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Antiplasmodial activity of the natural product compounds alstonine and himbeline

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ABSTRACT

Malaria, caused by *Plasmodium* parasites, continues to be a devastating global health issue. Despite a decline in malaria related deaths over the last decade, overall progress has plateaued. Key challenges to malaria prevention and control include the lack of a broadly effective vaccine and parasite drug resistance, including to the current gold standard artemisinin combination therapies (ACTs). New drugs with unique modes of action are therefore a priority for both the treatment and prevention of malaria. Unlike treatment drugs which need to kill parasites quickly to reduce or prevent clinical symptoms, compounds that kill parasites more slowly may be an option for malaria prevention. Natural products and natural product derived compounds have historically been an excellent source of antimalarial drugs, including the artemisinin component of ACTs. In this study, 424 natural product derived compounds were screened for *in vitro* activity against *P. falciparum* in assays designed to detect slow action activity, with 46 hit compounds identified as having >50% inhibition at 10 μM. Dose response assays revealed nine compounds with submicromolar activity, with slow action activity confirmed for two compounds, alstonine and himbeline (50% inhibitory concentration (IC_{50}) 0.17 and 0.58 μM, respectively). Both compounds displayed >140-fold better activity against *P. falciparum* versus two human cell lines (Selectivity Index (SI) >1,111 and > 144, respectively). Importantly, *P. falciparum* multi-drug resistant lines showed no cross-resistance to alstonine or himbeline, with some resistant lines being more sensitive to these two compounds compared to the drug sensitive line. In addition, alstonine displayed cross-species activity against the zoonotic species, *P. knowelsi* (IC_{50} ~1 μM). Outcomes of this study provide a starting point for further investigations into these compounds as antiplasmodial drug candidates and the investigation of their molecular targets.

1. Introduction

Malaria, caused by *Plasmodium* parasites, is one of the world's most lethal tropical infectious diseases, causing >200 million cases and 409,000 deaths in 2019 (WHO 2020). Of the six human-infecting species, including two zoonotic species (*P. knowlesi* and *P. cynomolgi*), *P. falciparum* causes the most mortality, mainly in children under five years of age (WHO 2020). There is currently no effective malaria vaccine for widespread use (de Vrieze 2019) and while antimalarial drugs play a key role in the prevention and treatment of malaria, all are susceptible to parasite drug resistance including the current gold standard artemisinin combination therapies (ACTs) (WHO 2020). The discovery of new drugs to prevent and treat malaria, especially those with different targets to existing drugs, is therefore critical to addressing the problem of antimalarial drug resistance.

Antimalarial drug discovery efforts have largely focused on the development of fast-action compounds that rapidly kill *Plasmodium* with the aim of reducing symptoms and disease severity. However, the drive to achieve malaria eradication means that new tools are needed, including drugs that focus on chemoprevention and blocking parasite transmission. In addition to compounds that target liver stage parasites, previously deprioritised compounds with 'slow-action' activity against asexual intraerythrocytic stage parasites are of interest as chemoprevention drug leads (Wu et al., 2015; Burrows et al., 2017). These compounds are of interest for chemoprevention as their slow onset of activity likely reflects different modes of action to fast action treatment drugs, a feature that should circumvent the selection of parasites resistant to treatment therapies.

Natural products have played a key role in the control and treatment of malaria. Quinine, a component of the bark of the Cinchona tree, was

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first used to treat malaria from as early as the 1600's and remained the mainstay for malaria treatment until the 1920's when the more effective synthetic derivative chloroquine became available (Slater 1993; Achan et al., 2011). Since the failure of chloroquine began in the 1960's (Harinasuta et al., 1965), several other synthetic naturally derived compounds have been developed for clinical use including, atovaquone, amodiaquine and mefloquine (Srivastava et al., 1997; Schlagenhauf 1999; Olliaro and Mussano 2003). Since the early 2000's, the sesquiterpene lactone artemisinin, or synthetic derivatives (e.g. dihydroartemisinin, artemether and artesunate), have been used as the foundation combination drug partner in the highly effective ACTs. Artemisinin is derived from the sweet wormwood *Artemisia annua*, a plant that was used for ~2,000 years in traditional Chinese medicine to treat malaria symptoms (Klayman 1985).

