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RESEARCH PAPER

Methnaridine is an orally bioavailable, fast-killing and long-acting antimalarial agent that cures *Plasmodium* infections in mice

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Background and Purpose: Malaria is one of the deadliest diseases in the world. Novel chemotherapeutic agents are urgently required to combat the widespread *Plasmodium* resistance to frontline drugs. Here, we report the discovery of a novel benzonaphthyridine antimalarial, methnaridine, which was identified using a structural optimization strategy.

Experimental Approach: An integrated pharmacological approach was used to evaluate the antimalarial profile of methnaridine. The pharmacokinetic properties of methnaridine were investigated along with the associated safety profile. Host immune response patterns were also analysed.

Key Results: Methnaridine exhibited potent antimalarial activity against *P. falciparum* (3D7: IC_{50} = 0.0066 μM; Dd2: IC_{50} = 0.0056 μM). In *P. berghei*-infected mice, oral administration effectively suppressed parasitemia (ED_{50} = 0.52 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$) and cured the established infection (CD_{50} = 10.13 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$). These results are equivalent to or better than those of other antimalarial agents in clinical use. Notably, a four-dose oral regimen at a dosage of 25 $mg{\cdot}kg^{-1}$ achieved a complete cure of *P. berghei* infection in mice. Methnaridine exhibited a rapid parasiticidal profile (PCT_{99} = 36.0 h) and showed no cross-resistance to chloroquine. Pharmacokinetic studies revealed that methnaridine is readily absorbed, long-lasting and slowly cleared. The safety profile of methnaridine is also satisfactory (maximum tolerated dose = 1,125 $mg{\cdot}kg^{-1}$). In addition, following methnaridine treatment, infection-induced Th1 immune response was almost fully alleviated in mice.

Conclusion and Implications: Methnaridine is an orally bioavailable, fast-acting and long-lasting agent with excellent antimalarial properties. Our study highlights the potential of methnaridine for clinical development as a promising antimalarial candidate.

KEYWORDS
antimalarial activity, malaria, methnaridine, *Plasmodium berghei*, *Plasmodium falciparum*

Abbreviations: cLogP, calculated octanol–water partition coefficient; CYPs, cytochrome P-450; FDA, Food and Drug Administration; PCT99, the time required to achieve a 99% reduction of parasitemia; PND, pyronaridine; RBCs, red blood cells; RI90, resistance index.

1 | INTRODUCTION

Malaria, one of the deadliest infectious diseases in the world, is caused by infection with protozoan parasites of the genus *Plasmodium*. There are over 200 million new cases of malaria each year, and in 2018, the disease resulted in 405,000 deaths (World Health Organization [WHO], 2019a). Children under 5 years old are the most vulnerable, accounting for over 60% of all deaths in the world (WHO, 2019a). In the absence of a clinically approved vaccine, chemotherapy still remains the cornerstone of malaria control, with artemisinin-based combination therapies (ACTs) predominantly being deployed as first-line antimalarial treatments (White et al., 2014). Currently, the widespread resistance of *Plasmodium* to available drugs represents one of the biggest global health issues. Of particular concerns are the widespread artemisinin resistance in the Greater Mekong Subregion and the *de novo* emergence of *Pf*kelch13-mediated artemisinin resistance in Africa (Conrad & Rosenthal, 2019; Phillips et al., 2017; Tilley, Straimer, Gnädig, Ralph, & Fidock, 2016; Uwimana et al., 2020; WHO, 2018). In addition, as this disease primarily affects poor populations in developing countries and low-income areas, the price of antimalarial treatment represents a vital factor in relation to the success of programmatic strategies for malaria control and elimination. Therefore, novel, efficacious, and affordable antimalarial drugs are urgently required to act as the next line of defence to combat the development of resistance to known drugs.

Pyronaridine (PND) is a China FDA-approved antimalarial agent that was discovered by the China National Institute of Parasitic Diseases in the 1970s. Pyronaridine is highly potent against the blood-stage of *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* and shows no cross-resistance to other antimalarials in clinical use (Chen & Zheng, 1992; Croft et al., 2012; Ringwald, Bickii, Same-Ekobo, & Basco, 1997). Resistance to pyronaridine has only gradually emerged in field populations, and the impact of resistance is ameliorated when the drug is utilized as a combination therapy (Croft et al., 2012). Recently, a pyronaridine/artesunate combination has been prequalified by WHO and included in WHO's Essential Medicines List (WHO, 2019b). This combination has also been registered in Africa and Southeast Asia for the treatment of *P. falciparum* and *P. vivax* malaria.

We were interested in the benzonaphthyridine scaffold, the core of pyronaridine, which is a unique pharmacophore that is not observed in other antimalarial agents. However, the cost of pyronaridine is well beyond the reach of most patients compared with other antimalarial drugs and this factor limits its use in low-income regions/countries. In this context, we performed a structural optimization of pyronaridine using a new strategy as follows:- (i) retaining the benzonaphthyridine core structure, which is essential for antimalarial activity and (ii) focusing on the modification at the dual basic amine side chains, replacing the expensive pyrrolidine ring with other lower-cost amine groups. A novel benzonaphthyridine derivative methnaridine (MND) was identified. Methnaridine proved to be highly potent against drug-sensitive and multidrug-resistant *P. falciparum* in vitro. Methnaridine is orally active at very low doses as demonstrated by the Peters' suppressive test and Rane's curative test in *P. berghei* rodent models. Furthermore, methnaridine did not exhibit cross-resistance to chloroquine. The safety profiles and the absorption, distribution, metabolism and excretion, and pharmacokinetic characteristics of methnaridine are also described in this report. In addition, immune response patterns in infected mice following methnaridine treatment were analysed.

What is already known

- Drug resistance and unaffordable drug prices threaten the success of malaria control and elimination programs.

What does this study add

- Methnaridine is a novel and low-cost orally active agent with excellent antimalarial properties.

What is the clinical significance

- Methnaridine could potentially be further developed as a promising drug candidate for the treatment of malaria.

