Blood schizontocidal activity of azithromycin and its combination with α/β arteether against multi-drug resistant *Plasmodium yoelii nigeriensis*, a novel MDR parasite model for antimalarial screening

R. TRIPATHI[1]*, S. DHAWAN[2] *and* G. P. DUTTA[3]

[1] *Division of Parasitology, P.O. Box No. 173, Central Drug Research Institute, Chattar Manzil Palace, Lucknow, 226001, India*
[2] *C.I.M.A.P., Lucknow, India*
[3] *Emeritus Scientist, C.S.I.R., C.D.R.I., Lucknow, India*

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SUMMARY

Many different drug-resistant lines of rodent malaria are available as screening models. It is obligatory to screen new compounds for antimalarial activity against a series of resistant lines in order to identify a compound with potential for the treatment of multi-drug resistant (MDR) malaria infections. Instead of using a battery of resistant lines, a single MDR *Plasmodium yoelii nigeriensis* strain that shows a wide spectrum of drug resistance to high doses of chloroquine, mepacrine, amodiaquine, mefloquine, quinine, quinidine, halofantrine as well as tetracyclines, fluoroquinolines and erythromycin, was used to assess the blood schizontocidal efficacy of a new macrolide azithromycin and other antibiotics. The present study shows that only azithromycin has the potential to control an MDR *P. y. nigeriensis* infection in Swiss mice, provided the treatment with a dose of 50–100 mg/kg/day by oral route is continued for a period of 7 days. Tetracycline, oxytetracycline, doxycyline, erythromycin, ciprofloxacin and norfloxacin, although active *in vitro*, failed to protect the mice. Tetracycline, ciprofloxacin and norfloxacin combinations with chloroquine did not control the infection. Additionally, the antimalarial efficacy of azithromycin can be potentiated with the addition of arteether, which is an ethyl ether derivative of artemisinin. A total (100%) curative effect has been obtained with a shorter regimen of 4 days only.

Key words: *Plasmodium yoelii nigeriensis*, multi-drug resistance, malaria, antimalarial, azithromycin, antibiotics, α/β arteether.

INTRODUCTION

According to a WHO (1999) report there are approximately 275 million malaria cases throughout the world, with nearly 1·1 million deaths occurring annually. Most deaths reported were among children, infants and pregnant women. Also, due to the global malaria burden, 40·213 million disability adjusted life years have been calculated (Remme *et al*. 2002). Emergence of drug-resistant *Plasmodium falciparum* is posing a serious threat to the National Malaria Control Programmes in tropical countries and a high level of resistance to chloroquine, mefloquine, pyrimethamine-sulfadoxin combination, quinine and halofantrine has made malaria control doubtful in some countries. According to Sharma (1998), the data available in the South East Asia Regional Office of WHO show an estimated 15 million malaria cases and 19 500 malaria-related deaths annually in India. Nearly one-third of the *P. falciparum* cases show some degree of chloroquine resistance (Sharma, 1998). The National Antimalaria Programme (NAMP) (2003) has reported 25·3% chloroquine-sensitive and 53·0, 14·1, 4·8, and 3·8% cases of S/RI, RI, RII and RIII levels of chloroquine resistance, respectively, out of total 16267 *P. falciparum* cases in 29 states of India.

Previous studies on the antimalarial efficacy of various antibiotics had been reviewed by Puri & Dutta (1982) and amongst the various groups of antibiotics, the macrolides had shown high potential for the control of malaria parasites. Antimalarial activity has been specifically reported for rifampicin (Strath *et al*. 1993), erythromycin (Gingras & Jensen, 1993), spiramcyin (Hill, 1975), midecamycin (Puri & Dutta, 1989), clindamycin (Lewis, 1968), minocycline (Dutta & Singh, 1979), fluoroquinolones (Singh & Puri, 1996), and azithromycin (Gingras & Jensen, 1993; Andersen *et al*. 1995). More recently Puri & Singh (2000) have shown blood schizontocidal activity of azithromycin in Swiss mice against *P. yoelii nigeriensis* N-67 strain that show a low level of resistance to chloroquine only. An azithromycin dose of 70 mg/kg per day × 4 days,

