Clarithromycin, a cytochrome P_{450} inhibitor, can reverse mefloquine resistance in *Plasmodium yoelii nigeriensis*-infected Swiss mice

RENU TRIPATHI*, SWAROOP KUMAR PANDEY *and* AMBER RIZVI

Division of Parasitology, Central Drug Research Institute (CSIR), Lucknow-226001, India

(*Received 15 February 2011; revised 15 April and 29 April 2011; accepted 29 April 2011; first published online 15 July 2011*)

SUMMARY

During the last 2 decades there have been numerous reports of the emergence of mefloquine resistance in Southeast Asia and nearly 50% resistance is reported in Thailand. A World Health Organization report (2001) considers mefloquine as an important component of ACT (artesunate + mefloquine) which is the first line of treatment for the control of uncomplicated/multi-drug resistant (MDR) *Plasmodium falciparum* malaria. In view of the emergence of resistance towards this drug, it is proposed to develop new drug combinations to prolong the protective life of this drug. Prior studies have suggested that mefloquine resistance can be overcome by a variety of agents such as ketoconazole, cyproheptadine, penfluridol, Icajine and NP30. The present investigation reports that clarithromycin (CLTR), a new macrolide, being a potent inhibitor of Cyt. P_{450} 3A4, can exert significant resistance reversal action against mefloquine resistance of plasmodia. Experiments were carried out to find out the curative dose of CLTR against multi-drug resistant *P. yoelii nigeriensis*. Mefloquine (MFQ) and clarithromycin (CLTR) combinations have been used for the treatment of this MDR parasite. Different dose combinations of these two drugs were given to the infected mice on day 0 (prophylactic) and day 1 with established infection (therapeutic) to see the combined effect of these combinations against the MDR malaria infection. With a dose of 32 mg/kg MFQ and 225 mg/kg CLTR, 100% cure was observed, while in single drug groups, treated with MFQ or CLTR, the cure was zero and 40% respectively. Therapeutically, MFQ and CLTR combinations 32 + 300 mg/kg doses cleared the established parasitaemia on day 10. Single treatment with MFQ or CLTR showed considerable suppression of parasitaemia on day 14 but neither was curative. Follow-up of therapeutically treated mice showed enhanced anti-malarial action as reflected by their 100% clearance of parasitaemia. The present study reveals that CLTR is a useful antibiotic to be used as companion drug with mefloquine in order to overcome mefloquine resistance in plasmodia.

Key words: *Plasmodium yoelii nigeriensis*, malaria, clarithromycin, mefloquine, drug resistance.

INTRODUCTION

Multi-drug resistant malaria is fast emerging in Southeast Asia, Africa and other continents. The national malaria control programme in tropical countries have reported high levels of resistance to chloroquine, amodiaquine, MFQ, quinine, halofantrine and combinations including pyrimethamine-sulfadoxin and LAPDAP. According to World Health Organization reports (2008), there are approximately 275 million malaria cases throughout the world with nearly 1·1million deaths occurring annually. In a recent report of World Health Organization (2010*a*), malaria causes about 2414 deaths a day, over 90% of those are in Sub-Sahara Africa. The mortality among the children and infants is highest in Africa with 5 children dying every minute.

During the last 2 decades MFQ (amino alcohol) alone or its combinations with artemisinin derivatives had been preferred over artemether, artesunate monotherapy because of higher cure rates with artesunate + MFQ and artemether + MFQ (Looareesuwan *et al*. 1997; Price *et al*. 1998) but now the resistance against MFQ has attained serious dimensions reaching a level of nearly 50% in Thailand (Nosten *et al*. 1991). Five fixed-dose ACT's combinations have been identified, namely artemether with lumefantrine, artesunate with MFQ, artesunate with pyronaridine, artesunate with amodiaquine and DHA with piperaquine for the control of uncomplicated *P. falciparum* (World Health Organization, 2010*b*, Sinclair *et al* 2010). Because of the emergence of resistance the efficacy of MFQ is being compromised and overall cure rates are declining.

CLTR, a new macrolide, is specifically used for the treatment of *Mycobacterium avium* and *M. intracellulare* (atypical mycobacteria) (Malhotra-Kumar *et al*. 2007). This macrolide has also been claimed to be effective against helminth *Echinococcus multilocularis* by virtue of its inhibition of mitochondrial ribosomes. Further the mitochondrial ribosome in *Acanthamoeba castellanii* has also been found to be a target for CLTR, resulting in inhibition of

* Corresponding author: Division of Parasitology, P.O. Box No. 173, Central Drug Research Institute (CSIR), Chattar Manzil Palace, Lucknow 226001, India. Tel: +91 522 2212411 18 Extn. 4461. Fax: +91 522 223405/223938. E-mail: renu1113@rediffmail.com

Parasitology (2011), **138**, 1069–1076.
doi:10.1017/S0031182011000850

encystment of amoebae (Mathis *et al*. 2004). *In vitro* anti-malarial activity of CLTR against schizont maturation had been studied in 2 strains of *P. falciparum* (chloroquine-sensitive *P.f*.3D7 and chloroquine-resistant *P.f*.K1) (Wisedpanichkij *et al*. 2009) but the IC_{50} values were very high i.e. >10 μM.

Being the cytochrome P_{450} inhibitor (Pinto *et al*. 2005), CLTR in the present communication has been used in combination with MFQ to reverse MFQ resistance of plasmodia.

