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Potent *ex vivo* activity of naphthoquine and methylene blue against drug resistant clinical isolates of *Plasmodium falciparum* and *Plasmodium vivax*

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Running title: NAPHTHOQUINE AND METHYLENE BLUE *EX VIVO* SUSCEPTIBILITY

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Abstract

The 4-aminoquinoline naphthoquine (NQ) and the thiazine dye methylene blue (MB) have potent *in vitro* efficacy against *P. falciparum*, but susceptibility data for *P. vivax* are limited. The species- and stage-specific *ex vivo* activities of NQ and MB were assessed using a modified schizont maturation assay on clinical field isolates from Papua, Indonesia, where multidrug resistant *P. falciparum* and *P. vivax* are prevalent. Both compounds were highly active against *P. falciparum* (median IC_{50} of NQ = 8.0 nM [Range: 2.6-71.8 nM] and MB = 1.6 nM [0.2-7.0 nM]) and *P. vivax* (NQ = 7.8 nM [1.5-34.2] and MB =1.2 nM [0.4-4.3]). Stage-specific drug susceptibility assays revealed significantly greater IC_{50}s in parasites exposed at trophozoite compared to ring stage for NQ in *P. falciparum* (26.5 versus 5.1 nM, p=0.021) and *P. vivax* (341.6 versus 6.5 nM, p=0.021), and for MB in *P. vivax* (10.1 versus 1.6 nM, p=0.010). The excellent *ex vivo* activity of NQ and MB against both *P. falciparum* and *P. vivax* highlights their potential utility for the treatment of multidrug resistant malaria in areas endemic for both species.

Introduction

Early diagnosis and effective treatment of malaria is central to the success of malaria control programs. However, this strategy is compromised by the emergence of drug resistant parasites that are well established in *P. falciparum* and increasingly recognised in *P. vivax* (1, 2). The emergence of multidrug resistant parasites throughout the malaria endemic world has been a driving force in the development and deployment of a variety of artemisinin-based combination therapies (ACT) for the treatment of falciparum malaria (3). Endorsed by the World Health Organization (WHO), ACT is now first-line anti-malarial policy in over 80 countries. Although chloroquine (CQ) remains the treatment of choice for vivax malaria, this position is increasingly under threat from the emergence and spread of CQ resistant *P. vivax* that, in some areas, have reached high levels requiring the revision of national guidelines (2).

The ability of parasites to develop resistance to anti-malarial drugs is relentless, as evidenced by reports of the emergence and spread of decreased *in vivo* and *in vitro* susceptibility to most anti-malarial agents including the artemisinin derivatives (4, 5) and ACT combination therapies (6, 7). It is imperative that the development of new and effective partner drugs is maintained to ensure an armamentarium of highly effective anti-malarial treatments.

Naphthoquine phosphate (NQ), a 4-aminoquinoline compound first synthesized in China in the 1980s, demonstrates excellent efficacy against the murine malaria parasite *P. berghei* and CQ resistant *P. falciparum* (8-10). NQ has been combined with artemisinin as a fixed-dose combination therapy (ARCO®) with proven *in vivo* efficacy against falciparum (11-13) and vivax malaria (14-16). Although approved and registered in more than ten countries, *in vitro* NQ susceptibility data in clinical isolates of *P. falciparum* are limited, with no published *ex vivo* data yet available for *P. vivax*.

The anti-malarial activity of the thiazine dye methylene blue (MB) was first reported at the end of the 19th century (17). Although MB's anti-malarial efficacy has been proven in

several studies over the next decades, MB was abandoned from the drug development pipeline due to its low tolerability which was the result of unpleasant, but reversible adverse effects (18). There is renewed interest in the use of MB in the modern era of malaria therapeutics, with studies highlighting its potent schizonticidal activity against CQ resistant *P. falciparum* (19-24), as well as gametocyticidal activity (25-27). Furthermore, clinical trials in Africa have shown that effective anti-plasmodial doses of MB are safe in both adults and children, including G6PD-deficient individuals (28, 29). MB has also been shown to have *ex vivo* activity against CQ sensitive *P. vivax* (30), but has yet to be assessed against CQ resistant *P. vivax*.