* Corresponding author: Division of Parasitology, P.O. Box No. 173, Central Drug Research Institute, Chattar Manzil Palace, Lucknow, 226001, India. Tel: +91 522 2212411 18, Extn. 4461. Fax: +01 522 223405/223938. E-mail: renu1113@rediffmail.com

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as well as 40 mg/kg per day × 7 days was reported to be curative in the above study (Puri & Singh, 2000). Andersen *et al*. (1995) had reported the curative action of azithromycin against *P. berghei* and only 71% cure rate was obtained at a dose of 128 mg/kg per day administered for 3 days. Andersen *et al*. (1995) had also reported its curative action against *P. falciparum* infection in *Aotus* monkeys at a dose of 100 mg/kg administered for 7 consecutive days, whereas a lower dose (30 mg/kg × 7 days) was able to clear the parasitaemia initially, but there was a recrudescence in 1 of the 2 monkeys. The curative dose of azithromycin against *P. cynomolgi B* in rhesus monkey had been reported to be 25 mg/kg per day × 7-day schedule (Puri & Singh, 2000).

The *in vitro* antimalarial activites of ciprofloxacin, norfloxacin and ofloxacin and other antibiotics against *P. falciparum* has been reported by Tripathi *et al*. (1993) and Mahmoudi *et al*. (2003). The *in vitro* antimalarial activity of a variety of other antibiotics such as cycloheximide, erythromycin, tetracycline, chloramphenicol, clindamycin, actinomycin D, rifampin and isoniazid against *P. falciparum* has also been reported earlier by Geary & Jensen (1983).

A battery of more than one dozen drug-resistant lines of rodent malaria, had been developed by Peters (1987) and Peters *et al*. (1993*a*, *b*, *c*) for an antimalarial screening programme which would need enormous time and expertise to obtain ED_{50}/ED_{90} values of the test compounds in *in vivo* systems. In preference to a battery of antimalarial screens, we use a single MDR strain of *P. yoelii nigeriensis* with a very wide spectrum of resistance to antimalarials/antibiotics, as a blood schizontocidal screen in random-bred Swiss mice. In the present study, the MDR strain of *P. yoelii nigeriensis*, which produces 100% mortality in mice, was used to evaluate the blood schizonotocidal activity of azithromycin alone, in combination with α/β arteether and several other antibiotics.

MATERIALS AND METHODS

Plasmodium yoelii nigeriensis which had a innate low level of resistance to chloroquine (at 16 mg/kg × 4 days), was subjected to interrupted subcurative chloroquine/mefloquine/quinine treatments through successive passages and the strain eventually developed resistance to chloroquine (64 mg/kg × 4), amodiaquine (64 mg/kg × 4), mefloquine (128 mg/kg × 4), quinine (400 mg/kg × 4 doses) and tetracycline (500 mg/kg × 4) (Dutta & Pande, 1986). Since then the strain has been maintained in the Swiss mice by serial blood passage. The strain shows a high level of virulence for Swiss mice (20–25 g) and produces 100% lethal infection.

Subsequently, the above strain was exposed to high oral doses of chloroquine, amodiaquine, mepacrine, halofantrine and mefloquine (at 128 mg/kg doses × 4 days), quinine/quinidine (400 mg/kg × 4 days), and tetracycline and oxytetracycline (500 mg/kg × 4 days), doxycycline (100 mg/kg × 4 days) and erythromycin (250 mg/kg × 4 days). Treatment was given orally from day 0 (day of infection) to day +3 i.e., for 4 consecutive days. Blood smear examination of the above groups of mice carried out 10–12 days after treatment, showed recrudescence of parasitaemia in all treatment groups as shown by Giemsa staining, indicating that the parasite designated as MDR *P. yoelii nigeriensis* had developed resistance to the above-mentioned doses of drugs/antibiotics. This MDR strain was cryopreserved for re-use; the level of drug resistance was maintained.