In this study, 424 pure compounds derived from natural products were screened against asexual intraerythrocytic stage *P. falciparum* parasites in fast (48 h) and slow action (96 h) *in vitro* growth inhibition assays designed to identify compounds with slow action activity. Two slow action hit compounds with submicromolar activity (alstonine and himbeline) were investigated further to determine their antiplamsodial potency and selectivity versus human cells. Cross-resistance studies with different *P. falciparum* lines suggests that alstonine and himbeline act differently to several currently used antimalarials, warranting further investigation of the mode of action of these compounds and their potential as chemoprevention drug starting points.

2. Materials and methods

2.1. Compounds

Pure compounds were derived from NatureBank housed at the Griffith Institute for Drug Discovery at Griffith University, Australia. NatureBank contains biota from ~30,000 plant and marine invertebrates prepared into extract and fraction libraries for screening, as well as ~5,000 pure compounds of which 2,000 are natural products and 3,000 are natural product analogues (Camp et al., 2014). Chloroquine, atovaquone and clindamycin were purchased from Sigma-Aldrich®, USA. Cycloguanil hydrochloride was purchased from Santa Cruz Biotechnology Inc, USA. Vorapaxar was purchased from AK Scientific Inc, USA. Stock solutions were prepared in 100% DMSO (Sigma-Aldrich®, USA), stored at −20 °C and diluted as required.

2.2. P. falciparum in vitro culture

P. falciparum infected erythrocytes were cultured in O positive human erythrocytes in RPMI 1640 media (Gibco, USA) containing 10% heat-inactivated pooled human sera and 5 μg/mL gentamicin (Sigma, USA). Cultures were maintained at 37 °C in a gas mixture composed of 5% O_2, 5% CO_2, and 90% N_2, essentially as described previously (Trager and Jensen 1976). The following *P. falciparum* lines were used: 3D7 (Walliker et al., 1987), Dd2 (Nguyen-Dinh and Trager 1978), FCR3 (Jensen et al., 1981), and C2B (Ohrt et al., 2002).

2.3. In vitro P. falciparum growth inhibition assays

The activity of compounds was tested against *P. falciparum* in assays that spanned either 48 h (one asexual intraerythrocytic developmental cycle; "fast action") 72 h or 96 h (two asexual intraerythrocytic developmental cycle; "slow action"). Growth inhibition was assessed in [^{3}H]-hypoxanthine incorporation assays, as previously described (Skinner--Adams et al., 2019). Briefly, control or test compounds were added to 96 well plates (3596 Corning®, Costar®, USA; 100 μL/well) followed by addition of synchronous ring-stage *P. falciparum* infected erythrocytes obtained by sorbitol treatment (100 μL/well) (Lambros and Vanderberg 1979). Starting parasitemias were 1%, 0.25% or 0.1% parasitemia for 48 h, 72 h and 96 h assays, respectively, with haematocrits of 1% (48 h), 2.5% (72 h) or 2% (96 h). Compound vehicle (0.5% final DMSO) and clinically used antimalarial drugs served as negative and positive controls, respectively. For 48 h assays, 0.5 μCi [^{3}H]-hypoxanthine (PerkinElmer®, USA) was added to each well at the start of the assay. For 72 h 0.5 μCi [^{3}H]-hypoxanthine was added at approximately 48 h. After incubating 96 h assays for approximately 72 h, 100 μL of culture media was removed and replaced with 100 μL fresh parasite media supplemented with 0.5 μCi [^{3}H]-hypoxanthine (PerkinElmer®, USA). In each case, plates were then incubated for a further 24 h and the assay stopped by freezing plates at −20 °C. After thawing, cells were harvested onto 1450 MicroBeta filter mats (Wallac, USA) and [^{3}H] incorporation determined using a 1450 MicroBeta liquid scintillation counter (PerkinElmer®, USA). Percentage inhibition of growth for compound treated versus matched vehicle only (0.5% DMSO) controls was determined and IC_{50} values calculated using log linear interpolation (Huber and Koella 1993). Each compound was assayed in triplicate wells, in 2–3 independent experiments for dose response assays, and in singlicate for the primary screen. Statistical difference between IC_{50} values was determined using an unpaired *t*-test with GraphPad Prism® data analysis software. Z-factors were calculated according to (Zhang et al., 1999) where a Z-factor between 0.5 and 1 was deemed an excellent assay, 0–0.5 is marginal and <0 unsuitable for screening purposes.

