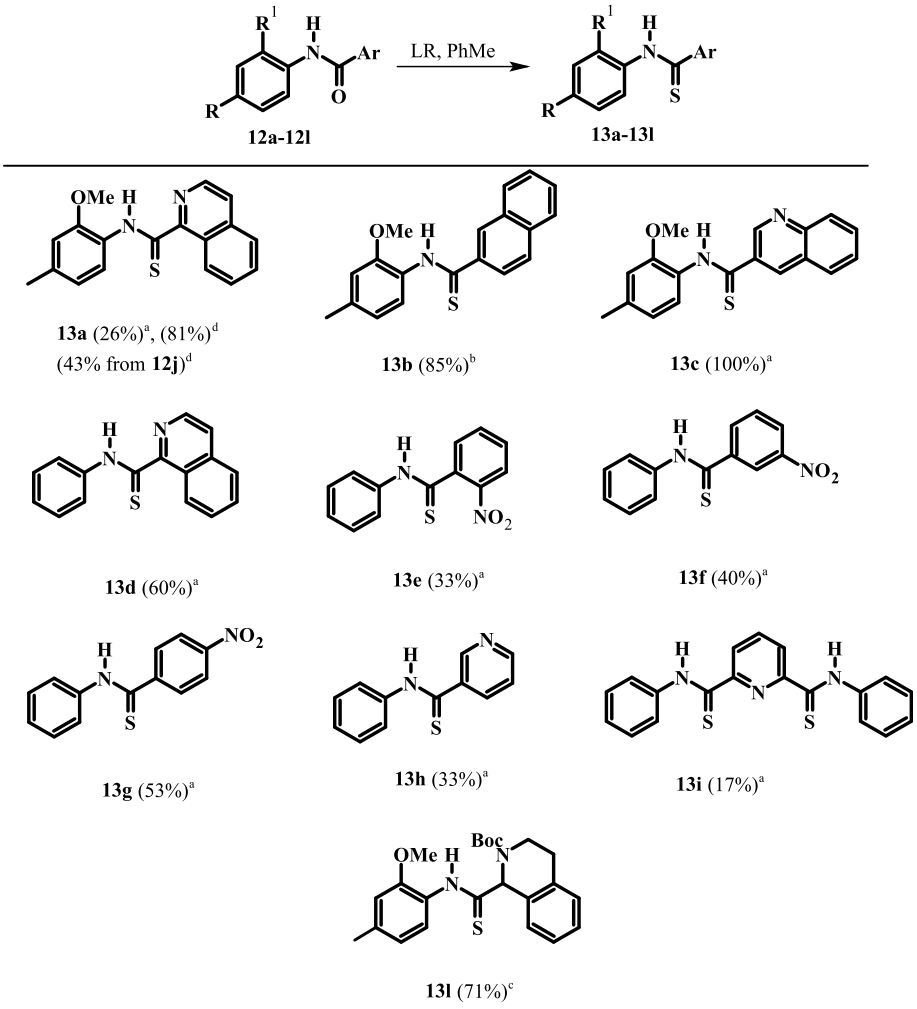


Scheme 2. a) conc. HCl, THF, **12j** (81%); b) $NaCNBH_3$, AcOH; c) Boc_2O, THF/H_2O (1:1), **12l** (44% over two steps).

The thionation of amide **12b** occurred in 85% yield using only 0.6 mol. equiv. of Lawesson's reagent (LR) (Scheme 3). By contrast, a low yield (26%) was obtained when amide **12a** was thionated using excess LR. This implied that the nitrogen of the isoquinoline moiety was either interacting with LR in a side reaction or it was electronically reducing the electron density of the amide functional group. There was a marked increase in the yield to 81% when the reaction was done under microwave conditions using 0.6 mol. equiv. of LR. Surprisingly, only starting material was obtained when we attempted to thionate salt **12j** using excess LR, likely owing to the electron deficiency of the isoquinolinium ring. However, 43% of thioamide **13a** was obtained when the salt was thionated using 1 mol. equiv. LR under microwave conditions. Thionation of amide **12k** using 3 mol. equiv. of LR gave only starting material. This was likely a consequence of the free amine forming adducts with the LR species.