MATERIALS AND METHODS

Animals and parasites

Outbred Swiss mice of either sex weighing 22–25 g were procured from the animal facilities at the institute and maintained on commercial pellet diet and water *ad libitum* under standard housing conditions. Ethical guidelines on handling and use of experimental animals were followed during the conduct of the study.

The rodent malaria parasite *P. yoelii nigeriensis* multi-drug resistant (MDR) was used in the study, which is resistant to chloroquine (250 mg/kg × 4 d), MFQ (128 mg/kg × 4 d) and quinine (400 mg/kg × 4 d). These are the maximum tested doses for the particular anti-malarials. The parasite was maintained in the animals through sequential passages from the blood of infected mice, obtained by cardiac puncture.

Curative dose of CLTR

To determine the curative dose of CLTR, it was given alone to groups of Swiss mice inoculated with 2×10^5 multi-drug-resistant rodent malaria parasite *P. yoelii nigeriensis*-infected erythrocytes. The concentrations at which the drug was administered were 150, 200, 250, and 400 mg/kg × 7 days. These doses were given every day in 2 divided doses for 7 days. The route of drug administration was oral. The drug was given twice in a day due to its short elimination half-life. Parasitaemia was monitored by Giemsa-stained thin blood smears on pre-determined days and the rate of survival was duly recorded.

Combination of MFQ and CLTR

To study the effect of the MFQ and CLTR combination, mice were inoculated with 2×10^5 *P. yoelii nigeriensis* MDR-infected erythrocytes. At 2–4 h after infection, they were treated with daily doses of MFQ alone (given orally once a day), CLTR alone (given orally twice a day) and with various combination groups of MFQ + CLTR (32 + 225 mg/kg, 48/32/16 + 150/75 mg/kg). For therapeutic efficacy of MFQ + CLTR different doses of this combination (64/32 + 300/150 mg/kg × 4 d) were administrated to infected mice with 0·9–2·5% established infection. Mice in the control group were injected with the parasites only. Parasitaemia was monitored by Giemsa-stained thin blood smears on pre-determined days and survival of the mice was duly recorded.

Assessment of anti-malarial interaction between mefloquine and clarithromycin

To obtain numeric values for the type of interactions, results were expressed as the sum of the fractional inhibitory concentrations (sum FIC) at the given effective concentrations by the formula (EC_x of agent A in the mixture/EC_x of agent A alone) + (EC_x of agent B in the mixture/EC_x of agent B alone) (Gupta *et al*. 2002). Sum FIC values indicate the type of anti-malarial interaction as follows: 'synergistic' if sum FIC < 1; 'fully additive' if sum FIC = 1; partially additive if sum FIC < 2 provided that both contributory FICs < 1; 'antagonistic' if any FIC > 1.

RESULTS

In the present communication anti-malarial assessment of MFQ with CLTR administered orally, has been evaluated against *P. yoelii nigeriensis* a multi-drug-resistant rodent infection, using random-bred Swiss mice following administration of individual drugs and their combinations. The parasite used in the study was resistant to orally administered chloroquine (250 mg/kg × 4 days), MFQ (128 mg/kg × 4 days) and quinine (400 mg/kg × 4 days). By oral administration of CLTR at doses of 150, 200, 250 and 400 mg/kg × 7days it was shown that the antibiotic was fully curative at 400 mg/kg × 7 days (Table 1).

For further studies MFQ dosing was evaluated at 16, 32 and 48 mg/kg × 4 days and these doses were found to be suppressive only, resulting in survival of 5 out of 5 mice each at 32 and 48 mg/kg while at 16 mg/kg only 2 mice survived beyond 28 days of observation. CLTR was also administered at 75, 150 and 225 mg/kg × 7days, these doses were also suppressive. At the highest dose of CLTR i.e. 225 mg/kg, 5 mice survived beyond 28 days, at 75 and 150 mg/kg doses, only 2 and 4 mice survived. Control untreated mice died of high parasitaemia by day 7.

When the MFQ and CLTR combinations were used at 32 mg/kg and 225 mg/kg respectively, there was 100% cure and none of the treated mice developed parasitaemia up to 28 days of observation, while drugs alone produced only 0 and 40% cure respectively during the observation period of 28 days. In another batch when CLTR dose was reduced to 150 mg/kg with MFQ 48 mg/kg, there was no parasitaemia until 24 days but 2 out of 5 mice developed a low level of parasitaemia on day 28. In the drug groups given MFQ or CLTR, most of the treated mice had developed infection on day 4. Further,

https://doi.org/10.1017/S0031182011000850 Published online by Cambridge University Press

Table 1. Curative dose of clarithromycin in Swiss mice infected with *Plasmodium yoelii nigeriensis* MDR