2.4. In vitro P. knowlesi growth inhibition assays

The *in vitro* antimalarial activity of compounds was tested against *P. knowlesi* infected erythrocytes using a modified [^{3}H]-hypoxanthine incorporation assay (Arnold et al., 2016). Briefly, asynchronous *P. knowlesi* A1H.1 parasites (0.25% parasitemia and 2% final haematocrit) were seeded into 96-well tissue culture plates (3596 Corning®, USA) containing serial dilutions of control or test compounds in parasite culture media (Moon et al., 2013). Assay plates were incubated for 24h or 48 h with 0.5 μCi [^{3}H]-hypoxanthine (PerkinElmer®, USA) added 24 h before the end of each assay. All other assay procedures, including data analysis, controls and independent replicates, were as described above for *P. falciparum*.

2.5. Human cell cytotoxicity assays

Neonatal foreskin fibroblast (NFF) and human embryonic kidney (Hek293) cells were cultured in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% FCS (CSL Biosciences, Parkville, Victoria, Australia), 1% streptomycin (Life Technologies, Inc., Rockville, MD) at 37 °C and 5% CO_2. Cytotoxicity assays were carried out as previously described (Andrews et al., 2013). Percentage inhibition of growth was compared to matched DMSO controls. IC_{50} values were calculated using log linear interpolation (Huber and Koella 1993). The mean IC_{50} (±SD) was determined for three independent experiments, each carried out in triplicate.

2.6. Isopentenyl pyrophosphate (IPP) rescue

In vitro Isopentenyl pyrophosphate (IPP) pathway rescue against *P. falciparum* 3D7 parasites was carried out as previously described (Skinner-Adams et al., 2019). Briefly, alstonine was tested in *P. falciparum* 96 h [^{3}H]-Hypoxanthine growth inhibition assays as previously described above with the following modifications. Two identical 96 h assays were performed simultaneously, one supplemented with 200 μM IPP and one without IPP. The antibiotic antimalarial compound clindamycin was used as a positive control. Each compound was assayed in triplicate wells, in at least three independent experiments. IC_{50} values were calculated using log linear interpolation (Huber and Koella 1993). Statistical difference between IC_{50} values was determined using an unpaired *t*-test with GraphPad Prism® data analysis software.

3. Results and discussion

3.1. Identification of slow action antiplasmodial compounds

Phenotypic assays have been developed to easily discern slow versus fast action antiplasmodial compounds *in vitro* (Skinner-Adams et al. 2016, 2019). Essentially these assays assess compound potency after one asexual intraerythrocytic cycle (48 h) and compare this to the potency of the compounds after two asexual intraerythrocytic cycles (96 h). This strategy was used in this study to identify natural product-derived compounds with slow action antiplasmodial activity. A panel of 424 compounds derived from NatureBank biota were screened in 48 h and 96 h assays against drug sensitive *P. falciparum* 3D7 at a single concentration (10 μM). A compound was defined as having slow action activity if growth inhibition was <50% in the 48 h assay and ≥50% in the 96 h assay. Data identified 46 compounds that met these criteria (**Fig. S1**). As expected, the fast action control drug chloroquine inhibited ~100% of parasite growth in both assays (**Fig. S1**).The quality of the primary screen assays was assessed by calculating Z-factors with Z-factors of 0.91 recorded for both assays, indicating excellent assays (Z-factor >0.5).