Thionation of amide **12l** was achieved using 1.2 mol. equiv. of LR in refluxing toluene for 24 h. The efficient conversion to thioamide **13l**, which has a Boc protecting group installed, suggests that steric hindrance is not a major factor in the reaction (Scheme 3). Thioamides **13c** and **13d** were obtained in 100 and 60% yields from the respective thionation of their amide precursors using excess (3 mol. equiv.) LR in refluxing toluene for 24 h (Scheme 3). Generally, the yields for thionation of the nitrobenzamides were comparable to those for thionation of amides **12a** and **12d**. Low yields (17–33%) were obtained when 3-picolinamide **12h** and pyridine-2,6-dicarboxamide **12i** were thionated using excess LR.

While no evidence was found to support any steric effect from the possible formation of pyridine-LR adduct, the presence of electron withdrawing atoms or groups attached to the carbonyl carbon can reduce the ability of the carbonyl functionality to act as a nucleophile and therefore reduce thionation yields. This effect is most dominant when the standard protocol of 0.6 mol. equiv. of LR is used.



Scheme 3. Thionation of amides **12a-l** using LR. [a]3 mol. equiv. LR, reflux, PhMe, 24 h; [b]0.6 mol. equiv. LR, reflux, PhMe, 24 h; [c]1.2 mol. equiv. LR, reflux, PhMe, 24 h; [d]0.6-1 mol. equiv. LR, MW, PhMe, 0.5 h.

3.2. Antiplasmodial activity

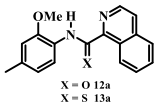
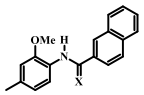
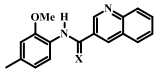
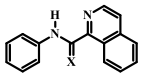
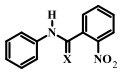
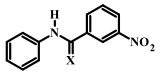
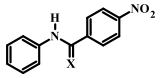
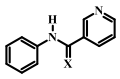
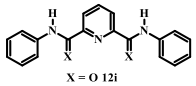
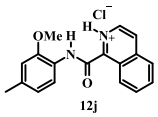
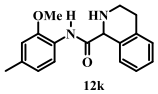
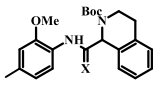
Initially the twenty-two (22) synthesized compounds were tested against intraerythrocytic stage *P. falciparum* parasites (strain 3D7) at a single concentration of 200 μM, or, where solubility did not permit, at a concentration between 10 and 100 μM. While compounds **12d**–**12i**, **13a**, **13d**, **13e** and **13h** showed less than 50% inhibition of parasite proliferation at the single concentration tested, compounds **12a** – **12c**, **12j** – **12l**, **13b, 13c**, **13f, 13g, 13i** and **13l** showed greater than 50% inhibition (Fig. S1), and we therefore generated dose-response curves for each to allow determination of the concentration causing 50% inhibition of *P. falciparum* proliferation (IC_{50} value; Fig. S2). As shown in Table 1, 2-methoxy-4-methylanilides **12a** and **12c** which bear isoquinoline and quinoline moieties, respectively, both showed antiplasmodial activity, with IC_{50} values of 69 and 62 μM, respectively. The corresponding naphthyl analogue (**12b**), however, showed slightly higher activity (IC_{50} = 38 μM, $p < 0.0079$, one-way ANOVA), consistent with the nitrogen-containing heterocycle not being required for activity.

The limited solubility of isoquinoline carboxamide **12d**, derived from aniline, made it difficult to assess the importance of the methyl and methoxy substituents on the aniline ring. The more soluble carboxamides without methyl and methoxy substituents and with nitrophenyl (compounds **12e** – **12g**) and pyridine (compounds **12h** and **12i**) moieties in place of the isoquinoline moiety also failed to inhibit parasite growth by more than 50% at the highest concentration tested. Although **12f**, **12g** and **12i** were previously tested for blood stage antiplasmodial activity by Wicht et al. (2016) using the NF54 and D10 strains of *P. falciparum*, they were retested here using the 3D7 strain. This was done to eliminate any strain-specific variations and in order to compare directly their activity with the rest of the compounds in the series which includes quinoline and isoquinoline carbothioamides as well as monopicolinamides.