The objectives of the current study were to examine the species- and stage-specific *ex vivo* susceptibilities of NQ and MB in clinical field isolates of *Plasmodium* from an area with known multidrug resistance in *P. falciparum* and *P. vivax*, and to define the cross-susceptibility patterns between NQ and MB and conventional anti-malarials.

Materials and Methods

Field location and sample collection

Plasmodium isolates were collected from patients attending malaria clinics in Timika (Papua, Indonesia), a region known to be endemic for *P. falciparum* isolates resistant to CQ, AQ, and sufadoxine-pyrimethamine (SP), and *P. vivax* isolates resistant to CQ (31-33). Unpublished clinical data from 2015 suggests that dihydroartemisinin (DHA) and PIP retain high efficacy against both *Plasmodium* species. Patients with symptomatic malaria were recruited into the study if singly infected with *P. falciparum* or *P. vivax*, with a parasitaemia of between 2,000 μL^{-1} and 80,000 μL^{-1}, and the majority (>70%) of parasites at ring stage of development. After written consent was obtained, venous blood (5 mL) was collected and after removal of host white blood cells by using cellulose filters, the packed infected red blood cells (iRBCs) were used for the *ex vivo* drug susceptibility assay.

Ex vivo drug susceptibility assay

Drug plates were pre-dosed with standard anti-malarials chloroquine (CQ), amodiaquine (AQ), piperaquine (PIP), mefloquine (MFQ), and artesunate (AS) (WWARN QA/QC Reference Material Programme) (34), plus NQ (=MMV17; Swiss Tropical and Public Health Institute, Basel, Switzerland on behalf of Medicines for Malaria Venture, MMV) and MB (Sigma-Aldrich, Australia).

Drug susceptibility of *P. vivax* and *P. falciparum* isolates was measured using a protocol modified from the WHO microtest as described previously (33, 35, 36). In brief, 200 µL of a 2% haematocrit Blood Media Mixture (BMM), consisting of RPMI 1640 medium plus 10% AB^+ human serum (*P. falciparum*) or McCoy's 5A medium plus 20% AB^+ human serum (*P. vivax*) was added to each well of pre-dosed drug plates containing 11 serial concentrations (2-fold dilutions) of the anti-malarials (maximum concentration shown in brackets) CQ

(2,993 nM), AQ (158 nM), PIP (1,029 nM), MFQ (338 nM), AS (49 nM), NQ (481 nM), and MB (51 nM). A candle jar was used to mature the parasites at 37.0°C for 35-56 hours. Incubation was stopped when >40% of ring stage parasites had reached mature schizont stage in the drug-free control wells.

Thick blood films made from each well were stained with 5% Giemsa solution for 30 minutes and examined microscopically. The number of schizonts per 200 asexual stage parasites was determined for each drug concentration and normalised to the control well.

To investigate the stage-specific drug susceptibility of anti-malarial action, a subgroup of isolates with initially greater than 90% ring stage parasites was exposed to the drugs for 24 hours. The iRBCs were then washed with phosphate buffered saline using centrifugation and were re-suspended in drug-free medium and cultured for another 20-24 hours before harvest (i.e., ring stage assay). The same isolate was cultured for 20-24 hours in the absence of drugs until 85-95% of parasites had reached trophozoite stage; these were then drug exposed for 24 hours until harvest (trophozoite stage assay) (33, 37).

Quality control procedures

Microscopy quality control was assured by randomly selecting recordings for two drugs per isolate which were read by a second microscopist. If the raw data derived by the two microscopists lead to a significant shift in the IC_{50} estimates of the selected drugs (i.e., more than one dilution difference), the whole assay (i.e., all standard drugs and experimental compounds) was re-read by a second reader and the results compared. Discrepant results were resolved by a third reading by an expert microscopist. Drug plate quality was assured by testing drug plates using the same methodology with *P. falciparum* laboratory strains K1 and FC27.