3.2. IC_{50}'s of slow action hit compounds against P. falciparum

Of the 46 slow action hits identified in the primary screen (**Fig. S1**), only 39 were available in sufficient quantity for retesting. All 39 compounds were evaluated in two independent 16-point dose response assays to determine their IC_{50} values and confirm their slow action phenotype. Chloroquine and clindamycin were used as fast and slow acting anti-malarial control compounds, respectively. As expected, there was no significant difference in the 48 h versus 96 h IC_{50} values for chloroquine (**Table 1**; $P > 0.05$), and clindamycin displayed slow action activity with no activity at 48 h ($IC_{50} > 50$ μM; **Table 1**) and potent activity at 96 h (IC_{50} 0.005 μM; **Table 1**). Compounds with an IC_{50} value ≤ 1 μM in the 96 h assay were classified as hits, with this level of *in vitro* antiplasmodial activity being one of several hit criteria defined by the Medicines for Malaria Venture (MMV) (MMV 2021), the global malaria product development partnership organization. Nine compounds met these criteria (**Table 1**) and of these, two compounds (compounds 13 and 6; **Fig. 1**) had >10-fold lower IC_{50}'s in 96 h versus 48 h assay, suggesting slow action activity. Compound 13 (known as alstonine (Elisabetsky and Costa-Campos 2006);) displayed a significantly lower 96 h IC_{50} (239-fold) versus 48 h IC_{50} ($P < 0.05$; **Table 1**) and compound 6 (known as himbeline (Chackalamannil et al., 1999) showed an 18-fold lower 96 h IC_{50} compared to 48 h ($P < 0.05$; **Table 1**). Alstonine and himbeline were further evaluated by assessing growth inhibition in 72 h assays, with no significant difference observed for either compound

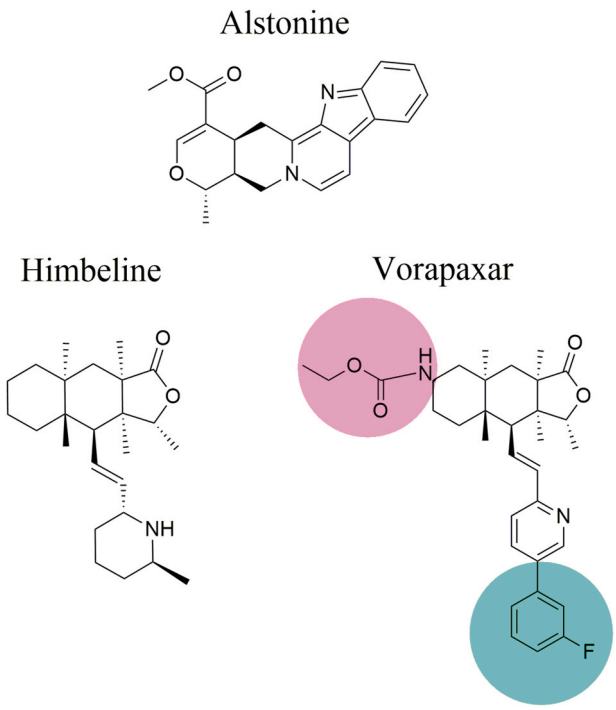

Fig. 1. Chemical structures of alstonine, himbeline and Vorapaxar.
Chemical structures of alstonine (top), himbeline (bottom left) and Vorapaxar (bottom right). Vorpaxar side chains; ethyl carbamate (pink) and flurobenzene (blue).

versus IC_{50}'s obtained in 96 h assays ($P > 0.05$; **Table 1**), indicating that these compounds act before the end of the second asexual intraerythrocytic cycle. Alstonine has been previously tested for anti-*Plasmodium* activity in a fast action assay (48 h) against *P. falciparum* K1 where it was found to be inactive ($IC_{50} > 30$ μM) (Wright et al., 1996). However, in a separate study, alstonine was found to have potent activity against *P. falciparum* D6 and W2 lines in a 72 h assay (IC_{50} 0.048 μM and 0.109 μM, respectively, (Okunji et al., 2005). Each of these previous reports support the findings of this study. To the best of our knowledge, the antiplasmodial activity of himbeline has not been previously reported.

The alstonine sourced for this study was isolated from a plant of the genus *Alstonia* that belongs to the family Apocynaceae. Interestingly, the bark of *Alstonia constricta* and *Alstonia scholaris* are used in African traditional medicine for a range of indications, including the treatment of malaria (Oliver-Bever 1983; Adotey et al., 2012). Alstonine has been investigated as a putative antipsychotic agent, with animal behavioural

Table 1
P. falciparum 3D7 IC_{50} values for slow action NatureBank hits and Vorapaxar.

