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Amodiaquine-Azithromycin Eradicates Blood and Liver Stages of *Plasmodium berghei* Infection in Mice

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Abstract

Amodiaquine (AQ) is used as a partner drug with artemisinins for malaria treatment. Azithromycin (AZ) is a macrolide antibiotic with potential antiplasmodial activity. This study assessed whether AZ can be used as a partner drug with AQ for malaria treatment in *Plasmodium berghei*-infected mice. Adult Swiss albino mice (30-35g) of both sexes were randomly grouped and used. The mice were inoculated with *Plasmodium berghei* and orally treated with AQ (10 mg/kg), AZ (10 mg/kg) and AQ-AZ, respectively. Chloroquine CQ (10mg/kg) was used as the standard. At the termination of treatment, blood samples were collected and assessed for percentage parasitamia, inhibition and hematological markers. Liver samples were examined for histological changes. The mice were also observed for mean survival time (MST). In the curative, prophylactic and suppressive tests, AQ-AZ significantly decreased percentage parasitamia with difference observed at $p<0.05$ when compared to AQ or AZ. Curatively, AQ, AZ and AQ-AZ produced 71.41 %, 66. 80% and 92.60% parasitamia inhibitions, respectively when compared to 88.20% produced by CQ. The curative, prophylactic and suppressive tests showed significant prolongation of MST by AQ-AZ with difference observed at $p<0.05$ when compared to AQ or AZ. AQ-AZ inhibitions of *Plasmodium berghei*-induced alterations in hematological makers were characterized by increased red blood cells, packed cell volume, hemoglobin and decreased white blood cells with difference observed at $p<0.05$ when compared to AQ, or AZ. AQ-AZ eradicates vascular congestion and inflammatory cells observed in the liver of *Plasmodium berghei*-infected mice. AZ can be used as a partner drug with AQ for the treatment of malaria.

Keywords: Amodiaquine, Azithromycin, Partner-Drug, *Plasmodium*, mice

1. Introduction

Globally, malaria represents a continual and relentless public health burden, which causes mortality especially among children below age five and pregnant women [1]. A reckoning 3.3 billion people were at jeopardy of malaria in 2010 [2]. In the world, people living in sub-Saharan Africa have the highest risk of acquiring malaria [2]. Significant progress has been made in the control of malaria over the past years through interventions such as early case identification and diagnosis and immediate management with artemisinin combination therapies [1].Additionally, advancement in providing trenchant treatment for malaria is facing challenges including parasite resistance to artemisinin combination therapies (ACTs), the mainstay for malaria treatment [3,4]. In order to overcome *Plasmodium* resistance, various combinations of antimalarial drugs and drugs with potential antimalarial activity are been explored [5].

Amodiaquine (AQ) was first added to the World Health Organization (WHO) Essential Drugs List in 1977 [6]. It is a semisynthetic 4-aminoqunoine, which is similar to chloroquine (CQ) used for the treatment of malaria. AQ is converted to its active metabolite desethylamodiaquine, which accumulates in parasite food vacuole and interferes with haem detoxification [7]. It is used in combination with artemisinins and sulphadoxine-pyrimethamine for malaria treatment [8]. It has also been suggested that it may be a less toxic alternative to sulphadoxine-pyrimethamine in people infected with human immunodeficiency virus in Sub Saharan Africa [9].

Azithromycin (AZ) is a macrolide used for the treatment of infections caused by Gram-positive bacteria and limited Gram-negative bacteria. Macrolides belong to the class of drugs that consist of a sizable macrocyclic lactone ring to which one or more deoxy sugars, usually cladinose and desosamine are attached [10]. AZ interferes and inhibits bacterial protein biosynthesis [11]. It has immunomodulatory and anti-inflammatory effects, which have applications in cystic fibrosis [9]. AZ has earlier been considered as a prospective antimalarial agent. It has proven activity against CQ-resistant *Plasmodium falciparum* [12] and *Plasmodium vivax* [13]. It was efficacious and well-tolerated in combination with artesunate [14] and dihydroartemisinin [15]. This study assessed whether AZ can be used with AQ for malaria treatment in *Plasmodium berghei*-infected mice.