Drug dose × no. of days, route of administration	Mean percentage parasitaemia ± S.D. (No. of surviving mice)							Cure rate
	Day 4	Day7	Day 10	Day 14	Day 18	Day 21	Day 28	
400 mg/kg × 7, oral	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	100%
250 mg/kg × 7, oral	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0·01 ± 0·02 (1/5)	0·18 ± 0·4 (1/5)	3·34 ± 7·47 (1/5)	0 ± 0 (0/4)	80%
200 mg/kg × 7, oral	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	7·95 ± 9·31 (2/5)	14·5 ± 16·95 (2/5)	20·98 ± 24·48 (2/4)	0 ± 0 (0/2)	40%
150 mg/kg × 7, oral	0·04 ± 0·09 (1/5)	0·34 ± 0·76 (1/5)	2·38 ± 4·34 (2/5)	7·82 ± 12·85 (2/5)	19·5 ± 17·23 (2/4)	25·69 ± 32·34 (2/4)	0 ± 0 (0/2)	40%
Control	25·1 ± 13·23 (5/5)	Died						–

lower doses of CLTR i.e.75 and 150 mg/kg combined with MFQ at 16, 32 and 48 mg/kg showed complete suppression of parasitaemia up to day 10 while from day 14 onwards, a low level of parasitaemia appeared, as shown in Table 2. Nevertheless, judging from percentage cure on day 28 in MFQ alone groups which was very low (ranging from 0 to 40%) while the combination groups of MFQ and CLTR showed better protection which ranged from 40 to 100%.

Therapeutic anti-malarial effect

Mice were infected with *P. yoelii nigeriensis* and treatment was started on day 1 after confirming the average parasitaemia which ranged from 0·9–2·5%. There was an increasing trend of parasitaemia up to 60 h in all the treated batches. In MFQ/CLTR-treated groups parasitaemia started declining after 60 h. In the combination groups (32 + 300 and 64 + 300 mg/kg doses), mice showed clearance of parasitaemia on day 10 whereas at the doses of 150 + 32 and 64 mg/kg, parasitaemia was cleared on day 14. Parasitaemia in single treatments with CLTR or MFQ showed considerable suppression. Follow-up of parasitaemia in the treated mice showed complete clearance in all the combination-treated groups. Amongst the monotherapy with CLTR at 300 mg/kg, parasitaemia was cleared by day 28. Since this parasite is resistant to MFQ, a low level of parasitaemia persisted in the MFQ alone treatment group beyond 28 days. Finally all 4 batches treated with the CLTR and MFQ combination showed enhanced anti-malarial action as reflected by their 100% clearance of parasitaemia (Table 3).

Anti-malarial interaction between MFQ and CLTR

The anti-malarial interaction of MFQ in combination with CLTR was assessed against *P. yoelii nigeriensis* infection in Swiss mice. The median (range) sums of the FICs for the anti-malarial interaction between MFQ and CLTR were <0·66 for prophylactic treatment and for therapeutic treatment it was <0·85. The sums of the FICs of <1·0 in both the experiments indicated synergistic interaction between the two drugs (Table 4).

The present study also reveals that the doses of both drugs administered orally were safe and did not produce any apparent side effects.

DISCUSSION

Our present study has shown the enhanced anti-malarial effect of a conventional anti-malarial MFQ with a new macrolide CLTR.

CLTR has recently been reported to exhibit *in vitro* anti-malarial activity against the chloroquine-sensitive strain of *P. falciparum* (3D7) and the chloroquine-resistant strain *P. falciparum* K1, and the IC_{50} was reported to be >10μM for both the strains (Wisedpanichkij *et al*. 2009). This antibiotic is specifically useful for treatment of *Mycobacterium avium* and *M. intracellulare* (atypical mycobacteria) with a dose schedule of 500 mg twice daily for 7 days. Its bioavailability is nearly 50% by the oral route and is the preferred treatment for children. This is preferred for oral use because of its basic stability in the stomach and its ability to achieve high therapeutic levels (Pai *et al*. 2000). CLTR is reported to be metabolized to 4-hydroxy CLTR, which has twice as active anti-bacterial profile compared to its parent drug. The half- life of CLTR has been reported to be 3–7 h while that of its active metabolite is nearly 7 h (Malhotra-Kumar *et al*. 2007). This antibiotic has activity against the helminths (*Echinococcus multilocularis*) and *Acanthamoeba castellanii* (Mathis *et al*. 2004, 2005).

After developing resistance against chloroquine and fansidar, MFQ was the only mainstay for *P. falciparum* treatment. During the last 2 decades there have been numerous reports of emergence of MFQ resistance in Southeast Asia (Price *et al*. 1995) and Nosten *et al*. 1991 had reported nearly 50% MFQ resistance in *P. falciparum* in Thailand. This drug

https://doi.org/10.1017/S0031182011000850 Published online by Cambridge University Press

Table 2. Prophylactic response of mefloquine and clarithromycin against MDR *Plasmodium yoelii nigeriensis* in Swiss mice