Data analysis

The dose-response data were analysed using nonlinear regression analysis (WinNonLn 4.1, Pharsight Corporation) and the IC_{50} value derived using an inhibitory sigmoid Emax model. *Ex vivo* IC_{50} data were only used from predicted curves where the E_{max} and E_0 were within 15% of 100 and 1, respectively. Data analysis was performed using STATA (version 13.1) and GraphPad Prism (version 6) software. The Mann-Whitney U test, Wilcoxon Signed Rank Test, and Spearman Rank correlation were used for nonparametric comparisons and correlations.

Ethical approval

Ethical approval for this study has been obtained from the Eijkman Institute Research Ethics Commission, Eijkman Institute for Molecular Biology, Jakarta, Indonesia, and the Human Research Ethics Committee of the Northern Territory (NT) Department of Health & Families and Menzies School of Health Research, Darwin, Australia.

Results

Drug susceptibility

A total of 147 clinical isolates from patients with single-species infections of either *P. falciparum* (n=80) or *P. vivax* (n=67) were assessed. The standard anti-malarials were assayed for all isolates, as well as NQ in 63 isolates (25 *P. falciparum* and 38 *P. vivax*) between June and October 2011 and again between June and September 2013. MB was tested in 113 isolates (63 *P. falciparum* and 50 *P. vivax*) between January and September 2013. NQ and MB were tested in parallel in only 8 *P. falciparum* and 21 *P. vivax* isolates. Adequate growth for harvest was achieved in 84% (21/25) of *P. falciparum* and 76% (29/38) of *P. vivax* isolates in which NQ was tested, and in 83% (52/63) of *P. falciparum* and 82% (41/50) of *P. vivax* isolates in which MB was tested. Baseline characteristics of the isolates processed are presented in Table 1.

The IC_{50} values for isolates which were successfully cultured and the comparison with IC_{50}s for *P. falciparum* laboratory strains FC27 and K1 are shown in table 2 and figure 1. Although NQ and MB were tested on different drug plate lots, quality control experiments using laboratory strains and the same assay method showed no difference between drug plate lots for either the standard anti-malarials, or the test compounds NQ and MB. In the MB group, the median IC_{50}s in *P. falciparum* were significantly higher compared to *P. vivax* for AS (3.8 versus 1.7 nM, p=0.002) and MB (1.6 versus 1.2 nM, p<0.001), but lower for CQ (93.7 nM versus 146.0, p<0.001), AQ (13.7 versus 23.4 versus, p<0.001), and MFQ (10.5 nM versus 16.8, p=0.015).

NQ showed very potent activity against both species (8.0 nM in *P. falciparum* and 7.8 nM in *P. vivax*), and this was significantly lower than the activities of all of the standard anti-malarials tested, with the exception of AS. MB exhibited excellent activity (median IC_{50}s of

1.6 nM against *P. falciparum* and 1.2 nM against *P. vivax*), exceeding the activity of all standard anti-malarials tested, including the artemisinin derivative AS (Table 2, Figure 1).

Stage-specific drug activity

In *P. vivax* isolates, the median IC_{50} of all drugs was significantly higher at the trophozoite stage compared to the ring stage, the ratio being 1.5 to 8-fold higher for the standard anti-malarials and MB, and 50-fold higher for NQ (Table 3 and Figure 2). The difference in stage specificity was much less in *P. falciparum* isolates with significance only reached for PIP (21.9 nM for rings versus 128.2 nM for trophozoites; p=0.045) and NQ (5.1 nM versus 26.5 nM; p=0.021).

Cross-susceptibility patterns

Growth inhibition by NQ was strongly correlated in *P. falciparum* and *P. vivax* with AQ, PIP and MFQ, but only with CQ in *P. vivax* and with AS in *P. falciparum* isolates (Table 4). For MB, moderate correlations were observed for CQ, AQ, PIP, and MFQ in *P. falciparum*, but none in *P. vivax*.