In order to assess the blood schizontocidal activity of ciprofloxacin, norfloxacin, tetracycline, oxytetracycline, doxycycline, erythromycin, and azithromycin, the *P. yoelii nigeriensis* MDR strain was passaged intraperitoneally into new batches of random-bred Swiss mice (20 ± 1 g) and the infected mice were administered orally different antibiotics individually as well as in combination with chloroquine.

Azithromycin was tested at 12·5, 25 and 50 mg/kg doses for 7 consecutive days (from day 0 to day + 6). Giemsa-stained blood smears were examined to monitor the parasitaemia (% mean ± S.D.). In a repeat experiment, the dose of 50, 100 and 200 mg/kg azithromycin was again evaluated using a 7-day treatment schedule. Observations were continued for up to 28 days, and the number of mice surviving beyond 28 days was recorded.

Intramuscular injection of arteether α/β (30 : 70 mixture of enantiomers) had been developed at the Institute as a fast-acting blood schizontocide (Dutta, Bajpai & Vishwakarma, 1989).

To study the combined effect of azithromycin and α/β arteether (dissolved in neutralized and sterilized groundnut oil), they were administered simultaneously to the infected mice. Doses of 30·15 and 3·75 mg/kg × 4 of azithromycin and 25, 16·6 and 5·5 mg/kg × 4 of α/β arteether were administered orally. The same doses of both drugs were also given individually for comparison.

Azithromycin capsules used in the study were obtained from Wockhardt Ltd, India.

RESULTS

The MDR strain of *P. yoelii nigeriensis* used in this study shows a high level of resistance to chloroquine, amodiaquine, mepacrine, mefloquine, quinine, quinidine and halofantrine.

Blood schizontocidal activity of azithromycin against MDR *P. yoelii nigeriensis* has been investigated in Swiss mice. Azithromycin was administered orally at doses of 12·5, 25 and 50 mg/kg for 7 consecutive days, starting from day 0 to day + 6. The lowest doses of 12·5 and 25 mg/kg were not

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Table 1. Response of multi-drug resistant *Plasmodium yoelii nigeriensis* (MDR) to azithromycin treatment in Swiss mice

Treatment	Dose (Oral) mg/kg × days	No. of mice	%Parasitaemia (mean ± S.D.)						No. of mice surviving for 28 days
			Day 5	Day 6	Day 8	Day 12	Day 16	Day 28	
Azithromycin	200 × 7	8	0·012 ± 0·03(8)	0·0 ± 0·0(8)	−ve (8)	−ve (8)	−ve (8)	−ve (8)	8
	100 × 7	8	0·01 ± 0·03 (8)	0·0 ± 0·0 (8)	−ve (8)	−ve (8)	−ve (8)	−ve (8)	8
	50 × 7	8	0·07 ± 0·17 (8)	0·25 ± 0·70 (8)	−ve (8)	−ve (8)	−ve (8)	−ve (8)	8
	50 × 7	8	N.D. (8)	0·02 ± 0·04 (8)	−ve (8)	−ve (8)	−ve (8)	−ve (8)	8
	25 × 7	8	N.D.	2·12 ± 5·55 (8)	6·37 ± 10·9 (7)	9·33 ± 11·27 (4)	1·66 ± 2·88 (4)	−ve (4)	4
	12·5 × 7	8	N.D.	17·14 ± 5·55 (5)	10·25 ± 7·58 (4)	5·0 ± 3·8 (4)	9·59 (4)	−ve (3)	3
Control	—	8	11·25 ± 2·86 (8)	51·75 ± 25·94 (8)	65·52 ± 34·39 (4)	Died	—	—	Nil
	—	8	3·13 ± 1·65 (8)	56·7 ± 18·06 (8)	Died	Died	—	—	Nil

curative and the number of animals surviving was 3 and 4 respectively out of 8 mice in each group. At 50 mg/kg, a transient parasitaemia (0·02 ± 0·04) was recorded on day 6 but the parasitaemia was finally cleared and all 8 mice survived beyond 28 days (Table 1). In the second experiment the doses of 50, 100 and 200 mg/kg were evaluated and in this experiment also, although the 50 mg/kg dose showed transient parasitaemia on days 5 and 6, the parasitaemia was cleared and all 8 mice survived. Similarly, at 100 and 200 mg/kg, parasitaemia showed a transient patency on day 5 and parasitaemia was cleared up to 28 days of observation. The study showed that the 50 mg/kg/day dose was curative by the oral route, in a 7-day treatment schedule in 2 experiments. Both of the control batches, which were untreated showed 100% mortality and the mice developed very high parasitaemia before death.