Drugs (mg/kg) Mefloquine × 4 d + Clarithromycin* × 7 d	Mean Parasitaemia ± S.D.								% Cure	MST (Days)
	Day 4	Day 7	Day 10	Day 14	Day 18	Day 21	Day 24	Day 28		
32 + 225	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	100	> 28
48 + 150	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0·004 ± 0·004 (2/5)	60	> 28
32 + 150	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0·08 ± 0·15 (2/5)	2·78 ± 5·12 (3/5)	0·2 ± 0·4 (1/5)	0·0002 ± 0·0004 (1/5)	0 ± 0 (0/5)	40	> 28
16 + 150	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0·0002 ± 0·0004 (1/5)	0 ± 008 ± .01 (1/5)	5·52 ± 11·04 (1/5)	0·02 ± 0·04 (1/5)	0·002 ± 0·004 (1/5)	80	> 28
48 + 75	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0·002 ± 0·003 (2/5)	0·72 ± 1·29 (2/5)	1·96 ± 2·46 (3/5)	0·0002 ± 0·0004 (1/4)	2·5 ± 4·32 (2/4)	40	27 ± 2
32 + 75	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0·0002 ± 0·0004 (1/5)	0·002 ± 0·004 (1/5)	0·0002 ± 0·0004 (1/5)	0 ± 0 (0/5)	0·002 ± 0·004 (1/5)	80	> 28
16 + 75	0 ± 0 (0/5)	0 ± 0 (0/5)	0·0002 ± 0·0004 (1/5)	0·002 ± 0·004 (1/5)	0·004 ± 0·007 (2/5)	1·22 ± 2·15 (3/5)	0·08 ± 0·16 (1/5)	0·002 ± 0·004 (1/5)	40	> 28
48 + —	0·39 ± 0·33 (5/5)	0·99 ± 1·46 (4/5)	2·58 ± 2·83 (4/5)	4·4 ± 8·79 (2/5)	0·76 ± 1·52 (1/5)	0·002 ± 0·003 (2/5)	0·01 ± 0·02 (1/5)	0·0002 ± 0·0004 (1/5)	20	> 28
32 + —	0·5 ± 0·49 (4/5)	2·8 ± 1·88 (5/5)	6·52 ± 9·85 (5/5)	2·1 ± 3·95 (3/5)	0·004 ± 0·004 (3/5)	0·08 ± 0·15 (2/5)	0·0002 ± 0·0004 (1/5)	0·07 ± 0·08 (2/5)	0	> 28
16 + —	0·26 ± 0·31 (5/5)	2·54 ± 2·66 (5/5)	0·88 ± 1·26 (3/5)	0·52 ± 0·84 (4/5)	0·09 ± 0·15 (3/5)	6·78 ± 12·6 (4/5)	0·05 ± 0·04 (2/3)	0·03 ± 0·04 (1/2)	0	25·2 ± 3·42
— + 225	0·0002 ± 0·0004 (1/5)	0 ± 0 (0/5)	0·14 ± 0·27 (2/5)	9·1 ± 147 (2/5)	0·24 ± 0·47 (3/5)	0·96 ± 1·9 (2/5)	6·4 ± 12·8 (1/5)	0·002 ± 0·004 (1/5)	40	> 28
— + 150	0·064 ± 0·11 (3/5)	0·18 ± 0·27 (3/5)	8·24 ± 14·4 (5/5)	9·0 ± 15·5 (3/4)	10 ± 9·69 (3/4)	2·75 ± 4·76 (2/4)	0 ± 0 (0/4)	0·025 ± 0·043 (1/4)	0	24·8 ± 5·03
— + 75	6·7 ± 6·95 (5/5)	12·5 ± 8·58 (3/4)	12·4 ± 6·96 (4/4)	10·5 ± 6·46 (4/4)	0·075 ± 0·082 (2/4)	0·005 ± 0·005 (2/4)	0·2 ± 0·2 (1/2)	0·9 ± 0·9 (1/2)	0	21·2 ± 7·30
Control	36·8 ± 13·04 (5/5)	Dead							–	5 ± 0

Number of positive mice/number of total live mice on particular day is given in parentheses.
* Clarithromycin was given in 2 divided doses.

Table 3. Therapeutic response of mefloquine and clarithromycin against MDR *Plasmodium yoelii nigeriensis* in Swiss mice