Cpd	[a]*P. falciparum* 3D7 IC_{50} (μM)			48 h/96 h IC_{50}	P value
	48 h	72 h	96 h		
Chloroquine	0.008 ± 0.002	0.006 ± 0.004	0.004 ± 0.002	2	>0.05
Clindamycin	>50	0.009 ± 0.002	0.005 ± 0.002	>10,000	*n.d.*
13 (alstonine)	40.6 ± 16.2	0.18 ± 0.08	0.17 ± 0.03	239	<0.05
9	0.66 ± 0.21	*n.d.*	0.10 ± 0.03	6	>0.05
6 (himbeline)	10.7 ± 0.7	0.77 ± 0.18	0.58 ± 0.22	18	<0.05
5	3.64 ± 0.40	*n.d.*	0.37 ± 0.30	10	<0.05
19	3.16 ± 0.06	*n.d.*	0.58 ± 0.30	5	<0.05
28	2.14 ± 0.21	*n.d.*	0.61 ± 0.07	4	<0.05
1	1.96 ± 0.38	*n.d.*	0.83 ± 0.08	2	>0.05
31	4.46 ± 0.93	*n.d.*	1.06 ± 0.47	4	<0.05
42	10.8 ± 2.2	*n.d.*	1.08 ± 0.24	10	<0.05
Vorapaxar	11.2 ± 3.0	3.93 ± 1.07	3.52 ± 0.25	3	>0.05

[a] IC_{50} values were determined using log linear interpolation with mean IC_{50} (±SD) shown for at least two independent replicates, each in triplicate wells. Compounds with a >10-fold lower IC_{50} value in 96 h versus 48 h assays were classified as having slow action activity in this study.

studies suggesting a possible association with the 5-HT2A/C receptor (a G protein-coupled receptor (GPCR)) (Linck et al. 2011, 2012), however no biochemical evidence exists in support of this theory. There are four genes encoding putative GPCR-like receptors in *P. falciparum* (Madeira et al., 2008). However, these proteins are unlikely to be the primary targets of alstonine in these parasites given current evidence suggests that they are not essential for *P. falciparum* survival (Zhang et al., 2018; Santos et al., 2020). Himbeline, is an analogue of himbacine and the clinically approved drug Vorapaxar, a known protease-activated receptor 1 (PAR-1) antagonist (a GPCR), approved for use in cardiovascular diseases (Chackalamannil et al., 2008; Gryka et al., 2017). Given Vorapaxar's structural similarity to himbeline, we hypothesised that if Vorapaxar had similar antiplasmodial activity to himbeline it may be of interest for repurposing for malaria. However, Vorapaxar was shown to be ~6-fold less active than himbeline in 96 h assays (96 h IC_{50} 3.5 μM; **Table 1**), thus does not meet the MMV hit criteria for *in vitro* antiplasmodial activity. Although Vorapaxar shares a similar core structure with himbeline, the addition of fluorobenzene and ethyl carbamate groups to the two side chains (**Fig. 1**) are substantial changes and may explain why Vorapaxar displays lower antiplasmodial activity than himbeline.

3.3. Selectivity of alstonine and himbeline for P. falciparum versus human cells

To determine whether alstonine and himbeline selectively kill *Plasmodium* parasites over human cells, the compounds were tested against neonatal foreskin fibroblasts (NFFs) and human embryonic kidney cells (HEK293). Alstonine showed <50% activity at the highest concentration tested (200 μM) for both cell lines, while himbeline displayed <50% activity against NFF and HEK293 at 200 μM and 111 μM, respectively (**Table 2**). These data were compared to the *P. falciparum* 3D7 72 h IC_{50} values to determine Selectivity Indices (SI; NFF or HEK293 IC_{50}/*P. falciparum* 3D7 72 h IC_{50}). Alstonine and himbeline were found to have selectivity indices of >1,111 and > 144, respectively. Thus they meet the "early lead" criteria for *in vitro* selectivity (SI > 100) as outlined by the Medicines for Malaria Venture (MMV 2021), one of the several required criteria for early lead compounds. The SI for the control drug chloroquine was similar to previous reports (Andrews et al., 2013; Fisher et al., 2014; Engel et al., 2015) (**Table 2**).