2. Materials and Methods

2.1 Drug, animal and care

Adult Swiss albino mice of both sexes (30-35g) were purchased from the Animal Facility of the Department of Pharmacology, Faculty of Basic Clinical Sciences, University of Port Harcourt, Nigeria. The mice were housed under a 12 hr light /dark cycle with free access to commercial food pellets and water. The mice were acclimated for two weeks prior to the study and were under standardized environmental conditions during the study. The principles of laboratory animal care (NIH publication No. 85-23, revised 1985) [16] were used. AQ, AZ and CQ used were of analytical grade. Doses of CQ (10 mg/kg) [17], AQ (10 mg/ kg) [18] and AZ (10 mg/kg) [19] were used.

2.2 Malaria parasite strain

Chloroquine (CQ) sensitive *Plasmodium berghei* (*P. berghei*) (NK65) was supplied in donor mice by the Nigerian Institute for Medical Research, Yaba, Lagos. The parasites were maintained in continuous blood passage in mice. A standard inoculum of parasitized erythrocytes (1×10^7) was prepared by diluting the blood collected from a donor mouse with normal saline and injected intraperitoneally (i.p) to each test mouse.

2.3 Evaluation of antiplasmodial activity

2.3.1 Evaluation of antiplasmodial curative activity of amodiaquine-azithromycin

The method described by Ryley and Peters (1970) [20] was used for the curative study. Twenty five Swiss mice were inoculated i.p with *P. berghei* (1×10^7) and grouped into 5 of 5 mice/group. The groups were orally treated daily for 4 days as follows: Negative control-Normal saline (0.2mL) and positive control- (CQ 10mg/kg). Other groups were treated with A Q (10 mg/kg), AZ (10mg/kg) and AQ-AZ, respectively. Tail blood samples from the mice were collected on day 5, thin blood films were produced on slides. The slides were stained with Giemsa stain and viewed with the aid of a

microscope. The evaluations of percentage parasitamia and percentage inhibitions were performed using the formula below:

$$\% \text{ Parasitamia} = \frac{\text{Number of parasitized red blood cells (RBCs)} \times 100\%}{\text{Total number of RBCs count}}$$

$$\% \text{ Inhibition} = \frac{(\%\text{Parasitamia of negative control} - \%\text{ Parasitamia of treated group}) \times 100\%}{\%\text{ Parasitamia of negative control}}$$

2.3.2 Evaluation of suppressive antiplasmodial activity of amodiaquine-azithromycin

Suppressive test was determined as described by Knight and Peters 1980 [21]. Twenty five Swiss albino mice grouped into 5 of 5 mice/ group were inoculated with *P. berghei* ($1x10^7$) i.p. Two hours later, the mice were orally treated daily for 4 days as follows: AZ (10 mg/kg), AQ (10 mg/kg) and AQ-AZ, respectively. The negative and positive controls were treated with normal saline (0.2mL) and CQ (10mg/kg), respectively. On day 5, percentage parasitamia and percentage inhibitions were determined from tail blood samples as shown above.

2.3.3 Evaluation of prophylactic antiplasmodial activity of amodiaquine-azithromycin

It was performed as described by Peter, 1967 [22]. Twenty five Swiss albino mice grouped into 5 of 5 mice /group were orally treated daily with AQ (10 mg/kg), AZ (10 mg/kg) and AQ-AZ for 4 days, respectively. The negative and positive controls were treated with normal saline (0.2mL) and CQ (10mg/kg) for 4 days, respectively. The mice were inoculated with *P. berghei* on day 5 and allowed for 72 hr. Thereafter, percentage parasitamia and percentage inhibitions were determined from tail blood samples collected from the mice as shown above.

2.4 Determination of mean survival time

The mortalities of the mice (in the control and experimental groups) were monitored daily and the number of days from the time of infection up to death were obtained and recorded. Mean survival time (MST) was calculated as shown below.

$$\text{MST} = \frac{\text{Sum of survival time of all mice in a group (Days)}}{\text{Total number of mice in that group}}$$

2.5 Evaluation of hematological indices

The mice in the curative group were anaesthetized, blood samples were collected from the heart in tubes containing anticoagulant. The blood samples were evaluated for packed cell volume (PCV), white blood cells (WBCs), red blood cells (RBCs), and hemoglobin (HB) using an automated hematology analyzer.

2.6 Statistical analysis

Data as mean ± standard error of mean (SEM). Data analysis was carried out using GraphPad Prism (GraphPad Prism Software, Inc., US). The means of the control and experimental groups were compared using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A P value<0.05 was regarded as statistically significant.