Drugs (mg/kg) Mefloquine × 4 d + Clarithromycin* × 7 d	Mean% Parasitaemia ± S.D.																Cure Rate	MST
	24 hr	30 hr	36 hr	42 hr	48 hrs	54 hrs	60 hrs	72 hrs	Day 4	Day 7	Day 10	Day 14	Day 18	Day 21	Day 24	Day 28		
64 + 300	1·68 ± 0·94 (5/5)	5·82 ± 3·76 (5/5)	6·14 ± 2·62 (5/5)	7·58 ± 3·73 (5/5)	9·5 ± 5·08 (5/5)	13·92 ± 6·3 (5/5)	11·84 ± 8·89 (5/5)	5·82 ± 4·6 (5/5)	0·64 ± 0·12 (5/5)	0·002 ± 0·004 (2/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	100	> 28
32 + 300	1·4 ± 0·49 (5/5)	3·26 ± 1·45 (5/5)	4·16 ± 1·59 (5/5)	5·64 ± 3·98 (5/5)	5·88 ± 3·81 (5/5)	7·56 ± 4·29 (5/5)	8 ± 4·06 (5/5)	2·96 ± 2·27 (5/5)	0·54 ± 0·42 (5/5)	0·004 ± 0·004 (1/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	100	> 28
64 + 150	0·94 ± 0·08 (5/5)	3 ± 1·2 (5/5)	4·12 ± 1·34 (5/5)	7 ± 2·7 (5/5)	6·78 ± 1·99 (5/5)	8·58 ± 2·54 (5/5)	8·94 ± 2·46 (5/5)	5·16 ± 1·61 (5/5)	0·36 ± 0·43 (5/5)	0·004 ± 0·0048 (2/5)	0·002 ± 0·003 (3/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	40	> 28
32 + 150	1·6 ± 0·57 (5/5)	2·68 ± 0·59 (5/5)	3 ± 0·72 (5/5)	4·64 ± 1·46 (5/5)	7·26 ± 2·52 (5/5)	5·24 ± 1·32 (5/5)	6·1 ± 0·96 (5/5)	3·5 ± 1·65 (5/5)	0·36 ± 0·43 (4/5)	0·02 ± 0·03 (3/5)	0·004 ± 0·0048 (2/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	0 ± 0 (0/5)	60	> 28
64 + —	1·62 ± 0·55 (5/5)	4·52 ± 0·69 (5/5)	8·64 ± 2·43 (5/5)	11·6 ± 3·16 (5/5)	15·6 ± 5·16 (5/5)	20·2 ± 5·15 (5/5)	28 ± 4·14 (5/5)	13·28 ± 4·86 (5/5)	10·88 ± 6·4 (5/5)	5·36 ± 9·83 (5/5)	0·41 ± 0·42 (5/5)	2·83 ± 5·08 (5/5)	0·008 ± 0·011 (3/5)	0·006 ± 0·01 (1/5)	0 ± 0 (0/5)	0·002 ± 0·004 (1/5)	0	> 28
32 + —	1·44 ± 0·39 (5/5)	3·84 ± 0·72 (5/5)	5·24 ± 1·43 (5/5)	6·82 ± 1·89 (5/5)	7·98 ± 3·9 (5/5)	12·6 ± 3·61 (5/5)	13·7 ± 4·51 (5/5)	7·84 ± 3·61 (5/5)	7·5 ± 2·87 (5/5)	0·62 ± 0·52 (5/5)	0·4 ± 0·55 (5/5)	0·052 ± 0·076 (4/5)	0·004 ± 0·004 (3/5)	0·52 ± 1·03 (2/5)	1·6 ± 3·2 (1/5)	0·002 ± 0·004 (1/5)	0	> 28
— + 300	1·36 ± 0·36 (5/5)	4·36 ± 0·42 (5/5)	11·26 ± 1·09 (5/5)	13·56 ± 2·39 (5/5)	17·52 ± 2·33 (5/5)	33 ± 6·26 (5/5)	36 ± 4·33 (5/5)	30 ± 8·09 (5/5)	7·62 ± 5·37 (5/5)	0·06 ± 0·07 (5/5)	0·0004 ± 0·0004 (2/5)	0·16 ± 0·31 (2/5)	4·86 ± 9·57 (2/5)	0·06 ± 0·12 (1/5)	0·0002 ± 0·0004 (1/5)	0·0002 ± 0·0004 (1/5)	60	> 28
— + 150	1·38 ± 0·37 (5/5)	4·62 ± 0·86 (5/5)	11·42 ± 5·03 (5/5)	17·4 ± 7·39 (5/5)	24·36 ± 9·47 (5/5)	32·4 ± 7·47 (5/5)	34·4 ± 6·97 (5/5)	27·26 ± 8·91 (5/5)	7·66 ± 1·93 (5/5)	0·05 ± 0·04 (5/5)	0·0006 ± 0·0004 (2/5)	0·06 ± 0·07 (3/5)	0·1 ± 0·15 (2/5)	1·36 ± 2·71 (4/5)	0·02 ± 0·03 (2/5)	0·0002 ± 0·0004 (1/5)	20	> 28
Control	1·44 ± 0·54 (5/5)	5·26 ± 1·25 (5/5)	11·8 ± 4·65 (5/5)	22 ± 6·22 (5/5)	23·94 ± 5·71 (5/5)	39 ± 6·51 (5/5)	50·6 ± 9·49 (5/5)	58 ± 18·9 (5/5)	40·6 ± 5·24 (3/3)	8 ± 0 (1/1)	D						0	6 ± 0·89

Treatment was started at 24 h post-infection.
**Clarithromycin was given in 2 divided doses.

Table 4. Median (range) sum of FICs for the interaction of mefloquine – clarithromycin against *Plasmodium yoelii nigeriensis* MDR.

Drug combination	Dose	Median (range) sum of FICs of *P. yoelii nigeriensis* MDR
Mefloquine + clarithromycin (Prophylactic)	32 mg/kg × 4 + 225 mg/kg × 7	< 0·66
Mefloquine + clarithromycin (Therapeutic)	32 mg/kg × 4 + 300 mg/kg × 7	< 0·85

Maximum tolerated dose i.e. 300 mg/kg × 4 days and 400 mg/kg × 7days were given for mefloquine and clarithromycin respectively. FICs- Synergy < 1; addtivity 1; antagonism > 1.

was considered as the backbone for anti-malarial operation because of its high level of activity and prolonged period of plasma clearance (more than 42 days). In view of the emergence of resistance towards this drug the World Health Organization (2004, 2006) proposed new drug combinations to prolong the protective life of this drug but MFQ in combination with artesunate or artemether could not produce 100% cure (Price *et al*. 1995; Looareesuwan *et al*. 1997).

Preliminary studies reported earlier suggested that it could be possible to reverse MFQ resistance of *P. falciparum*. Kyle *et al*. (1988) and Odoula *et al*. (1993) reported that MFQ resistance of *P. falciparum* could be partly reversed *in vitro* by reversal agents such as penfluridol. Peters and Robinson (1991) reported that MFQ resistance could be reversed in rodents through penfluridol. Cyproheptadine and its derivatives had also been reported to reverse this resistance of *P. falciparum in vitro* (Baldwin *et al*. 1991). Further studies on MFQ resistance reversal by Frederich *et al*. (2001) showed the resistance reversal action of icajine against MFQ-resistant *P. falciparum in vitro*. Icajine was isolated as a menoindole alkaloid from the plant *Strychnous myrtoides*.