In another experiment azithromycin was administered with *α/β* arteether by the oral route of administration. Doses of 30, 15 and 3·75 mg/kg of azithromycin were given with 25, 16·6 and 5·5 mg/kg doses of *α/β* arteether respectively for 4 days. A dose of 30 mg/kg azithromycin with 25 mg/kg *α/β* arteether provided 100% curative effect against MDR strain. There was no trace of parasitaemia during 28 days of observation, whereas the lower dose i.e. 15 + 16·6 mg/kg produced only 60% cure. Results show that with the appropriate dose combination, these two drugs can produce 100% protection and cure of MDR strain (Table 2).

Blood schizontocidal activity of other antibiotics against the MDR parasite

Tetracycline (500 mg × 4), oxytetracycline (500 mg × 4), doxycycline (25 mg × 4), erythromycin (250 mg × 4), ciprofloxacin (50 mg × 4) and norfloxacin (50 mg × 4), were evaluated for blood schizontocidal activity against *P. yoelii nigeriensis* MDR strain and the treatment was given orally for 4 consecutive days. The results presented in Table 3 show that the strain is fully resistant to high doses of these antibiotics and the mortality after treatment was 100% in all the groups. Even combinations of tetracycline and ciprofloxacin with chloroquine (32 mg/kg × 5) were not protective. However, norfloxacin and chloroquine combination protected 3 out of 10 mice.

DISCUSSION

Besides the antimalarial action, azithromycin possesses a wide spectrum of antimicrobial activity against *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Chlamydia trachomatis*, *Mycobacterium avium*, *M. intracellulare* complex, *Helicobacter pylori*, *Campylobacter* sp., *Bordetella pertusis* (Tarlow *et al.*

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Table 2. Chemotherapeutic response of azithromycin with α/β arteether against MDR *Plasmodium yoelii nigeriensis* in Swiss mice

Sl. no.	Drugs/doses mg/kg × 4 days	%Mean Parasitaemia ± S.D. Day 4	Day 7	Day 14	Day 21	Day 28	% Cure rate
	Azithromycin						
1	30·0	0·30 ± 0·4 (5)	0·0 (5)	5·5 ± 2·86 (5)	0·0 (4)	0·0 (4)	80·0
2	15·0	1·22 ± 0·97 (5)	8·0 ± 7·77 (5)	4·3 ± 2·8 (4)	0·0 (3)	0·0 (3)	60·0
3	3·75	34·6 ± 12·7 (5)	25·0 ± 0·0 (1)	D			0·0
4	Arteether						
5	25·0	0·0 (5)	0·0 (5)	2·90 ± 2·85 (5)	0·0 (2)	0·0 (2)	40·0
6	16·6	0·0 (5)	0·0 (5)	0·0 (3)	0·0 (1)	0·0 (1)	20·0
7	5·5	1·54 ± 0·92 (5)	17·8 ± 9·62 (4)	15·0 ± 0·0 (1)	D		0·0
	Azithromycin + Arteether						
8	30·0 + 25·0	0·0 (5)	0·0 (5)	0·0 (5)	0·0 (5)	0·0 (5)	100·0
9	15·0 + 16·6	0·0 (5)	0·0 (5)	0·0 (3)	0·0 (3)	0·0 (3)	60·0
10	3·75 + 5·5	36·0 ± 0·49 (5)	3·5 ± 3·74 (5)	8·50 ± 3·50 (2)	3·50 ± 4·7 (2)	0·0 (2)	40·0
11	Control	35·0 ± 7·90 (5)	75·0 ± 0·0 (1)	D			0·0

1997; Zuckermann and Kaye, 1995), *Entamoeba histolytica*, *Giardia lamblia* (Lode *et al*. 1996), and *Toxoplasma gondii* (Degerli *et al*. 2003).