Introducing a formal positive charge to the nitrogen atom of isoquinoline carboxamide **12a**, as in **12j**, reduced antiplasmodial activity by about 2-fold (IC_{50} value of **12j** = 136 μM; $p = 0.0002$, one-way ANOVA), while saturating the nitrogen-containing ring, to generate tetrahydroquinoline **12k**, resulted in an ~ 8-fold increase in activity (IC_{50} value of **12k** = 8.8 μM; $p < 0.0001$, one-way ANOVA). The corresponding Boc-protected compound (**12l**, IC_{50} = 32 μM) also showed improved activity compared with isoquinoline **12a** ($p < 0.0001$, one-way ANOVA) but was less active compared to **12k** ($p < 0.0001$, one-way ANOVA).

The thiocarboxamide analogue of isoquinoline **12a** (compound **13a**) did not inhibit proliferation of *P. falciparum* by >50% at concentrations up to 200 μM. However, the quinoline thiocarboxamide **13c** showed similar activity to its carboxamide analogue **12c** (IC_{50} value of compound **13c** = 61 μM; $p > 0.9999$, one-way ANOVA). As for the carboxamides, higher activity was associated with the naphthyl thiocarboxamide derivative (compound **13b,** IC_{50} = 23 μM; $p < 0.0001$, one-way ANOVA). The Boc-protected tetrahydroquinoline thiocarboxamide (**13l**) was five-fold less active than its carboxamide equivalent (**12l**) ($p < 0.0001$, one-way ANOVA). While the carboxamides without

Table 1
Effect of carboxamides **12a-12l** and thioamides **13a-13l** on proliferation of intraerythrocytic stage *P. falciparum* (strain 3D7) *in vitro* over 96 h.

Compound	IC_{50} against *P. falciparum* (μM)[a]	
	X = O	X = S
 X = O 12a X = S 13a	69.2 ± 5.0	>200
 X = O 12b X = S 13b	37.6 ± 2.1	23.3 ± 1.6
 X = O 12c X = S 13c	61.6 ± 1.1	61.2 ± 1.6
 X = O 12d X = S 13d	>10	>50
 X = O 12e X = S 13e	>200	>200
 X = O 12f X = S 13f	>200	109 ± 1
 X = O 12g X = S 13g	>50	80.3 ± 6.0
 X = O 12h X = S 13h	>200	>200
 X = O 12i X = S 13i	>50	0.142 ± 0.028
 12j	136 ± 13	ND [b]
 12k	8.81 ± 0.30	ND
 X = O 12l X = S 13l	31.9 ± 1.2	172 ± 12

[a] Data are averages from two or three independent experiments. IC_{50} values below the highest concentration tested are presented as mean ± standard error of the mean (SEM, $n = 3$).
[b] ND, not determined because target compound not obtained.

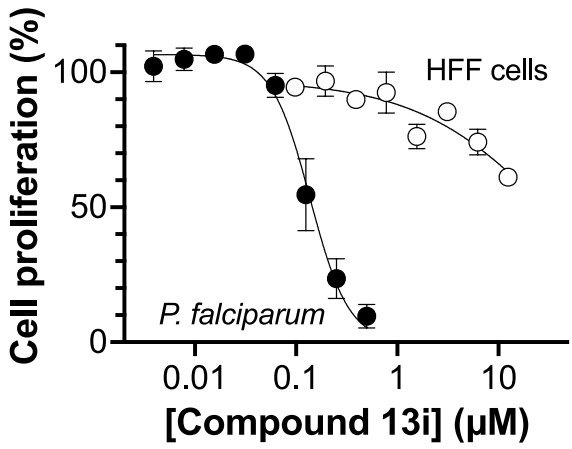


Fig. 2. Effect of thiocarboxamide **13i** on the proliferation of intraerythrocytic stage *P. falciparum* (strain 3D7) and HFF cells *in vitro* over 96 h. The data are from three independent experiments and error bars represent standard error of the mean (SEM). Where not visible, error bars are smaller than the symbol.