Ketoconazole pharmacokinetic studies had indicated that this anti-fungal had shown inhibition of cytochrome P_{450} 3A, which inhibited the biotransformation of MFQ into 2 major metabolites namely carboxy MFQ and hydroxyl MFQ. The authors had proposed that this inhibition led to the enhanced bioavailability of MFQ resulting in increasing the plasma levels of active drug MFQ through slowing down its conversion to its metabolites. Cytochrome 3A4 inhibitor ketoconazole was reported to potentiate the action against MFQ resistance of the malaria parasite (Awasthi *et al*. 2004).

Earlier studies had also reported that a combination of γ-interferon and MFQ was able to partially reverse the MFQ resistance of MDR *P. yoelii nigeriensis*, and this combination was reported to give 62% protection compared to MFQ alone which protected 25% in mice (Dhawan *et al*. 2000). The resistance reversal effect of NP30 (an uncharged polyethoxylated non-phenol surfactant) against MFQ resistance of *P. falciparum* had been documented in an *in vitro* study (Ciach *et al*. 2003). In a subsequent study, the resistance reversal action of ketoconazole, a cytochrome P_{450} inhibitor, against MFQ-resistant simian malaria *P. knowlesi* strain W_1 had been established (Tripathi *et al*. 2005). The strain W1 of *P. knowlesi* has innate resistance against MFQ up to a total dose of 80–160 mg/kg administered over 3–4 days. Combined treatment with 75 mg/kg ketoconazole for 10 days, together with a low dose of MFQ (20 mg/kg × 4 days), cured the infection in rhesus monkeys. It was reported that ketoconazole besides having a suppressive anti-malarial action against *P. knowlesi*, was also effective in reversing the MFQ resistance of the parasite resulting in the curative effect of the combination.

Wisedpanichkij *et al*. (2009) studied the *in vitro* anti-malarial activity of Cyp 3A4 inhibitors namely ketoconazole and CLTR against *P. falciparum* K1 and 3D7 strains and demonstrated that both the Cyp 3A4 inhibitors showed anti-malarial activity as demonstrated by their IC_{50} of 3·8 and 4·8 μM for ketoconazole and >10 μM for CLTR. As compared to these Cyp inhibitors, MFQ tested *in vitro* showed IC_{50} 8·6 and 12·1 nM against *P. falciparum* K1 and 3D7 isolates respectively. They provided preliminary *in vitro* evidence to support the concept that Cyp inhibitors could have a role in reversing MFQ resistance of *P. falciparum* also. They supported our earlier finding that ketoconazole – a Cyp 3A4 inhibitor could provide a higher concentration of MFQ in plasma due to inhibition of metabolism of MFQ to carboxy MFQ (inactive metabolite) (Fontaine *et al*. 2000). Ridtitid *et al*. (2005) had also reported an increased plasma concentration of MFQ in healthy human volunteers administered MFQ together with ketoconazole.

The present paper deals with the anti-malarial action of a new macrolide antibiotic CLTR, an inhibitor of hepatic microsomal Cyp_{450} enzyme (Brophy *et al*. 2000; Westphal, 2000; Suzuki *et al*. 2003; Zhou, 2008) that was evaluated in combination with MFQ to assess the reversal of MFQ resistance in *P. yoelii nigeriensis in vivo*. The anti-malarial action of CLTR has been reported earlier by Wisedpanichkij *et al*. (2009) against *P. falciparum* with IC_{50} of >10 μM. Based on *in vitro* results of the combination of MFQ and ketoconazole (Wisedpanichkij *et al*. 2009) and *in vivo* results of *P. yoelii nigeriensis* (Awasthi *et al*. 2004) and *P. knowlesi* (Tripathi *et al*. 2005), Wisedpanichkij *et al*. (2009) speculated that being a cyp inhibitor ketoconazole could exert a synergistic effect when tested in combination with MFQ. They also postulated that cyp 3A4 inhibitors,

https://doi.org/10.1017/S0031182011000850 Published online by Cambridge University Press

in general, could have a role in reversing MFQ resistance.

The present study also reports that CLTR can exert a curative action against MDR/MFQ-resistant *P. yoelii nigeriensis* at 400 mg/kg dose × 7days schedule. The lower dose 250 mg/kg × 7 days produces a suppressive effect with 80% cure. When MFQ was combined with CLTR, a synergistic anti-malarial effect and complete suppression of parasitaemia were observed up to 10 days. Further studies with MFQ 48 mg/kg combined with CLTR 150 mg/kg showed synergistic action as these combinations completely eliminated the *P. yoelii nigeriensis* parasitaemia beyond 24 days, though trace parasitaemia was observed. When a higher dose of CLTR (225 mg/kg) was combined with a lower dose of MFQ (32 mg/kg), there was complete cure beyond 28 days, indicating synergistic and curative actions of both these drugs in combination.