2 | METHODS

2.1 | In vitro antimalarial drug screening

P. falciparum 3D7 (drug-sensitive) and Dd2 (chloroquine-, mefloquine-, and pyrimethamine-resistant) were cultured according to the method described by Trager and Jensen (1976; Xu et al., 2013). Parasites were maintained in fresh human erythrocytes at 2% haematocrit in complete culture medium (Thermo Fisher Scientific Cat# 22400089) containing 0.5% AlbuMAX II (Thermo Fisher Scientific Cat# 11021045). In vitro drug screening was performed using the SYBR green I fluorescence assay (Smilkstein, Sriwilaijaroen, Kelly, Wilairat, & Riscoe, 2004). Synchronous ring-stage parasites (>80%) were plated in triplicate at 2% haematocrit and 0.5% parasitemia in 100 μl and treated with serial dilutions of methnaridine and pyronaridine (concentrations: 0.78, 1.56, 3.1, 6.3, 12.5, 25, and 50 nM) at 37°C for 72 h. In vitro antimalarial activity was expressed as the compound concentration that inhibited parasite growth by 50% (IC_{50}). The experiments were repeated three times.

2.2 | Animals

Healthy male and female Kunming mice (aged 4–6 weeks, 20 ± 2 g) were purchased from Shanghai SLAC Laboratory Animal Company Limited. Animals were housed in sterilized polycarbonate cages

(18 cm × 35 cm) with sawdust bedding and maintained in the animal care facility under controlled conditions (24 ± 2°C, 45–60% humidity, 12-h light–dark cycles). The mice were acclimatized for 1 week after arrival and fed with standard rodent chow and water *ad libitum*. Animal care and experimental procedures complied with the Chinese Laboratory Animal Administration Act (2017). All animal experiments and procedures were approved by the Laboratory Animal Welfare & Ethics Committee of the National Institute of Parasitic Diseases (approval number: IPD-2015-9). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). Drug administration was performed unblinded due to the distinctive orange colour of methnaridine and pyronaridine, compared to the vehicle. Experimenters were blinded to the preparation of blood smears and parasitemia examination.

2.3 | Peters' 4-day suppressive test

The Peters' 4-day suppressive test (Meyers et al., 2019; Peters, 1965) was employed to evaluate the chemo-suppressive activity of methnaridine against *P. berghei* infection. Mice were intraperitoneally infected with 1×10^7 *P. berghei*-parasitized erythrocytes (ANKA strain, drug-sensitive); the mice were then randomly assigned to groups (n = 10 per group; each group was composed of five male and five female individuals). All the treatments were initiated at 4 h post-infection (day 0). Animals in medicated groups were administrated with 0.4 ml of methnaridine solution in deionized water (0.39, 0.49, 0.61 0.77, 0.96 and 1.20 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$) by gavage once daily for four successive days (day 0 to day 3). The control group received an equal volume of deionized water. On day 4, tail blood was collected from each mouse and blood smears were prepared, fixed with methanol, and stained with Giemsa. The number of parasite infected red blood cells was counted using a microscope. The per cent parasitemia was calculated using the formula:- $\%\text{Parasitemia} = \frac{\text{Number of parasitized RBCs}}{\text{Total number of RBC count}} \times 100$ (Misganaw, Engidawork, & Nedi, 2019). For positive smears, a minimum of 1,000 RBCs was examined. No smear was considered as negative until at least 25,000 RBCs had been examined. The per cent suppression of parasite growth was calculated using the following equation: $\%\text{Suppression} = \frac{\%\text{Parasitemia control} - \%\text{Parasitemia treated}}{\%\text{Parasitemia control}} \times 100$ (Forkuo et al., 2016). A dose–response curve was subsequently fitted and 50% and 90% effective doses (ED_{50} and ED_{90}) were calculated using Probit analysis with SPSS 19.0 software. Survival was assessed on a daily basis over an observation period of 24 days (day 0 to day 23).

2.4 | Rane's curative test

The Rane's test (Ryley & Peters, 1970) was carried out to evaluate the curative potential of methnaridine against established infection. On day 0, mice were intraperitoneally inoculated as described in Peters' 4-day test and randomly assigned to groups (n = 10 per group, five male and five female individuals per group). The mice in the latter groups remained untreated until the fourth day of the study (day 3). On day 3, blood smears were made using the tail blood of each mouse to confirm the infection and determine the parasite counts. Animals in medicated groups were then orally administrated with respective doses (3.5, 4.7, 6.3, 8.4 11.3, 15.0, 20.0, and 25.0 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$) of methnaridine once a day for 4 days (from day 3 to day 6). The control individuals received an equal volume of deionized water. All animals were checked daily for survival and potential infection symptoms from day 0 to day 28. Mice were considered to have been cured upon survival after 28 days with no parasitemia (negative blood smears) being detected on day 28.

2.5 | Parasite reduction ratio study

On day 0, mice were intraperitoneally infected as described in section 2.3 and randomly assigned to two groups (n = 10 per group, five male and five female individuals per group). On day 3, the pre-treatment parasitemia of each animal was measured, and animals were then orally administrated with a single dose of methnaridine or pyronaridine at a dosage of 20× ED_{90}. Parasitemia was monitored every 4 h for 48 h. The parasite reduction ratios at each time point with respect to the initial population were calculated.

2.6 | In vivo cross-resistance test

The experimental procedures of parasite infection and medication were followed for the cross-resistance test as described for the preceding Peters' 4-day test, except that mice were infected with a chloroquine-resistant *P. berghei* R_{CH} strain for the former test. The degree of cross-resistance was determined by comparing the activity of the drug-sensitive and drug-resistant strains using the following formula: Resistance index (RI_{90}) = $ED_{90}^{\text{resistant}}/ED_{90}^{\text{sensitive}}$ (Peters & Robinson, 1992).