Prophylactic efficacy of azithromycin against *P. falciparum* infection was reported to be 83% by de Vries *et al*. (1999) and 71·6% by Taylor *et al*. (1999, 2003). Andersen *et al*. (1998) had reported that the daily administration of 250 mg azithromycin for 10 weeks, gave 82·7% prophylactic protection against *P. falciparum* while Andersen *et al*. (1995) had shown only 40% protection (protecting 4 out of 10 volunteers) in the first batch treated with 250 mg daily dose, while the second batch of 10 volunteers treated with above dose was fully protected against *P. falciparum* infection.

An earlier report had shown that a short 3-day azithromycin therapy was inadequate for achieving complete cure of *P. falciparum* cases even when azithromycin was combined with artemether. Combination of 300 mg artemether together with 500 mg azithromycin followed by 250 mg azithromycin at 24 and 48 h was evaluated for treatment of MDR *P. falciparum* infection, and the recrudescence rate of drug-resistant falciparum infection was 53·3% (Na Bangchang *et al*. 1996).

Clinical studies on the blood schizontocidal efficacy of azithromycin in combination with artesunate and dihydroartemisinin against multi-drug resistant *P. falciparum* have also been conducted (Krudsood *et al*. 2000, 2002). In one of the trials, artesunate 200 mg together with azithromycin 50 mg once daily for 3 days gave a partial cure rate of 56% among uncomplicated *P. falciparum* cases in Bangkok (Krudsood *et al*. 2000). In another clinical trial dihydroartemisinin (80 mg dose daily ×3 days) was given in combination with azithromycin (500 mg dose daily ×3 days) which resulted in 69·7% cure rate among MDR *P. falciparum* cases in Thailand (Krudsood *et al*. 2002). Partial radical cures reported in the above studies were mainly due to inadequate dosages of azithromycin and short-term treatment.

In earlier studies azithromycin in combination with artemisinin derivatives did not prove to be very effective and 52%, 56% and 69% cure rates were achieved with azithromycin + artmether, artesunate and dihydroartemisinin (DQHS) respectively. In the present study we achieved 100% cure with the combination of azithromycin with α/β arteether. α/β arteether is a very potent antimalarial and its LD_{50} (i.e. <1250 mg) is more than artemether, artesunate and DQHS. We are convinced that doses used in earlier combination studies in humans were inadequate and that the number of doses was also not sufficient. Four days treatment with azithromycin and α/β arteether will certainly improve the cure rate in *P. falciparum*-infected humans.

In vitro antimalarial studies with azithromycin against *P. falciparum* carried out by Yeo & Rieckman (1995) also showed that the antibiotic was slow acting and maximum activity was obtained at 96 h, with a MIC ranging from 0·04 to 0·08 μg/ml. In the present study also the action of azithromycin on clearance of asexual blood stages of *P. yoelii nigeriensis* was slow at the curative dose i.e. 50 mg/kg, as well as at 100 and 200 mg/kg doses and a slide-positive parasitaemia was observed up to days 5–6 of treatment. Because of the slow blood schizontocidal action of azithromycin against the MDR rodent strain, treatment was extended to a 7-day course in order to achieve complete cure. The curative action of tetracycline, oxytetracycline, doxycycline, ciprofloxacine, norfloxacine and erythromycin against MDR *P. yoelii nigeriensis* could not be achieved in the present study even when high doses of these antibiotics were used for treatment of mice. The MDR strain developed in the study exhibits a high level of resistance to the above antibiotics excepting azithromycin to which the strain is still sensitive. Combinations of several

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Table 3. Response of multi-drug resistant strain of *Plasmodium yoelii nigeriensis* (MDR) to antibiotics and antibiotic-chloroquine combinations in Swiss mice