In order to monitor the therapeutic response of the MFQ and CLTR combination, the treatment was started 24 h after infection in mice, and doses of CLTR 300 mg/kg combined with MFQ at 32 and 64 mg/kg × 4 days, the parasitaemia was completely suppressed by day 10 post-infection, and this combination completely cured *P. yoelii.nigeriensis* infection in both groups beyond 28 days. Even the lower doses of CLTR 150 mg/kg with 32 or 64 mg/kg of MFQ resulted in complete suppression of parasitaemia beyond 14 days of observation.

This study has established that MFQ and CLTR alone exert only suppressive effects on MFQ-resistant *P. yoelii.nigeriensis*. However, a combination of CLTR 150–300 mg/kg with MFQ 32–64 mg/kg exerts a synergistic effect, resulting in clearing parasitaemia beyond 14 days, and survival of all the treated mice.

The new macrolide CLTR exerts a resistance reversal effect on the MFQ-resistant *P. yoelii nigeriensis* infection. The data establish that CLTR, being a Cyp3A4 inhibitor, exerts an inhibitory effect on MFQ metabolism to carboxyMFQ. The MFQ-CLTR combination seems to produce significant pharmacokinetic (metabolic) drug interaction leading to a higher serum concentration of MFQ (Ridtitid *et al*. 2005). The CLTR–MFQ combination seems to slow down the metabolic conversion of MFQ to carboxy MFQ (inactive), thus helping to maintain a higher concentration of MFQ. These findings suggest that MFQ + CLTR combination therapy could provide elevated levels of MFQ in the blood and it may benefit human anti-malarial treatment of MFQ-resistant *P. falciparum* infection.

ACKNOWLEDGEMENTS

Authors are thankful to the Director, C.D.R.I. and Head, Division of Parasitology, C.D.R.I. for their continuous support throughout the study. Amber Rizvi and Swaroop K. Pandey acknowledge the financial support from CSIR, New Delhi. The funding for the present study was provided by CSIR-CDRI, India. CDRI communication No. 8065.

REFERENCES

Awasthi, A., Dutta, G. P., Bhakuni, V. and Tripathi, R. (2004). Resistance reversal action of ketoconazole against mefloquine resistance of *Plasmodium yoelii nigeriensis* (MDR). *Experimental Parasitology* **107**, 115–119.

Baldwin, J., Gabriel, E. F., Friedman, P. A., Remy, D. C. (1991). Method of treating malaria with cyproheptadine derivatives. *United States Patent* 5021426.

Brophy, D. F., Isreal, D. S., Pastor, A., Gillotin, C., Chittick, G. E., Symonds, W. T., Lou, Y., Sadler, B. M. and Polk, R. E. (2000). Pharmacokinetic interaction between Amprenavir and clarithromycin in healthy male volunteers. *Journal of Antimicrobial Agents and Chemotherapy* **44**, 978–984.

Ciach, M., Zong, K., Kain, K. C. and Crandall, I. (2003). Reversal of mefloquine and quinine resistance in *Plasmodium falciparum* with NP30. *Antimicrobial Agents and Chemotherapy* **47**, 2393–2396.

Dhawan, S., Awasthi, A., Tripathi, R., Puri, S. K. and Dutta, G. P. (2000). Reversal of chloroquine/mefloquine resistance of *Plasmodium yoelii nigeriensis* (MDR) by IFN-gamma and chloroquine resistance by Poly ICLC. *Journal of Parasitic Diseases* **24**, 195–201.

Fontaine, F., Sousa, G.de., Burcham, P. C., Duchene, P. and Rahmani, R. (2000). Role of cytochrome P450 3A in the metabolism of mefloquine in human and animal hepatocytes. *Life Sciences* **66**, 2193–2212.

Frederich, M., Hayette, M. P., Tits, M., Mol, P. D. and Angenot, L. (2001). Reversal of chloroquine and mefloquine resistance in *Plasmodium falciparum* by the two monoindole alkaloids, Icajine and Isoretuline. *Planta Medicine* **67**, 523–27.

Gupta, S., Thapar, M. M., Wernsdorfer, W. H. and Bjorkman, A. (2002). *In vitro* interactions of artemisinin with atovaquone, quinine and mefloquine against *plasmodium falciparum*. *Antimicrobial Agents and Chemotherapy* **46**, 1510–1515.

Kyle, D. E., Milhous, W. K. and Odula, M. M. J. (1988). Reversal of mefloquine resistance in Plasmodium falciparum *in vitro*. *37th Annual Meeting of The American Society of Tropical Medicine and Hygiene, Washington, D.C. 4–8 December, 1988*. p. 218.

Looareesuwan, S., Wilariatana, P., Viravan, C., Vanijanonta, S., Pitisutithum, P. and Kyle, D. E. (1997). Open randomized trial of oral artemether alone and a sequential combination with mefloquine for acute uncomplicated falciparum malaria. *American Journal Tropical Medicine & Hygiene* **56**, 613–617.

Malhotra-Kumar, S., Lammens, C., Coenen, S., Herck, K. V. and Goossens, H. (2007). Effect of azithromycin and clarithromycin therapy on pharyngeal carriage of macrolide-resistant streptococci in healthy volunteers: A randomised, double-blind, placebo-controlled study. *Lancet* **369**, 482–490. doi:10.1016/S0140-6736(07)60235-9. PMID 17292768.

Mathis, A., Wild, E. C., Boettger, E. C., Kapel, C. M. O. and Deplazes, C. P. (2005). The mitochondrial ribosome as target for macrolide antibiotic clarithromycin in the helminth Echinococcus multiocularis. *Antimicrobial Agents and Chemotherapy* **49**, 3251–3255.