2.7 | In vivo pharmacokinetics

The in vivo pharmacokinetic properties of methnaridine were investigated in Kunming mice (n = 6 per time point, three male and three female individuals were randomly assigned to per time point) following a single intravenous (2 $mg{\cdot}kg^{-1}$) or oral (5 $mg{\cdot}kg^{-1}$) administration as described previously (Wang et al., 2017). Blood samples were collected at 0.08, 0.25, 0.5, 1, 2, 4, 8, 24, 30 and 48 h post i.v. dose and 0.25, 0.5, 1, 2, 4, 6, 8, 10, 24, 30 and 48 h post p.o. dose. Plasma samples were subsequently isolated by centrifugation at 2,000× g for 15 min and prepared for analysis. The drug concentration of each sample was determined by LC–MS/MS, and pharmacokinetic parameters were analysed using Phoenix WinNonlin 7.0 software (non-compartmental model).

2.8 | Flow cytometric analysis and serum cytokine assay

Mice were intraperitoneally infected with 5×10^6 *P. berghei*-parasitized erythrocytes and randomly divided into two groups (n = 6 per group, three male and three female individuals per group). For the methnaridine-treated group, the individuals were treated as described for the Peters' 4-day test, and mice were orally administered with methnaridine at a dosage equivalent to the ED_{90} value. The vehicle-treated group received the same volume of deionized water. Uninfected healthy mice were used as controls (n = 6, three male and three female mice). On day 4, blood was collected from each mouse by heart puncture, and serum was isolated following centrifugation at 2,000× g for 15 min. The serum levels of **IL-2**, **IL-4**, **IL-6**, **IL-10**, **TNF-α** and **IFN-γ** were measured using a Cytometric Bead Array mouse Th1/Th2 cytokine kit (BD Biosciences Cat# 551287) according to the manufacturer's protocol.

Spleen was dissected out from each animal and single cell suspensions were prepared. Lymphocytes were subjected to immunofluorescent staining. To measure $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}4^+$ T cells, a Cytofix/Cytoperm™ Plus Fixation/Permeabilization Solution Kit with BD GolgiStop™ (BD Biosciences Cat# 554715) was used. Briefly, fresh splenocytes were stimulated with phorbol myristate acetate (50 ng·ml^{-1}) and ionomycin (0.5 μg·ml^{-1}), and BD GolgiStop™ (2 μM) was added to block cytokine export. Next, the cells were surface stained with anti-CD4-FITC (Thermo Fisher Scientific Cat# 11-0042-85, **RRID:AB_464897**) and then fixed, permeabilized, and stained with anti-IFN-γ-BV421 (BioLegend Cat# 505830, **RRID:AB_2563105**) or anti-IL-4-PE-cy7 (BioLegend Cat# 504118, **RRID:AB_10898116**) antibodies and analysed by flow cytometry (BD FACS Aria II, **RRID:SCR_018091**). $CD4^+CD25^+Foxp3^+$ T cells were measured using a Mouse Regulatory T Cell Staining Kit (Thermo Fisher Scientific Cat# 88-8111-40) according to the manufacturer's protocol.

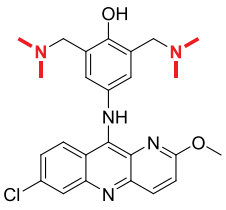
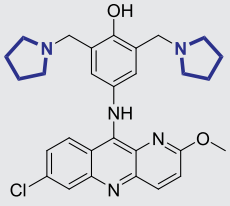
2.9 | Data and statistical analyses

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). Data are represented as mean ± SEM. Group size in the study was based on our previous studies and that of others. The group size is the number of independent values, and the statistical analysis was done using these independent values. Outliers were included in data analysis. All analyses were performed using SPSS 19.0 software. Comparisons between two groups were performed with the Student's t-test. And one-way ANOVA followed by Tukey's post hoc test was conducted for comparisons among multiple groups. Post hoc tests were run only if F achieved $P < 0.05$ and there was no significant variance inhomogeneity. In the case of heterogeneity of variance, a nonparametric Kruskal–Wallis test was performed. Comparison of survival rates was performed by log-rank test. P values less than 0.05 were considered statistically significant. Statistical analysis was undertaken only for studies where each group size was at least n = 5.

2.10 | Materials

Methnaridine tetraphosphate (4-((7-chloro-2-methoxybenzo[*b*][1,5]naphthyridin-10-yl)amino)-2,6-bis((dimethylamino)methyl)phenol tetraphosphate) and pyronaridine tetraphosphate (4-((7-chloro-2-methoxybenzo[*b*][1,5]naphthyridin-10-yl)amino)-2,6-bis(pyrrolidin-1-ylmethyl)phenol tetraphosphate) were synthesized in-house (Figures S1 and S2). Methnaridine tetraphosphate and pyronaridine tetraphosphate were dissolved in deionized water to prepare drug solutions for all in vitro and in vivo assays. All chemicals were obtained from Sigma-Aldrich unless otherwise specified.

TABLE 1 In vitro antimalarial activity and cytotoxicity of methnaridine

Drugs	Structure	*Pf*3D7 IC_{50} (μM)[a]	*Pf*Dd2 IC_{50} (μM)[a]	WI-38 CC_{50} (μM)[a]	SI[b]
Methnaridine		0.0066 ± 0.0009	0.0056 ± 0.0001	5.94 ± 0.22	900
Pyronaridine		0.0114 ± 0.0060	0.0076 ± 0.0070	8.54 ± 0.13	749

[a]Mean ± SEM of three replicates.
[b]SI, selectivity index = CC_{50} (WI-38)/IC_{50} (*Pf*3D7).

2.11 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3 | RESULTS

3.1 | Methnaridine is highly potent against drug-sensitive and multidrug-resistant *P. falciparum*

A series of pyronaridine analogues containing modifications at the amine side chains but maintaining the benzonaphthyridine scaffold were designed and synthesized. Replacement of the pyrrolidine ring of pyronaridine with other lower cost amines maintained or improved the in vitro potency (Table S1). Among the derivatives tested, the most potent compound, methnaridine demonstrated excellent antimalarial activities against both drug-sensitive (3D7) and multidrug-resistant (Dd2) strains of *P. falciparum* with IC_{50} values in the single-digit nanomolar range; these values were superior to those exhibited by the parent drug pyronaridine (Table 1).