While this selectivity is encouraging, these *in vitro* data should be considered with caution, particularly given that alstonine and himbeline analogues have known/putative targets in humans. For example, himbeline's clinical analogue Vorapaxar exerts antiplatelet effects which can cause bleeding in susceptible patients (Gryka et al., 2017). However, other studies suggest that Vorapaxar may prove to be beneficial in reducing brain swelling in cerebral malaria (Storm et al., 2020), therefore the associated risks versus benefits need to be considered. In addition, adverse effects in humans may be dependent on the therapeutic dose required for clearance of *Plasmodium* parasites.

Table 2
P. falciparum selectivity profile of alstonine and himbeline.

Name	*Pf*3D7 IC_{50} (μM)[a]	NFF IC_{50} (μM)	HEK^{293} IC_{50} (μM)	*Pf*SI[b]
Chloroquine	0.006 ± 0.004	35.4 ± 3.6	27.3 ± 13.3	4555–5896
Alstonine	0.18 ± 0.08	>200	>200	>1111
Himbeline	0.77 ± 0.18	>200	111 ± 26	144 - >260

[a] 72 h IC_{50} values were determined using log linear interpolation with mean IC_{50} (±SD) shown for at least three independent experiments, each in triplicate wells.

[b] Selectivity Index (SI) – mammalian cell IC_{50}/*P. falciparum* IC_{50}.

3.4. P. falciparum alstonine and himbeline slow action activity is not apicoplast dependent

Compounds with a slow action "delayed death" phenotype such as clindamycin inhibit normal function of the parasite's apicoplast organelle which leads to the inability to produce isoprenoid precursors such as IPP (Burkhardt et al., 2007). *In vitro* supplementation with IPP can rescue the effect of apicoplast inhibitors (Yeh and DeRisi 2011) and reveal "delayed death" apicoplast related activity. Using this approach alstonine and himbeline were tested in 96 h growth inhibition assays against *P. falciparum* 3D7 parasites with and without addition of IPP. Control compounds included the fast action control drug chloroquine and the "delayed death" compound clindamycin. As expected, upon supplementation with 200 μM IPP, the *in vitro* activity of chloroquine was not rescued ($P > 0.05$; **Table S1**). In contrast, and as expected, supplementation with IPP rescued the growth inhibitory effect of clindamycin (IC_{50} 0.004 ± 0.001 μM without IPP and $IC_{50} > 0.5$ μM with IPP; **Table S1**). Alstonine and himbeline activity was not rescued by IPP supplementation ($P > 0.5$; **Table S1**), suggesting that alstonine and himbeline slow action activity is different to delayed death compounds like clindamycin.

3.5. Profiling alstonine and himbeline activity against multi-drug resistant P. falciparum

To prolong the clinical lifespan of new antimalarial drugs it is imperative that cross-resistance studies are carried out on new drug leads to ensure that they do not succumb to current parasite resistance mechanisms. Therefore to confirm that there was no cross-resistance, alstonine and himbeline activity was investigated against the *P. falciparum* multi-drug resistant lines Dd2 (resistant to chloroquine, mefloquine, pyrimethamine and sulfadoxine (Wang et al., 1997; Rathod et al., 2002)), C2B (resistant to chloroquine, cycloguanil, pyrimethamine and atovaquone (Ohrt et al., 2002)) and FCR3 (resistant to chloroquine and cycloguanil (Nguyen-Dinh and Trager 1978; Banyal and Inselburg 1986). As previously seen for the drug sensitive *P. falciparum* line 3D7, alstonine and himbeline retained a slow-action phenotype against Dd2, C2B and FCR3, displaying significantly less activity in the 48 h assay compared to 72 h and 96 h assays (**Fig. 2**; **Table S2**; $P < 0.05$). There was no significant difference in activity seen between 72 h and 96 h assays (**Fig. 2**; **Table S2**; $P > 0.05$).