Mathis, A., Wild, P., Deplazes, P. and Boettger, E. C. (2004). The mitochondrial ribosome of the protozoan *Acanthamoeba castellanii* is the target for macrolide antibiotics. *Molecular and Biochemical and Parasitology* **135**, 225–229.

Nosten, F., Kuile, F. T., Chongsaphajaisiddhi, T., White, N. J., Kuile, F. T., Luxemburger, C., Webster, H. K., Edstein, M., Phajpun, L., Thew, K. L. and White, N. J. (1991). MFQ resistant falciparum malaria on the Thailand- Burmese border. *Lancet*, **337**, 1140–1143.

Odoula, A. M., Omitowoju, G. O., Genera, L., Kyle, D. E., Milhouse, W. K., Sowunmi, A. and Salako, L. A. (1993). Reversal of mefloquine resistance with penfluoridol in isolates of Plasmodium falciparum from southwest Nigeria. *Transaction of Royal Society of Tropical Medicine and Hygiene* **87**, 81–83.

Pai, M. P., Graci, D. M. and Amesden, G. W. (2000). Macrolide-drug interaction: An update. *Annals of Pharmacotherapy* **34**, 495–513.

Peters, W. and Robinson, B. L. (1991). The chemotherapy of rodent malaria. XLVI Reversal of mefloquine resistance in rodent *Plasmodium*. *American Journal of Tropical Medicine and Parasitology* **88**, 5–10.

Pinto, A. G., Wanq, Y. H., Chalasani, N., Skaar, T., Kolwankar, D., Gorski, J. C., Lianqpunsaqul, S., Hamman, M. A., Arefayene, M. and

Hall, S. D. (2005). Inhibition of human intestinal wall metabolism by macrolide antibiotics: effect of clarithromycin on cytochrome P450 3A4/5 activity and expression. *Clinical Pharmacological Therapy* **77**, 178–188.

Price, R. N., Luxemburger, C., van Vugt, M., Simpson, J., Phaipun, L., Chongsuphajaisiddhi, T., Nosten, F. and White, N. J. (1998). Artesunate and mefloquine in the treatment of uncomplicated multidrug- resistant hyperparasitaemic falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **92**, 207–211.

Price, R. N., Nosten, F., Luxemburger, C., Kham, A. M., Brockman, A., Chongsuphajaisiddhi, T. and White, M. N. J. (1995). Artesunate versus artemether in combination with mefloquine for the treatment of multidrug resistant falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **89**, 523–527.

Ridtitid, W., Wongnawa, M. W., Mahatthanatrakul, W., Raungsri, N. and Sunbhanich, M. (2005). Ketoconazole increases plasma concentrations of antimalarial mefloquine in healthy human volunteers. *Journal of Clinical Pharmacy and Therapeutics* **30**, 285–290.

Sinclair, D., Olliaro, P. and Garner, P. (2010). Guest Editorial: Artemisinin-based combination therapy for malaria, but which one? *Clinical Evidence* **8**, 1–4.

Suzuki, A., Ilda, I., Hirota, M., Akimoto, M., Higuchi, S., Suwa, T., Tani, M., Ishizaki, T. and Chiba, K. (2003). Cyp isoforms invoved in the metabolism of CLTR in vitro: Comparison between the identification from disappearance rate and that from formation rate of metabolites. *Drug Metabolism and Pharmacokinetics* **18**, 104–113.

Tripathi, R., Awasthi, A. and Dutta, G. P. (2005). Mefloquine resistance reversal action of ketoconazole- A cytochrome P_{450} inhibitor, against mefloquine resistant malaria. *Parasitology* **130**, 475–479.

Westphal, J. F. (2000). Macrolide induced clinically relevant drug interactions with cytochrome P450 A (CYP) 3A4: an update focused on clarithromycin, azithromycin and dirithromycin. *British Journal of Clinical Pharmacology* **50**, 285–295.

Wisedpanichkij, R., Chaijaroenkul, W., Sangsuwan, P., Tantisawat, J., Boonprasert, K. and Bangchang, K. N. (2009). *In vitro* antimalarial interactions between mefloquine and cytochrome P450 inhibitors. *Acta Tropica* 112, 12–15.

World Health Organization (2001). Antimalarial drug combination therapy: Report of WHO Technical Combination, Geneva, Switzerland. (WHO/CDS/RBM/2001·35)

World Health Organization (2004) *The World Health Report 2004 – Changing Story*. World Health Organization, Geneva, Switzerland.

World Health Organization (2006). *Facts on ACTs (Artemisinin Based Combinations): An Update*. World Health Organization, Geneva, Switzerland.

World Health Organization (2008). *Malaria Report*. World Health Organization, Geneva.

World Health Organization (2010*a*). *Malaria – Disease Burden in SEA Region*. World Health Organization, Geneva, Switzerland.

World Health Organization (2010*b*). *Guidelines for the Treatment of Malaria*. 2nd Edn. World Health Organization, Geneva, Switzerland.

Zhou, S. F. (2008). Drugs behave as substrates, inhibitors and inducers of human cytochrome P450 3A4. *Current Drug Metabolism* **9**, 310–322.

https://doi.org/10.1017/S0031182011000850 Published online by Cambridge University Press