3.2 | Methnaridine is orally efficacious in a mouse model of malaria

Motivated by the in vitro results, in vivo studies were performed in a *P. berghei* murine model using the standard Peters' 4-day suppressive test. Methnaridine was orally active and elicited dose-dependent chemo-suppressive activity against parasite growth in mice (Figure 1a). Oral treatment of methnaridine exhibited very promising in vivo efficacy with ED_{50} and ED_{90} values of 0.52 and 0.71 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$, respectively; these results were slightly better than those of pyronaridine (ED_{50} = 0.60 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$, ED_{90} = 0.84 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$, Figure S3). Following administration at 0.71 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$ (ED_{90}), methnaridine effectively prevented parasite growth, with treated mice exhibiting significantly improved survival rates over a 24-day observation period compared with vehicle-treated mice (Figure 1b). Additionally, methnaridine treatment increased animal survival relative to that of pyronaridine group, although there was no statistical difference between the two groups.

3.3 | Methnaridine cures mice infected with *P. berghei*

We utilized Rane's curative test to evaluate the ability of methnaridine to treat established infections in mice (Figure 2). Animals were orally administered with a four-dose regimen of

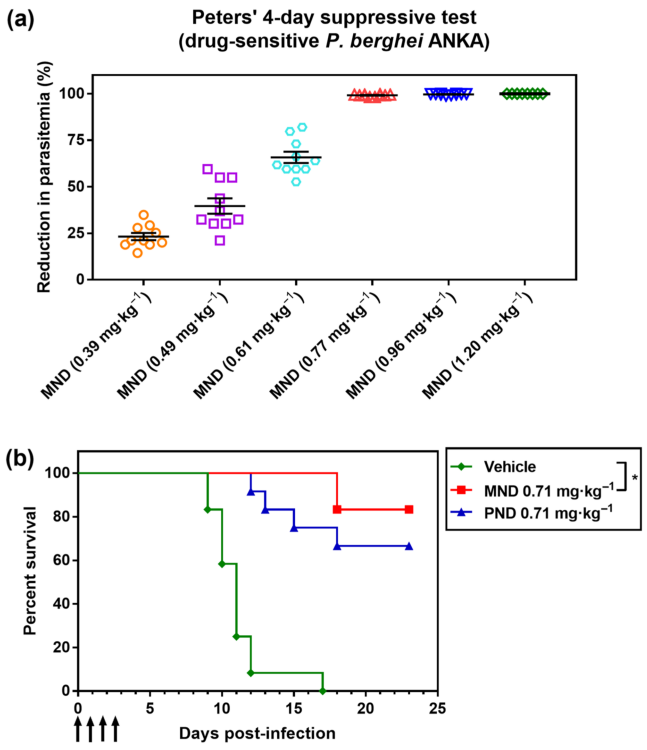


FIGURE 1 In vivo efficacy of methnaridine (MND) against *P. berghei* ANKA infection using Peters' 4-day test. Mice (n = 10 per group for efficacy test, n = 12 per group for survival study) were infected with 1×10^7 parasitized erythrocytes on day 0. Oral treatment of MND or pyronaridine (PND) was initiated at 4 h post-infection at indicated dosages once daily for 4 days (indicated by arrows). Parasitemia levels were measured on day 4. (a) Reduction in parasitemia levels and (b) survival rate curves are shown. Data are represented as mean ± SEM. *P < 0.05 (log-rank test)

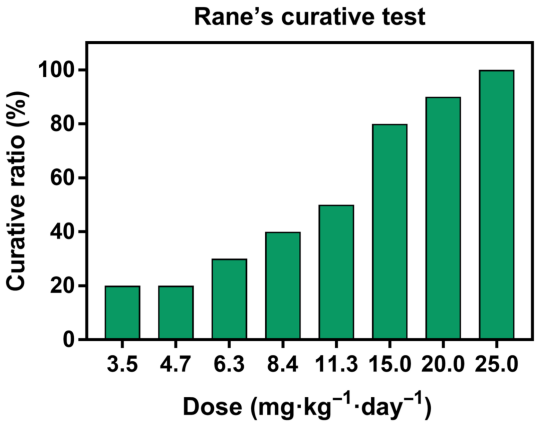


FIGURE 2 Curative efficacy of methnaridine (MND) against established *P. berghei* ANKA infection using Rane's test. Mice (n = 10 per group) were infected with 1×10^7 parasitized erythrocytes on day 0 and remained untreated until day 3. Oral treatment of MND was initiated after the confirmation of infection on day 3 at indicated dosages once daily for 4 days. Mice were considered to have been cured if no parasitemia being detected on day 28

methnaridine after confirmation of *P. berghei* infection. At doses ≥15 mg·kg^{-1}·day^{-1}, methnaridine effectively suppressed both the increase in parasitemia and the occurrence of severe symptoms of disease within the 28-day observation period. Notably, at a dosage of 25 mg·kg^{-1}·day^{-1}, methnaridine completely cured all of the infected mice, that is, these mice survived for >28 days, and no parasitemia was detected on day 28 (the last day of observation period). The CD_{50} and CD_{90} values obtained for oral treatment of methnaridine were 10.13 and 18.96 mg·kg^{-1}·day^{-1}, respectively. The curative efficacy of methnaridine was fourfold greater than for chloroquine (CD_{50} = 45.6 mg·kg^{-1}·day^{-1} × 4 days, p.o.) and almost equivalent to pyronaridine (CD_{50} = 6.8 mg·kg^{-1}·day^{-1} × 4 days, p.o.) (Chen, Tang, & Chun, 1992). No obvious clinical toxicity and/or behaviour changes were observed for any of the regimens.