Discussion

The current study highlights the potent *ex vivo* activity of NQ and MB against multidrug resistant isolates of *P. falciparum* and *P. vivax*. The IC_{50} values of NQ in *P. falciparum* (median IC_{50}: 8.0 nM, interquartile range: 26.5 nM) were similar to those reported in culture-adapted *P. falciparum* isolates from Papua New Guinea (geometric mean: 7.0, 95% CI: 5.5-8.8 nM) (38). Our *ex vivo* study presents additional evidence for NQ's potent activity against highly CQ resistant isolates of *P. falciparum* and *P. vivax*, revealing lower IC_{50}s for NQ than all of the standard drugs with the exception of AS. The *ex vivo* activity of MB was greater than that of NQ, in line with previous observations in *P. falciparum* (19-24, 39) and *P. vivax* (30), with lower IC_{50}s than all standard drugs including AS for both species.

Inter-species comparisons of drug susceptibilities revealed slightly different patterns in the two subgroups of isolates. Whilst these observations might suggest underlying species differences in pharmacodynamic activity, the differences were of marginal magnitude and may also reflect statistical chance.

Positive correlations of drug susceptibilities are potentially indicative of either similar modes of action, or similar pharmacokinetic properties of these drugs. Alternatively, they can represent acquired resistance on the background of previous anti-malarial resistance phenotypes. Previous studies of *P. falciparum* have documented positive correlations between the IC_{50} values for the quinoline class of compounds and related drugs (38, 40). The correlation of NQ with AQ, PIP, MFQ, and AS observed in *P. falciparum* in the present study was similar to that reported from neighbouring Papua New Guinea (38). Surprisingly, we observed no correlation between NQ and CQ IC_{50}s in *P. falciparum*. However, in *P. vivax* isolates, NQ IC_{50} values were positively correlated with all the quinoline-based drugs, but not AS. In contrast, the only significant correlations for MB were with CQ, AQ and PIP in *P. falciparum*, a pattern similar to that observed in previous studies (22, 24). MB's

parasitocidal activity is postulated to be mediated by the inhibition of haem polymerization (39, 41) and glutathione reductase in *P. falciparum* (42, 43). Assuming a similar mode of cytotoxicity in *P. vivax*, the contrasting correlation most likely indicates differences in drug uptake and partition between the two species.

We observed a marked stage-specific action for NQ. Compared to parasites at ring stage, the trophozoites of both *P. falciparum* and *P. vivax* were resistant to NQ, with 5-fold and 50-fold higher IC_{50}s, respectively. The stage-specificity was less apparent for MB, only reaching significance for *P. vivax* trophozoites, which had 6-fold higher IC_{50}s compared to ring stages. The findings for NQ are at odds with previous data for other conventional anti-malarials such as CQ and AQ which manifest a far greater difference in stage-specific activity for *P. vivax*, although the number of isolates tested was small (33, 37). Further studies are needed to validate these findings and investigate the phenomenon of innate versus acquired drug tolerance in *Plasmodium*.

The potent NQ *ex vivo* activity demonstrated against both *P. falciparum* and *P. vivax* suggests that NQ may be a suitable candidate as an ACT partner drug in regions where these species are co-endemic. When combined with artemisinin derivatives, NQ has achieved adequate clinical efficacy against falciparum malaria (11-13), as well as CQ sensitive and CQ resistant vivax malaria (14-16). However, concerns have been raised that the currently available single-dose regimen (ARCO™) provides inadequate reduction of the early parasite biomass, leaving the more slowly eliminated NQ vulnerable to the selection of drug resistance. Our data demonstrate a correlation in anti-malarial activity with other 4-aminoquinolines and together with a report of the induction of NQ resistance in an experimental rodent model (8), highlights the importance of closely monitoring drug efficacy, particularly in areas where quinoline-based ACTs have been in widespread use.