The IC_{50} values of the *P. falciparum* 3D7 drug sensitive line were compared to activity against drug resistant lines (Dd2, C2B and FCR3) to calculate a Resistance Index (RI; resistant line IC_{50}/sensitive line (3D7) IC_{50}; the higher the RI the higher the resistance level). RI's were compared for assays carried out over 48 h, 72 h and 96 h and the antimalarial drugs chloroquine, atovaquone and cycloguanil were used as positive controls for Dd2, C2B and FCR3, respectively. As expected, comparing the drug sensitive 3D7 line to Dd2 resulted in significant differences in IC_{50} values for the control drug chloroquine ($P < 0.05$) for all time points (RI's 3, 3 and 7 for 48 h, 72 h and 96 h assays, respectively; **Table S2**). In contrast, at 72 h and 96 h, the Dd2 IC_{50} for alstonine was not significantly different than against 3D7 (**Fig. 2**; **Table S2** $P > 0.05$) and at 48 h Dd2 was 8-fold more sensitive to alstonine (**Table S2**; $P < 0.05$). Similarly, Dd2 displayed no cross-resistance to himbeline (RI's < 1; **Table S2**), but had increased sensitivity to himbeline reflected by a 4–8 fold decrease in IC_{50} values at 48 h and 72 h ($P < 0.05$; **Fig. 2**; **Table S2**).

The atovaquone resistant line C2B was also not cross-resistant to alstonine and himbeline as reflected by RI's of <1 in 48 h, 72 h and 96 h assays (**Table S2**). Interestingly, C2B parasites displayed increased sensitivity to alstonine in 48 h assays, with a 27-fold lower IC_{50} for C2B in comparison to 3D7 (1.50 μM and 40.60 μM, respectively; **Fig. 2**; **Table S2**). A similar trend was seen for himbeline, however the IC_{50} fold change was considerably less (~2-fold lower C2B IC_{50} for 48 h and 72 h assays; **Fig. 2**; **Table S2**). As previously reported (Skinner-Adams et al.,

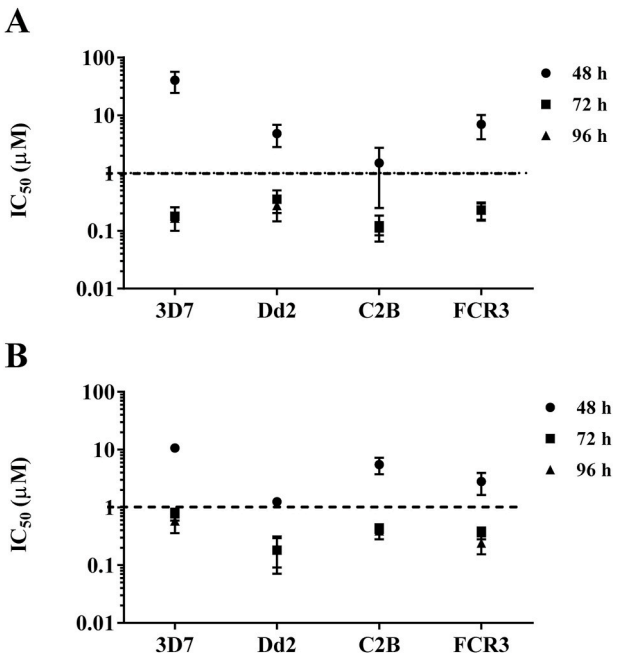

Fig. 2. IC_{50} of alstonine and himbeline against a panel of drug resistant *P. falciparum* lines.
Mean IC_{50} values (±SD) of *in vitro* cultured *P. falciparum* 3D7, Dd2, FCR3 and C2B against (A) alstonine and (B) himbeline at 48 h (circle), 72 h (square) and 96 h (triangle) were calculated using log linear interpolation. In each case the data was derived from at least two independent biological assays, each carried out in triplicate wells.

2019) the control drug atovaquone displayed RI's of >2000 for all time points against C2B (**Table S2**).

For the *P. falciparum* FCR3 line versus 3D7, no cross-resistance was observed to alstonine or himbeline in any assay (RI's < 1.4; **Table S2**). Lower IC_{50} values (2–6 fold) were observed for alstonine (48 h) and himbeline (48 h and 72 h) indicating low to moderate increase in sensitivity of FCR3 versus 3D7 (**Fig. 2**; **Table S2**). As expected, cycloguanil displayed significantly higher IC_{50} values for FCR3 compared to 3D7 ($P<0.05$, RIs >140; **Fig. 2**; **Table S2**).