3.4 | Methnaridine exhibits a fast-acting antimalarial profile

To best determine the parasite killing rate of methnaridine, we performed an in vivo parasite reduction ratio analysis that facilitated quantification of the decrease in viable parasites over 48 h, corresponding to one asexual parasite life cycle (Hastings, Kay, & Hodel, 2015). Methnaridine exhibited a rapid-killing profile without any lag phase (fast onset within 4–8 h after administration), essentially eliminating all viable parasites (>99%) within 48 h (Figure 3). The PCT_{99} value (the time required to achieve a 99% reduction of parasitemia) of methnaridine was 36.0 h. Although the latter value is slightly higher than that for pyronaridine (PCT_{99} = 27.1 h), methnaridine was still able to kill all parasites within the 48-h blood stage development of plasmodia.

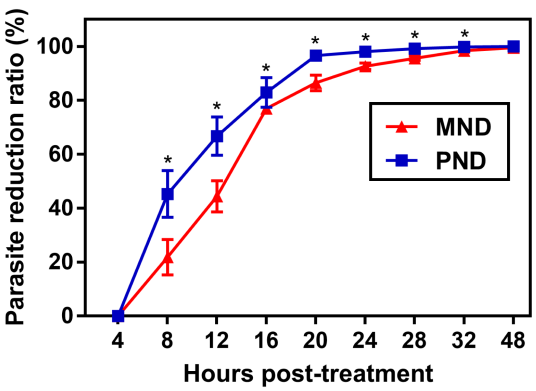


FIGURE 3 In vivo parasite reduction ratio time course in response to methnaridine (MND). Mice (n = 10 per group) were infected with 1 × 10^7 *P. berghei*-parasitized erythrocytes on day 0. On day 3, after confirmation of parasitemia, animals were orally administered with a single dose of 20 × ED_{90} MND or pyronaridine (PND). Parasitemia was monitored every 4 h for 48 h. Data are represented as mean ± SEM. *P < 0.05 (Student's t test)

3.5 | Methnaridine has no cross-resistance to chloroquine

New chemotypes are likely to offer new mechanisms of action and exhibit high antimalarial potencies across existing drug-resistant parasite lines. In vitro assays indicated that methnaridine was equally effective against both drug-sensitive and chloroquine-resistant parasites. In order to rule out the potential for cross-resistance, methnaridine was further evaluated in mice infected with a chloroquine-resistant strain, *P. berghei* R_{CH} (Figure 4). As expected, methnaridine elicited antimalarial activity against the two parasite strains. The ED_{50} and ED_{90} values of methnaridine against R_{CH} line were 0.54 and 0.70 mg·kg^{-1}·day^{-1}, respectively; these values were equivalent to those observed for the ANKA line. The resistance index, RI_{90}, calculated as the ratio of $ED_{90}^{resistance}$ to $ED_{90}^{sensitive}$, was 0.99, indicating that methnaridine was very unlikely to show cross-resistance to chloroquine.

3.6 | Methnaridine exhibits high selectivity index towards human host cells and is well tolerated in vivo

The bioactivity of methnaridine was further analysed using a cytotoxicity screening assay that measured the effects of methnaridine against normal embryonic lung fibroblasts (WI-38). Methnaridine resulted in relatively low cytotoxicity levels and had a very high selectivity index (>900) (Table 1). Next, the maximum tolerated dose of

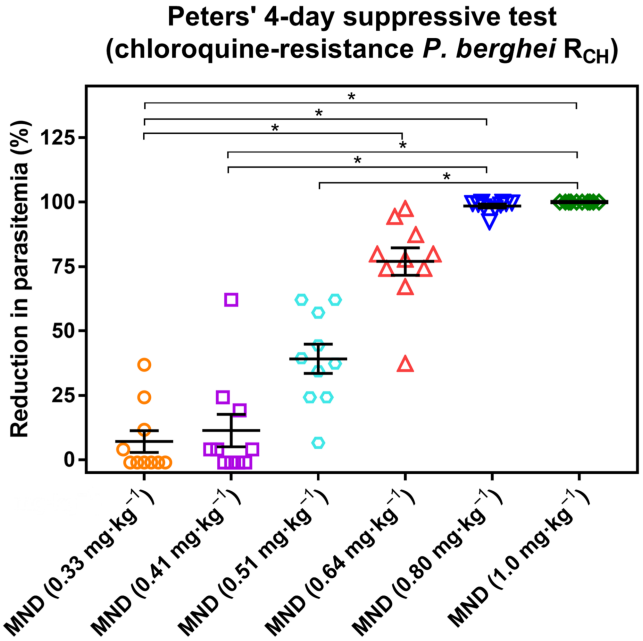


FIGURE 4 Antimalarial efficacy of methnaridine (MND) in a chloroquine-resistant *P. berghei* R_{CH} murine model using Peters' 4-day test. Mice (n = 10 per group) were infected with 1 × 10^7 parasitized erythrocytes on day 0. Oral treatment of MND was initiated at 4 h post-infection at indicated dosages once daily for 4 days. Parasitemia levels were measured on day 4. Data are represented as mean ± SEM. *P < 0.05 (nonparametric Kruskal–Wallis test)

TABLE 2 In vitro absorption, distribution, metabolism and excretion properties of methnaridine

Drugs	Solubility (μM)[a]	cLogP[b]	CYP450 IC_{50} (μM)[a]					Human liver microsomes[a]		Human hepatocyte[a]	
			1A2	2C9	2C19	2D6	3A4	$T_{1/2}$ (h)	Cl_{int} ($ml \cdot min^{-1} \cdot kg^{-1}$)	$T_{1/2}$ (h)	Cl_{int} ($ml \cdot min^{-1} \cdot kg^{-1}$)
Methnaridine	96.1	5.09	>10	>10	>10	>10	>10	4.8	6.01	9.9	2.96
Pyronaridine	—	5.89	>10	>10	>10	1.53	>10	—	—	7.8	3.78

[a]Mean of two replicates.
[b]Predicted by Molinspiration.

methnaridine was evaluated over an oral dosage range of 267 to 2,000 $mg \cdot kg^{-1}$ (Table S2). At a dosage level of 1,125 $mg \cdot kg^{-1}$, all mice survived up to 7 days (day 7 was the last day of observation) and did not exhibit apparent signs of toxicity (only one treated mice was less active, the others behave normally, no signs of emaciation, hunched back, loss of appetite, etc.), while 30% and 40% of the mice died at dosage levels of 1,500 and 2,000 $mg \cdot kg^{-1}$, respectively. Thus, the maximum tolerated dose of methnaridine was 1,125 $mg \cdot kg^{-1}$, suggesting an attractive safety profile.