The current study also confirms MB's remarkable *ex vivo* efficacy against resistant strains of *P. falciparum* and *P. vivax*, showing high potency, a broad stage-specificity of action, including gametocyte stages (25), and synergism with other anti-malarials (19, 21, 44). Although researchers and drug developers have been aware of the anti-malarial properties of MB against *P. falciparum* for almost a century, it has never been widely used in clinical practise, largely due to its poor tolerability profile. The latter includes headaches, nausea, and discolouration of urine and sclera, all of which are reversible. Neurotoxicity and mutagenicity have also been observed in animal models (45). However, recent clinical studies have demonstrated MB's ability to elicit adequate cure against *P. falciparum* with acceptable tolerability in young children (46, 47) and MB plasma levels have been shown to be safe at concentrations ten-fold higher than the IC_{50}s observed in our *ex vivo* experiments (48). In areas where artemisinin resistance is emerging, the partner drugs in combination therapies are under increasing pressure for the selection of resistance and the therapeutics available are limited. In this context, the utility of MB in the treatment of multidrug resistant malaria warrants further investigation.

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434 **Figure Legends**

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436 **Figure 1**: *Ex vivo* drug susceptibility (median IC_{50}s) to standard anti-malarials, NQ (A) and
437 MB (B) in *P. falciparum* (closed symbols) and *P. vivax* (open symbols) clinical isolates (*p*,
438 Wilcoxon rank sum test).

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440 **Figure 2**: *Ex vivo* drug susceptibility (median IC_{50}s) for NQ (A) and MB (B) according to
441 species tested and for paired ring (closed symbols) versus trophozoite (open symbols) stage
442 parasites (*p*, Wilcoxon rank sum test).

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446 **Table 1: Baseline characteristics of isolates for which *ex vivo* assay was accomplished**

Group	Naphthoquine		Methylene blue	
Baseline Characteristics	***P. falciparum* n=25**	***P. vivax* n=38**	***P. falciparum* n=63**	***P. vivax* n=50**
Total number of isolates reaching harvest (%)	21 (84.00%)	29 (76.32%)	52 (82.54%)	41 (82.00%)
Median (range) delay from venepuncture to start of culture (minutes)	160 (85-310)	178 (55-330)	125 (60-310)	148 (55-330)
Median (range) duration of assay (hours)	44 (32-48)	47 (42-50)	45 (32-50)	46 (32-50)
Geometric mean (95% CI[a]), parasitaemia (asexual parasites/µL)	24,102 (15,856-36,637)	25,001 (17,487-35,742)	13,343 (11,059-16,099)	22,454 (16,531-30,501)
Median initial % (range) of parasites at ring stage	100[b]	96 (71-99)	100[b]	96 (70-99)
Mean (95% CI) schizont count at harvest	50 (16-76)	45 (13-70)	42 (14-75)	43 (13-70)

447 [a] CI, confidence interval; [b] No range given (all values were 100%)
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Table 2: *Ex vivo* drug susceptibility for each drug according to species tested

	P. falciparum lab lines[%]		*P. falciparum* clinical field isolates			*P. vivax* clinical field isolates			
Drug	**FC27 (CQ^S)**	**K1 (CQ^R)**	**n* (%)**	**Median IC_{50} (range), nM**	***p*[#]**	**n* (%)**	**Median IC_{50} (range), nM**	***p*[#]**	***p*[@]**
CQ	24.1	140.5	21 (100)	80.9 (10.9-158.7)	<0.001	29 (100)	106.4 (9.0-334.6)	<0.001	0.485
AQ	20.3	26.9	21 (100)	22.5 (4.6-54.2)	<0.001	29 (100)	21.2 (8.8-49.2)	<0.001	0.549
PIP	31.7	47.2	21 (100)	36.9 (10.6-121.2)	<0.001	29 (100)	23.0 (3.5-51.9)	<0.001	0.014
MFQ	53.8	13.7	21 (100)	19.0 (3.2-53.3)	0.030	29 (100)	16.8 (4.9-61.5)	<0.001	0.791
AS	10.1	7.7	21 (100)	3.8 (0.8-15.7)	[0.001]	29 (100)	1.8 (0.9-13.5)	[0.001]	0.012
NQ	13.1	15.3	21 (100)	8.0 (2.6-71.8)		29 (100)	7.8 (1.5-34.2)		0.7015
CQ	16.3	121.4	52 (100)	93.7 (28.4-279.0)	<0.001	41 (100)	146.0 (29.2-383.4)	<0.001	<0.001
AQ	14.2	21.3	50 (96)	13.7 (4.6-28.3)	<0.001	40 (98)	23.4 (3.7-49.5)	<0.001	<0.001
PIP	45.9	38.1	52 (100)	25.0 (5.2-58.1)	<0.001	40 (98)	21.5 (3.4-75.3)	<0.001	0.440
MFQ	72.0	16.3	52 (100)	10.5 (2.8-38.0)	<0.001	41 (100)	16.8 (2.4-81.4)	<0.001	0.015
AS	9.7	5.1	51(100)	3.8 (0.7-31.0)	<0.001	40 (98)	1.7 (0.4-19.7)	<0.001	0.002
MB	7.2	5.7	51 (100)	1.6 (0.2-7.0)		40 (98)	1.2 (0.4-4.3)		<0.001