methyl and methoxy substituents lacked activity, three of the corresponding thiocarboxamides showed activity; compounds **13f** and **13g**, with nitro-substituted phenyl moieties, inhibited parasite growth with IC_{50} values of 109 and 80 μM, respectively, and pyridine **13i** was the most potent of all compounds tested ($IC_{50} = 0.142$ μM; $p < 0.0001$, one-way ANOVA; Fig. 2). The importance of the thiocarboxamide moieties for this activity is demonstrated by compound **12i** – the carboxamide analogue - possessing >350-fold lower activity. To assess the selectivity of the potent antiplasmodial effect of compound **13i**, the compound was also tested for its activity against human foreskin fibroblast (HFF) cells (Fig. 2), a low-passage number cell line. The compound inhibited proliferation of HFF cells by less than 50% at the highest concentration tested (12.5 μM), corresponding to a selectivity index of >88. To determine whether parasites resistant to chloroquine (**4**) were cross-resistant to **13i,** we tested the compound against Dd2, a chloroquine-resistant strain of *P. falciparum* (full chloroquine dose-response curves against Dd2 and 3D7 parasite strains are shown in Fig. S3). We found that **13i** was equally as effective against Dd2 ($IC_{50} = 0.146 \pm 0.018$ μM; Fig. S3) as it was against 3D7 ($IC_{50} = 0.142 \pm 0.028$ μM; $p = 0.9$, unpaired student's t-test).

3.3. Mechanism of action studies

In light of the structural similarity between some of our compounds and standard antimalarials known to inhibit parasite proliferation by inhibiting hemozoin formation (Sullivan et al., 1996), we decided to test a selection of the compounds with the highest antiplasmodial activity (**12b**, **12k**, **12l**, **13b**, **13i**) for their ability to inhibit β-hematin formation in a cell-free detergent mediated assay (de Sousa et al., 2020). Whilst chloroquine (included as a control) inhibited β-hematin formation as expected, none of the five compounds tested, including the most potent antiplasmodial derivative identified in this study (**13i**), had any effect on β-hematin formation (Fig. S4). This suggests that these compounds do not interact with the β-hematin crystal and therefore would be unlikely to interact with hemozoin in the parasite. Given this, the compounds were not taken forward for testing in the cell fractionation assay which can be used to test the ability of a compound to inhibit hemozoin formation within the parasite (Combrinck et al., 2015).

Numerous compounds kill *P. falciparum* parasites by inhibiting *Pf*ATP4 (Rottmann et al., 2010; Spillman et al., 2013; Jiménez-Díaz et al., 2014; Lehane et al., 2014; Vaidya et al., 2014; Flannery et al., 2015; Hewitt et al., 2017; Dennis et al., 2018; Gilson et al., 2019; Ashton et al., 2023), a protein believed to efflux Na^+ ions from the parasite cytosol whilst importing H^+ equivalents (Spillman et al., 2013). These