3.7 | Methnaridine shows excellent absorption, distribution, metabolism and excretion and pharmacokinetic properties

Methnaridine displayed significant aqueous solubility (96.1 μM), along with a calculated octanol–water partition coefficient (cLogP) of 5.09 (Table 2). The characteristics suggest that methnaridine should exhibit favourable drug-likeness and good oral bioavailability properties in vivo. A preliminary evaluation of in vitro metabolism properties was also performed for methnaridine. This analysis revealed that methnaridine exhibits good metabolic stability with a reasonable half-life and slow clearance rate both in human liver microsomes and human hepatocytes (Table 2); these results suggest a long-lasting profile in humans. Cytochrome P-450 (CYPs) are a superfamily of enzymes involved in drug metabolism. Pyronaridine is a known CYP2D6 inhibitor. Therefore, we investigated whether methnaridine facilitates the inhibition of CYPs. No significant inhibition was observed for five important CYP isoforms at concentrations of up to 10 μM (Table 3).

TABLE 3 In vivo pharmacokinetic profile of methnaridine

Parameters	i.v. (2 $mg \cdot kg^{-1}$)	p.o. (5 $mg \cdot kg^{-1}$)
C_{max} (μM)	—	0.22
T_{max} (h)	—	0.25
$T_{1/2}$ (h)	22.3	25.1
V_z ($L \cdot kg^{-1}$)	37.3	—
Cl ($ml \cdot min^{-1} \cdot kg^{-1}$)	20.9	—
$AUC_{0\text{-}t}$ ($nmol \cdot h \cdot ml^{-1}$)	2.2	2.7
Oral bioavailability (%)	—	49.1

In vivo pharmacokinetic profile of methnaridine was subsequently determined in mice after a single i.v. (2 $mg \cdot kg^{-1}$) or p.o. (5 $mg \cdot kg^{-1}$) dose was administered (Figure 5, Table 3). Methnaridine exhibited *good* oral bioavailability (F = 49.1%), a *long* elimination *half-life* ($T_{1/2}$ = 25.1 h) and low plasma clearance. Interestingly, methnaridine was rapidly absorbed and exhibited high blood exposure following a single oral dose; peak plasma concentration was achieved only 0.25 h after administration.

3.8 | Methnaridine restores the Th1 immune response

Plasmodium infection usually induces strong immune responses and marked changes in cellular composition. Analysis of T cell frequency in splenocytes isolated on day 4 in vehicle-treated mice revealed significant $CD4^+$ T cell lymphopenia compared with uninfected controls (Figure 6a). Early leukopenia, particularly for $CD4^+$ T cells, is a well-known phenomenon during *Plasmodium* infection (Hviid & Kemp, 2000; Wangoo, Kaur, Ganguly, Bhatti, & Mahajan, 1990). Following methnaridine treatment, the level of $CD4^+$ T cells was significantly elevated, indicating that a sufficient number of T cells was raised inside the host to fight the associated infection (Figure 6a). In addition, *P. berghei* infection resulted in a significant increase in the percentages of $CD4^+IFN\text{-}\gamma^+$ T cells (Th1) as well as

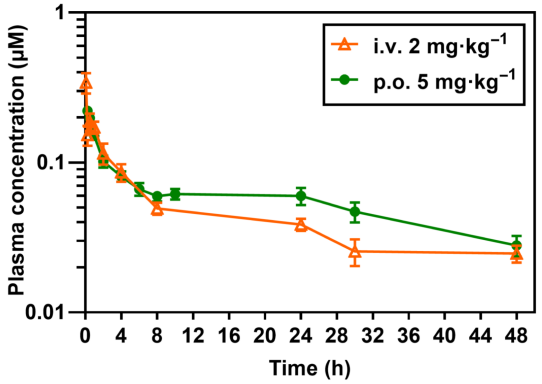


FIGURE 5 Plasma concentrations of methnaridine following intravenous (2 $mg \cdot kg^{-1}$) and oral (5 $mg \cdot kg^{-1}$) administration. Each data point represents the mean ± SEM (n = 6 per time point)