Taken together, these data suggest that the multi-drug resistant lines Dd2, C2B and FCR3 are not resistant to alstonine and himbeline indicating that these compounds are likely to be active against drug resistant parasites in the field. They also suggest that these compounds are likely to possess different modes of action to commonly used antimalarial drugs including atovaquone and cycloguanil. However, one noteworthy observation was the significant improvement in fast action activity (27-fold) for alstonine against the C2B line. The atovaquone resistant C2B line has a mutation in cytochrome *bc*1, an essential component of the parasites mitochondrial electron transport chain (mtETC). Thus, it may be that alstonine has a mode of action that is associated with the mtETC that is different to atovaquone. *P. falciparum* parasites expressing yeast dihydroorotate dehydrogenase (yDHODH) are resistant to *Pf*DHODH inhibitors and have reduced susceptibility to compounds that target the mtETC, including atovaquone (Painter et al., 2007). Future studies could include assessing alstonine's activity against these parasites to determine if alstonine targets the mtETC or *Pf*DHODH.

Whilst increased activity was also observed for alstonine and himbeline against Dd2 (4 to 8-fold) and FCR3 (2 to 6-fold), it is difficult to comment on what is causing this phenotype due to the complex resistance mechanisms associated with these multi-drug resistant lines.

3.6. *P. knowlesi in vitro* activity of alstonine

One of the desired traits of new antimalarial leads is the capacity to target multiple human infecting *Plasmodium* species. *P. knowlesi* is a zoonotic species that can cause death in humans (Barber et al., 2017) and is a potential model for *P. vivax* which causes significant malaria morbidity (Craig et al., 2012; WHO 2020). The most potent and selective slow action hit compound, alstonine, was chosen to carry out preliminary studies against *P. knowlesi*. Alstonine was tested *in vitro* for activity against *P. knowlesi* infected erythrocytes in 24 h and 48 h growth inhibition assays (equivalent to approximately one and two asexual intraerythrocytic developmental cycles, respectively). Similar to *P. falciparum*, alstonine displayed slow action activity against *P. knowlesi* reflected by > 9-fold increase in IC_{50} values at 48 h compared to 24 h assays (**Table S3**). However, the activity of alstonine was 8-fold lower against *P. knowlesi* compared to *P. falciparum* (**Table S3**), an observation that is not surprising given that differences in drug susceptibility has been previously reported for these two species (van Schalkwyk et al., 2019). The antimalarial drug clindamycin also displays slow action *in vitro* activity against *P. knowlesi* (Arnold et al., 2016), however this activity is only seen after 72 h exposure (equivalent to approximately three asexual intraerythrocytic developmental cycles). Therefore, it is possible that we may not be capturing alstonine's full activity against *P. knowlesi* at 48 h and future work should include assessing alstonine activity against *P. knowlesi* in 72 h assays. As previously reported (Arnold et al., 2016), the control drug chloroquine showed no significant difference ($P > 0.05$) in IC_{50} values after 24 h or 48 h exposure (**Table S3**).

4. Conclusion

In this study two slow action antiplasmodial natural product compounds, alstonine and himbeline, were identified via a screen of pure compounds derived from the NatureBank compound library. The activity of these compounds was not rescued by IPP, indicating a mode of action different to apicoplast-targeting slow action delayed death compounds like clindamycin. Both compounds displayed *in vitro* hit-like potency (<1 μM) against *P. falciparum* and lead like-selectivity (SIs >100), with no cross-resistance observed against multi-drug resistant lines. Furthermore, alstonine was shown to have activity against *P. knowlesi* (IC_{50} ~ 1 μM), demonstrating multi-species targeting. While further studies are needed to further assess the *in vivo* antiplasmodial potential of these compounds, this work supports additional studies, including the synthesis and testing of additional analogues, target identification studies and *in vivo* efficacy trials in rodent malaria models.

Declaration of competing interest

None.

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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.2021.04.003.

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