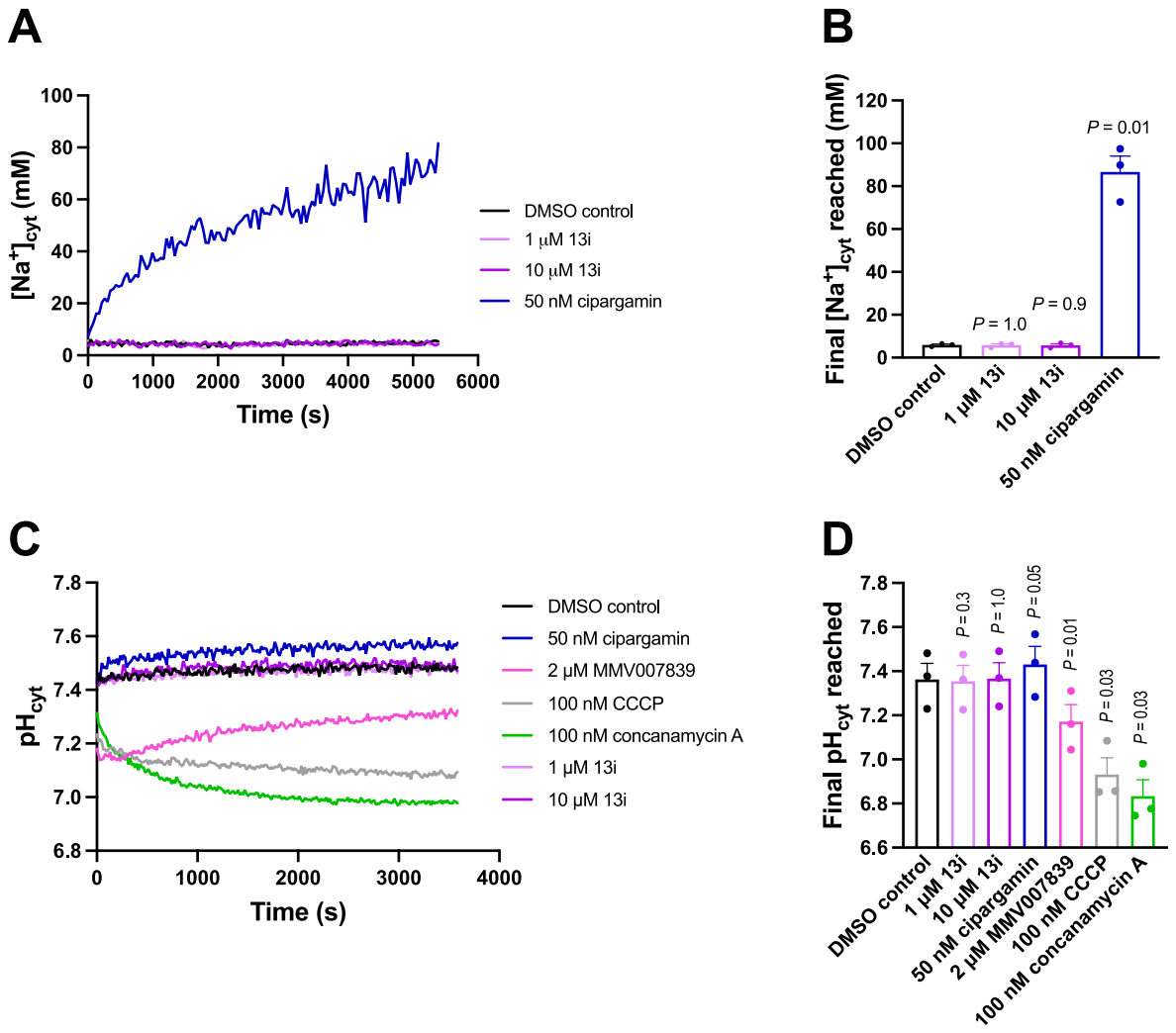


Fig. 3. Compound 13i does not affect parasite $[Na^+]_{cyt}$ or pH_{cyt}. (A,B) Isolated 3D7 parasites loaded with SBFI were exposed to **13i** (purple; 1 μM or 10 μM), solvent alone (black; DMSO control), or cipargamin (blue; 50 nM; positive control for *Pf*ATP4 inhibition). **(C,D)** BCECF-loaded 3D7 trophozoites were exposed to **13i** (purple; 1 μM or 10 μM), cipargamin (blue; 50 nM; positive control for *Pf*ATP4 inhibition), MMV007839 (pink; 2 μM; positive control for *Pf*FNT inhibition), CCCP (grey; 100 nM; protonophore), concanamycin A (green; 100 nM; positive control for V-type H^+-ATPase inhibition) or solvent alone (black; DMSO control). Panels **A** and **C** show representative traces from a single Na^+ (**A**) or pH (**C**) experiment. Panels **B** and **D** show data averaged from three independent experiments performed on different days in which all compounds/concentrations were tested concurrently. The bars and error bars show the mean + SEM and the symbols show the data from each individual experiment. The data for each compound and concentration were compared to those for the DMSO control using one-way ANOVA with Dunnett's multiple comparisons test, with the *p* values shown on the figure. The final $[Na^+]_{cyt}$ (**B**) and pH_{cyt} (**D**) reached were averaged from the data points obtained 85–90 min and 55–60 min after parasites were first exposed to the compound/solvent, respectively. The DMSO concentration was 0.1% v/v in pH experiments and 0.1–0.2% v/v in Na^+ experiments.