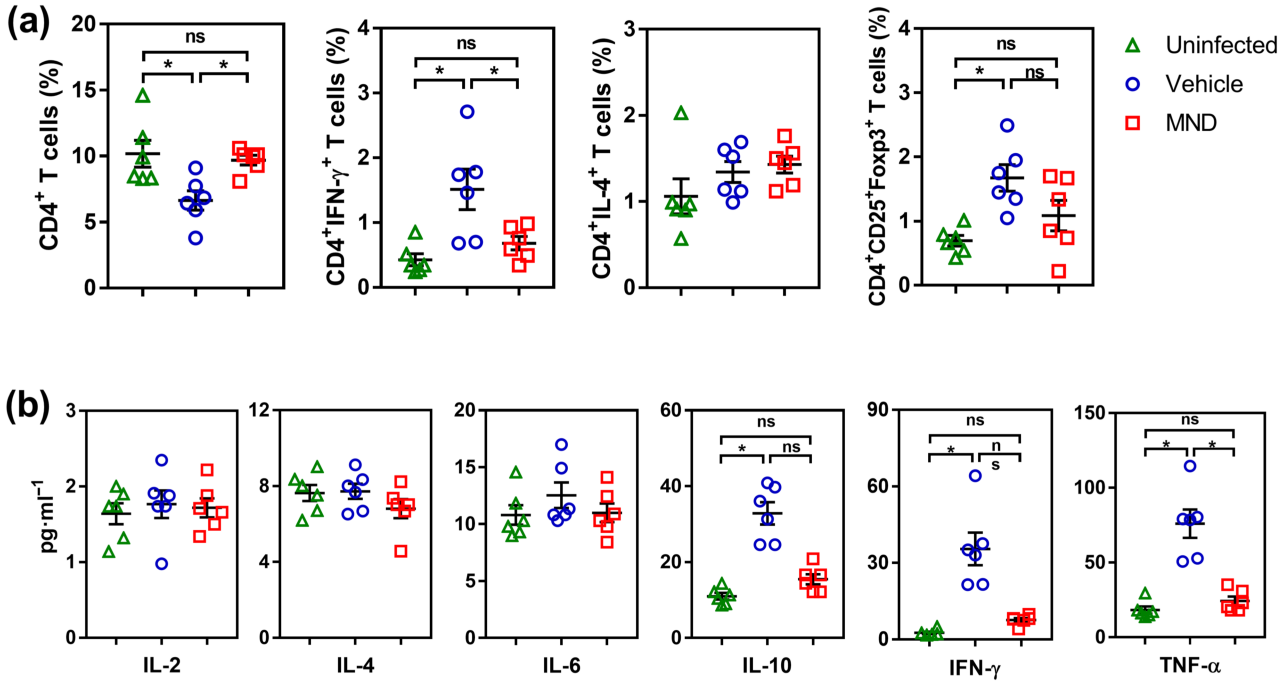


FIGURE 6 Effect of methnaridine (MND) on immune response in *P. berghei*-infected mice. (a) Flow cytometric analysis of the frequency of $CD4^+$, $CD4^+IFN\text{-}\gamma^+$ (Th1), $CD4^+IL\text{-}4^+$ (Th2), and $CD4^+CD25^+FoxP3^+$ (Treg) T cells in the spleen of uninfected mice and *P. berghei*-infected mice treated with MND (0.71 $mg{\cdot}kg^{-1}{\cdot}day^{-1} \times 4$ days, p.o.) or vehicle. (b) Serum levels of cytokines IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α determined by cytometric bead array. Data are represented as mean ± SEM ($n = 6$ per group). *$P < 0.05$; ns, no significance (one-way ANOVA or nonparametric Kruskal–Wallis test)

$CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg), while methnaridine treatment restored the levels of both cell populations to nearly normal baseline levels compared with non-infected individuals (Figure 6a). No significant difference in the population of splentic $CD4^+IL\text{-}4^+$ T cells (Th2) was observed in all groups.

The serum cytokine levels of IFN-γ and TNF-α, which are mainly derived from Th1 cells, were significantly higher in vehicle-treated mice than in uninfected individuals (Figure 6b). The levels of cytokines produced mainly by Th2 cells (IL-4 and IL-6) showed no significant change compared with uninfected controls except for IL-10 (Figure 6b). Following methnaridine treatment, as expected, the cytokine profiles returned to normal levels.

4 | DISCUSSION

Emerging parasite resistance to antimalarial drugs, if left unaddressed, could render some of the current treatment strategies ineffective and trigger a rise in global malaria incidence and mortality. Although there have been some promising developments in the identification of new compounds (Phyo et al., 2016; Wells, Hooft, & Van Voorhis, 2015), the high attrition rate encountered during clinical development and the inevitability of resistance demand continued efforts in relation to the discovery and development of new leads and candidates. In addition, in malaria-endemic countries, the patients' choice for self-treatment is driven predominantly by drug price (Mpanya, Tshefu, & Likwela, 2017; O'Connell et al., 2011). Although artemisinin-based combination therapy represents the most recommended antimalarial treatment strategy, high drug prices are often blamed for low artemisinin-based combination therapy uptake. Most patients prefer inexpensive medicines (e.g. chloroquine, **sulfadoxine-pyrimethamine**), and this is the most likely reason why less expensive, ineffective therapies persist in the local market. Therefore, efficacious and affordable new antimalarial drugs are urgently required.

The price of artesunate/pyronaridine is US$ 2.05 per adult treatment; this is much more expensive than non-artemisinin therapies (e.g., chloroquine, US$ 0.15) and other artemisinin-based combination therapies (e.g. artesunate/**amodiaquine**, US$ 0.40; **artemether**/**lumefantrine**, US$ 1.38) (The Global Fund to Fight AIDS, Tuberculosis and Malaria, 2020). The exorbitant price of the artesunate/pyronaridine combination is not only because of the expensive artemisinin active pharmaceutical ingredient but also due to the high synthetic cost of pyronaridine. Using our optimization strategy, we replaced the costly pyrrolidine ring (approximately US$ 13,000/ton) in the parent drug with the relatively inexpensive dimethylamine group (approximately US$ 800/ton); the resultant compound was identified as methnaridine. Employment of the latter strategy would reduce the chemical synthesis costs of methnaridine by 20–30% compared with the parent drug pyronaridine (Tables S3 and S4), and we estimate that the cost of methnaridine/artemisinins combinations is likely to be less than US$ 1.0 per adult treatment.

Methnaridine is highly active against both drug-sensitive and multidrug-resistant *P. falciparum*. Using *P. berghei* rodent *models*, we demonstrated that methnaridine is orally active and exhibits significant suppressive and curative properties against parasite infections. In *P. berghei* ANKA models, the suppressive ED_{90} (0.71 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$) of methnaridine was much lower than that of chloroquine (ED_{90} = 4.7 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$), **dihydroartemisinin** (ED_{90} = 4.2 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$), or **piperaquine** ($ED_{90}$ = 6.7 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$) (Birrell et al., 2015). Notably, in the clinically relevant curative murine model, the curative potency of methnaridine (CD_{50} = 10.13 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$, 4-day regimen, ANKA) against established infection was almost equivalent to that of artesunate (CD_{50} = 6.0 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$, 5-day regimen, NK-65) (Forkuo et al., 2016). In addition, based on the human equivalent dose (HED) formula (Nair & Jacob, 2016), the curative regimen (CD_{90}) in mice is equivalent in humans to 92 $mg{\cdot}day^{-1}$ × 4 days of methnaridine per adult treatment (reference body weight = 60 kg). The predicted methnaridine regimen for human is promising when compared with the recommended regimen for the best-selling artemisinin-based combination therapy, artemether/lumefantrine (80 mg + 480 mg/dose, twice daily × 3 days).