[%] Mean IC_{50} values (derived from three independent experiments) assessed by *in vitro* schizont maturation quantified by microscopy; CQ^S, chloroquine sensitive laboratory strain; CQ^R, chloroquine resistant laboratory strain; * Total number of assays with acceptable IC_{50} (percentage of samples which fulfilled criteria for successful culture); [#] Comparison of each drug (Wilcoxon rank sum test) with either NQ (top) or MB (bottom); [@] Significant difference in median drug IC_{50} between species (Wilcoxon rank sum test).

458 **Table 3: *Ex vivo* susceptibilities for paired isolates exposed for 24 hours at ring and trophozoite stage**

Drug	*P. falciparum*				*P. vivax*			
	n[a]	Median IC_{50} (range), nM		p^b	n[a]	Median IC_{50} (range), nM		p^b
		Rings	Trophozoites			Rings	Trophozoites	
CQ	6	107.9 (60.2-163.6)	142.7 (25.5-163.6)	0.855	11	81.7 (27.6-190.2)	705.6 (99.9-4,033.3)	<0.001
AQ	7	16.2 (4.9-20.8)	18.0 (7.8-25.4)	0.570	11	20.0 (2.1-39.5)	47.7 (7.6-89.8)	0.031
PIP	6	21.9 (7.8-33.3)	128.2 (13.8-359.3)	0.045	11	18.0 (3.5-69.6)	30.3 (18.1-204.5)	0.042
MFQ	7	15.3 (4.7-46.9)	17.3 (4.6-30.6)	0.570	11	21.4 (4.9-41.0)	34.9 (11.7-85.5)	0.042
AS	6	2.1 (0.7-5.1)	3.9 (1.3-8.1)	0.584	11	1.9 (0.9-5.7)	7.2 (0.8-19.3)	0.045
NQ	4	5.1 (2.5-7.0)	26.5 (16.6-36.5)	0.021	4	6.5 (4.0-18.8)	341.6 (150.8-622.6)	0.021
MB	4	4.1 (3.1-6.9)	4.4 (3.5-6.8)	0.773	6	1.6 (0.6-3.7)	10.1 (1.9-36.5)	0.010

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460 [a] n, number of paired isolates, [b] Comparison of drugs tested at ring and trophozoite stage
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463 **Table 4: Correlation of *ex vivo* anti-malarial susceptibilities in *P. falciparum* and *P.***

464 ***vivax* clinical field isolates**

Anti-malarial combination	*P. falciparum*			*P. vivax*		
	r_s[a]	*p*	df[b]	r_s[a]	*p*	df
NQ-CQ	0.179	0.437	21	0.528	0.003	28
NQ-AQ	0.583	0.006	21	0.418	0.024	28
NQ-PIP	0.607	0.004	21	0.413	0.026	27
NQ-MFQ	0.495	0.023	21	0.380	0.042	28
NQ-AS	0.609	0.003	21	0.015	0.937	28
MB-CQ	0.422	0.002	51	0.021	0.900	40
MB-AQ	0.393	0.005	50	-0.105	0.520	40
MB-PIP	0.385	0.005	51	0.116	0.481	39
MB-MFQ	0.327	0.020	51	0.021	0.897	40
MB-AS	0.170	0.239	50	0.107	0.516	39

465 [a] Spearman rank correlation, [b] df, degrees of freedom

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