compounds, which include the clinical candidate cipargamin, formerly known as NITD609 and KAE609 (Rottmann et al., 2010), give rise to a variety of physiological perturbations including an increase in the parasite's cytosolic $[Na^+]$ ($[Na^+]_{cyt}$) and pH (pH_{cyt}) (Spillman et al., 2013; Lehane et al., 2014; Vaidya et al., 2014; Hewitt et al., 2017; Dennis et al., 2018). Various other antiplasmodial compounds give rise to a decrease in the parasite's pH_{cyt}. These include inhibitors of the parasite's lactate: H^+ transporter *Pf*FNT (e.g. MMV007839 (Golldack et al., 2017; Hapuarachchi et al., 2017)) and V-type H^+-ATPase (e.g. concanamycin A (van Schalkwyk et al., 2010)).

We investigated whether **13i** had any effect on $[Na^+]_{cyt}$ (Fig. 3A and B) or pH_{cyt} (Fig. 3C and D) in isolated trophozoite-stage parasites (3D7 strain). Fig. 3A and C show representative traces from a single Na^+ (A) or pH (C) experiment, and Fig. 3B and D show averaged data for the $[Na^+]_{cyt}$ (B) and pH_{cyt} (D) reached in the final 5 min of each experiment. Compound **13i** had no effect on $[Na^+]_{cyt}$ at either of the concentrations tested (1 μM and 10 μM), whereas the *Pf*ATP4 inhibitor cipargamin gave rise to a significant increase in $[Na^+]_{cyt}$ (Fig. 3A and B), as observed previously (e.g. Lehane et al., 2014). Compound **13i** was also without effect on pH_{cyt} (Fig. 3C and D). Consistent with previous results (van Schalkwyk et al., 2010; Lehane et al., 2014; Hapuarachchi et al., 2017), cipargamin gave rise to a small increase in pH_{cyt} while MMV007839 and concanamycin A both gave rise to a significant decrease in pH_{cyt} (Fig. 3C and D). The well-characterised protonophore CCCP also caused a significant decrease in pH_{cyt}, as observed previously (Saliba and Kirk, 2001). Our data therefore suggest that **13i** does not kill parasites via inhibition of *Pf*ATP4, *Pf*FNT or the V-type H^+-ATPase, or by increasing the permeability of membranes to H^+. Taken together, although our studies do not establish a mechanism of action for **13i**, the fact that it is not one of the frequently encountered mechanisms of inhibition of hemozoin formation or *Pf*ATP4 inhibition is an important finding as drugs with novel mechanisms of action are desired.

Although thiocarboxamide **13i** is included in the PubChem database (Kim et al., 2022), the potent antiplasmodial activity we report

constitute its first documented biological activity. Given its potency and selectivity, further investigation into its antiplasmodial mechanism of action and structure-activity relationships is warranted. Here we showed that replacement of the two thiocarboxamide moieties with carboxamide groups is not tolerated. Further investigations should focus on preparing analogues of thiocarboxamide **13i** for antiplasmodial testing.

In summary, ten new compounds possessing quinoline or isoquinoline scaffolds were synthesized. Additionally, we have identified 13 compounds showing antiplasmodial activity, with two compounds possessing IC_{50} values below 10 μM, including one with a submicromolar IC_{50} of 142 nM and greater than 88-fold selectivity over a human cell line. β-Hematin formation was not inhibited in our hands, and as such, **13i** may act through a novel mechanism.

CRediT authorship contribution statement

Alexa Redway: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Christina Spry:** Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Ainka Brown:** Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization. **Ursula Wiedemann:** Investigation. **Imam Fathoni:** Investigation, Writing – review & editing. **Larnelle F. Garnie:** Investigation, Methodology, Writing – review & editing. **Deyun Qiu:** Formal analysis, Investigation. **Timothy J. Egan:** Methodology, Supervision. **Adele M. Lehane:** Formal analysis, Methodology, Visualization, Writing - review & editing. **Yvette Jackson:** Conceptualization, Funding acquisition, Project administration, Visualization. **Kevin J. Saliba:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing. **Nadale Downer-Riley:** Conceptualization, Data curation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2024.100536.

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