The potent growth-suppressive properties of methnaridine against blood-stage parasites is most likely attributable to the rapid onset of drug activity. Methnaridine (PCT_{99} = 36 h) exhibits rapid parasiticidal effects equivalent to those of chloroquine or lumefantrine (both PCT_{99} values are 32 h), the top-ranking antimalarials after artemisinins (Sanz et al., 2012). Methnaridine killed all parasites within a 48-h blood stage life cycle. Rapid-killing antimalarial agents are extremely beneficial in reducing initial parasite counts, particularly in severe and critical cases; these rapid-onset antimalarial agents are also useful for lowering the potential for the development of resistance to the drug itself or any partner drug in combination therapies.

Unlike other aminoquinoline antimalarial agents (Davis, Hung, Sim, Karunajeewa, & Ilett, 2005; Durrani et al., 2005), methnaridine does not exhibit cross-resistance to chloroquine both in vitro and in chloroquine-resistant *P. berghei*-infected mice. Resistance to the parent drug, pyronaridine, has only slowly emerged in clinical populations even during the 1980s–1990s when the drug was extensively used as a monotherapy. As an analogue of pyronaridine, it is likely that resistance to methnaridine will exhibit a similarly slow resistance profile and the development of resistance will most likely be retarded when it is used in combination with other antimalarials, particularly, artemisinins.

Pharmacokinetic analysis revealed that methnaridine has a long half-life, low clearance rate, and good oral bioavailability, suggesting that it could be used as a long-acting partner drug with artemisinins. Furthermore, methnaridine is readily absorbed due to a favourable balance between *lipophilicity* and *hydrophilicity*. Interestingly, unlike its parent compound, methnaridine did not present any issues in relation to CYP450 inhibition (Morris et al., 2014). The safety profile of methnaridine is also satisfactory. Methnaridine exhibited a low risk of cytotoxicity and a very high maximum tolerated dose of 1,125 $mg{\cdot}kg^{-1}$, indicating a high therapeutic index >2,000 (calculated by maximum tolerated dose/ED_{50}). All regimens investigated in maximum tolerated dose study were well tolerated by mice. Throughout the treatments, the behaviour and appearance of the animals were normal, and no obvious adverse effects or toxic reactions were observed.

$CD4^+$ T cells play a central role in the immune response to malaria (Reece et al., 2004). T lymphopenia is a well-established feature of *P. falciparum* malaria in early infection (also seen in murine malaria), although its cause remains unclear (Helmby, Jonsson, & Troye-Blomberg, 2000; Hviid et al., 1997; Hviid & Kemp, 2000; Wangoo, Kaur, Ganguly, Bhatti, & Mahajan, 1990). In this study, following the treatment with methnaridine, the $CD4^+$ T cell ratio was significantly elevated compared with the vehicle-treated controls. Protective immunity against blood-stage malaria is particularly complex. Timely and appropriate Th1 responses are mainly responsible for controlling parasitemia in the early phases of infection (Keegan & Dushoff, 2013). Indeed, we observed a significantly elevated frequency of $CD4^+IFN\text{-}\gamma^+$ T cells in infected mice. Further, cytokine profiling confirmed the Th1 immune response in the host. We also observed that methnaridine treatment normalized the cytokine levels. $CD4^+CD25^+Foxp3^+$ Tregs are *potent suppressors* of the adaptive immune system and help malaria parasites to escape from host immune responses (Hisaeda et al., 2004; Shevach et al., 2006). We observed that $CD4^+CD25^+Foxp3^+$ Tregs were up-regulated in response to acute parasite infection, and following methnaridine-mediated parasitemia clearance, the Tregs level was restored to almost normal, suggesting that methnaridine treatment contributed to breaking parasite immune escape. More detailed host immune response following treatment with methnaridine requires further investigation.

Although methnaridine showed compelling activity against *Plasmodium* infection, this study had some limitations. First, our findings were validated in murine malaria, while the antimalarial efficacy in humans has been unpredictable. Further studies evaluating the in vivo profiles of methnaridine in suitable preclinical models (e.g. humanized mouse model infected with *P. falciparum* or macaque models for malaria) will greatly aid the development of this compound. Second, there is little knowledge of the mechanism of action of methnaridine, a common problem for candidates discovered with phenotypic screening. Further drug pressuring studies for resistance selection are needed to give insight to the potential target(s) of methnaridine. In addition, as artemisinin-based combination therapy is the recommended first-line treatment by the WHO, we expect that a combination of methnaridine and artemisinins would obtain synergistic benefit on the efficacy, allowing the administration of a lower daily dose of one or both drugs. Investigations on methnaridine/artemisinins combination therapy are in progress.

5 | CONCLUSIONS

Methnaridine exhibited high potency against both drug-sensitive and multidrug-resistant *P. falciparum*. Methnaridine is orally active and able

to cure established infections while suppressing parasite growth in *P. berghei*-infected mice at very low doses. Methnaridine has desirable attributes for a novel antimalarial agent, including new chemotype, rapid killing, long-lasting effects, lack of cross-resistance, good safety profile, and, in particular, potentially low cost. Overall, the findings of this study indicate that methnaridine could be a promising drug candidate for malaria treatment.

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AUTHOR CONTRIBUTIONS

L.D. and W.W. designed and coordinated the project. W.W. and S.L. synthesized and identified the compounds. J.Y., Z.C., Y. Sun, Y. Shi, and Y.W. carried out the drug efficacy tests. W.W. and Y.Y. performed the pharmacokinetic studies. J.Y., H.Z., and Y.W. conducted the immunological experiments. W.W. and L.D. wrote the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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