Treatment	Dose (Oral) mg/kg × days	No. of mice	% Parasitaemia (mean ± S.D.)						No. of mice surviving for 28 days
			Day 3	Day 5	Day 7	Day 9	Day 11	Day 14	
Tetracycline	500 × 4 (Day 0–3)	6	1·36 ± 0·51	7·60 ± 1·76	15·0 ± 0·0	Died	—	—	Nil
Oxytetracycline	500 × 4 (Day 0–3)	6	1·30 ± 0·94	6·95 ± 3·0	8·70 ± 0·28	8·70 ± 0·28	11·0 ± 0·0	Died	Nil
Doxycycline	25 × 4 (Day 0–3)	6	3·28 ± 0·99	8·90 ± 2·22	16·0 ± 0·0	Died	—	—	Nil
Erythromycin	250 × 4 (Day 0–3)	6	5·86 ± 0·14	15·55 ± 4·0	16·0 ± 0·0	Died	—	—	Nil
Ciprofloxacin	50 × 5 (Day 3–7)	8	11·75 ± 5·1	64·2 ± 28·0	Died	—	—	—	Nil
Norfloxacin	50 × 5 (Day 3–7)	8	11·75 ± 5·1	74 ± 18·27	Died	—	—	—	Nil
Tetracycline	50 × 5 (Day 3–7)	8	11·75 ± 5·1	69·25 ± 4·78	Died	—	—	—	Nil
Ciprofloxacin + Chloroquine	50 mg/kg × 5 32 mg/kg × 5 (Day 3–7)	10	11·75 ± 5·1	9·8 ± 3·25	8·6 ± 4·12	—	—	1·5 ± 4·9	Nil
Norfloxacin + Chloroquine	50 mg/kg × 5 32 mg/kg × 5 (Day 3–7)	10	11·75 ± 5·1	8·7 ± 3·3	10·3 ± 12·8	—	—	40·0 ± 36·0	3
Tetracycline + Chloroquine	50 mg/kg × 5 32 mg/kg × 5 (Day 3–7)	8	11·75 ± 5·1	15 ± 9·5	12·6 ± 6·4	—	—	42·0 ± 7·5	Nil
Chloroquine	32 mg/kg × 5 (Day 3–7)	8	11·75 ± 5·1	12·5 ± 4·17	25·1 ± 33·7	—	—	44·5 ± 15·1	Nil
Control	—	8	11·75 ± 5·1	63·75 ± 17·9	Died	—	—	—	Nil
Control	—	6	4·23 ± 0·75	22·33 ± 5·70	36·0 ± 0	Died	—	—	Nil

antibiotics and chloroquine (32 mg/kg) were tested but norfloxacine and chloroquine combination was only partially protective (3/10 mice).

The *P. yoelii nigeriensis* MDR strain selected in this study would be a useful antimalarial screening model to identify new compounds/antibiotics, which could be developed to tackle the problem of multi-drug resistant malaria. The MDR strain has acquired the wide spectrum of drug resistance similar to the MDR *Plasmodium falciparum* isolates from the field.

We emphasize that out of several antibiotics screened against *P. yoelii nigeriensis* (MDR) only azithromycin was found to eliminate the multi-drug resistant parasites, although the rate of clearance of parasitaemia was slow. Besides azithromycin, minocycline is another antibiotic, which is potentially very active against malaria and has been reported by Dutta & Singh (1979) to cure fulminating *P. knowlesi* infection in rhesus monkeys.

The present study also shows that the single MDR line of *P. yoelii nigeriensis*, which produces 100% mortality in mice, could be a useful model for anti-malarial screening and it has obvious advantage over the conventional method of screening compounds against a battery of resistant lines of rodent malaria infections as has been proposed by earlier investigators (Peters, 1987; Peters *et al.* 1993*a*, *b*, *c*). Instead of determining ED_{50}/ED_{90} values of the potential antimalarials, the determination of 100% curative dose in mice infected with a wide spectrum of MDR *P. yoelii nigeriensis* seems to be a more rigorous and realistic criterion for selection of highly active compounds/antibiotics for further drug development.

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