3. Results

3.1 Curative antiplasmodial effect of amodiaquine-azithromycin on *Plasmodium berghei*-infected mice

AQ-AZ significantly decreased percentage parasitamia when compared to individual doses of AZ and AQ at $p<0.05$. AZ, AQ and AQ-AZ produced parasitamia inhibitions, which represent 66.80%, 71.41%, and 92.60%, respectively, while CQ produced 88.20% parasitamia inhibition (Table 1). AQ-AZ prolonged MST when compared to AQ or AZ with significance observed at $p<0.05$ (Table 1).

3.2 Suppressive antiplasmodial effect of amodiaquine-azithromycin on *Plasmodium berghei*-infected mice

AQ-AZ significantly decreased percentage parasitamia at $p<0.05$ when compared to individual doses of AZ and AQ (Table 2). Parasitamia inhibitions which represent 77.48%, 84.33%, 98. 48%, and 96. 70% were produced by AZ, AQ, AQ-AZ and CQ, respectively. MST was significantly prolonged by AQ-AZ when compared to individual doses of AZ and AQ with difference observed at $p<0.05$ (Table 2).

3.3 Prophylactic antiplasmodial effect of amodiaquine-azithromycin on *Plasmodium berghei*-infected mice

AQ-AZ decreased percentage parasitamia with significant difference observed at $p<0.05$ when compared to individual doses of AZ and AQ (Table 3). AQ-AZ prolonged MST when compared to individual doses of AQ and AZ with significance observed at $p<0.05$ (Table 3).

3.4 Effect of amodiaquine-azithromycin on hematological indices of *Plasmodium berghei*-infected mice

RBCs, PCV and HB were significantly ($p<0.05$) decreased whereas WBCs were significantly ($p<0.05$) increased in *P. berghei*-infected mice when compared to normal control (Table 4). But treatment with AQ-AZ significantly increased RBCs, PCV and HB and significantly decreased WBCs when compared to individual doses of AQ and AZ at $p<0.05$ (Table 4).

3.5 Effect of amodiaquine-azithromycin on liver histology of *Plasmodium berghei*-infected mice

The liver of the control mice showed normal hepatocytes, sinusoids and central vein (Figure A) whereas the liver of parasitized mice showed inflammatory cell infiltration, central vein congestion and congested sinusoids (Figure B and C). Liver of CQ (10mg/kg) treated mice (Figure D) and the liver of AQ (10mg/kg) treated mice (Figure E) showed central vein congestion, normal hepatocytes and sinusoids. Liver of AZ-treated mice showed central vein congestion, congested sinusoids, and normal hepatocytes (Figure F) while the liver of AQ-AZ treated mice showed normal sinusoids and hepatocytes (Figure G).

Table 1: Curative antiplasmodial effect of amodiaquine-azithromycin on *Plasmodium berghei*-infected mice

Treatment	% Parasitamia	% Inhibition	MST (Days)
NC	31.09±3.00	0.0	9.15±0.89
CQ	3.67±0.89[a]	88.20	33.63±3.39[a]
AZ	10.32±0.33[b]	66. 80	25.03±2.73[b]
AQ	8.89±0.16[c]	71.41	29.10±2.92[c]
AQ-AZ	2.30±0.23[d]	92.60	34.66±4.77[a]

NC: Negative control, CQ: Chloroquine, AZ: Azithromycin, AQ: Amodiaquine, MST; Mean survival time, n= 5, Data as mean ± SEM (Standard error of mean). Values with different superscripts down the column significantly differ at p<0.05 (ANOVA).

Table 2: Suppressive antiplasmodial effect of amodiaquine-azithromycin on *Plasmodium berghei*-infected mice

Treatment	% Parasitamia	% Inhibition	MST (Days)
NC	28.20±2.22	0.0	9.31±0.46
CQ	0.91±0.01[a]	96.70	35.12±3.44[a]
AZ	6.35±0.57[b]	77.48	24.38±2.68[b]
AQ	4.42±0.25[c]	84.33	29.23±3.16[c]
AQ/AZ	0.43±0.07[d]	98.48	45.04±4.37[d]

NC: Negative control, CQ: Chloroquine, AZ: Azithromycin, AQ: Amodiaquine, MST: Mean survival time, n= 5, Data as mean ± SEM (Standard error of mean). Values with different superscripts down the column significantly differ at p<0.05 (ANOVA).

Table 3: Prophylactic antiplasmodial effect of amodiaquine-azithromycin on *Plasmodium berghei*-infected mice

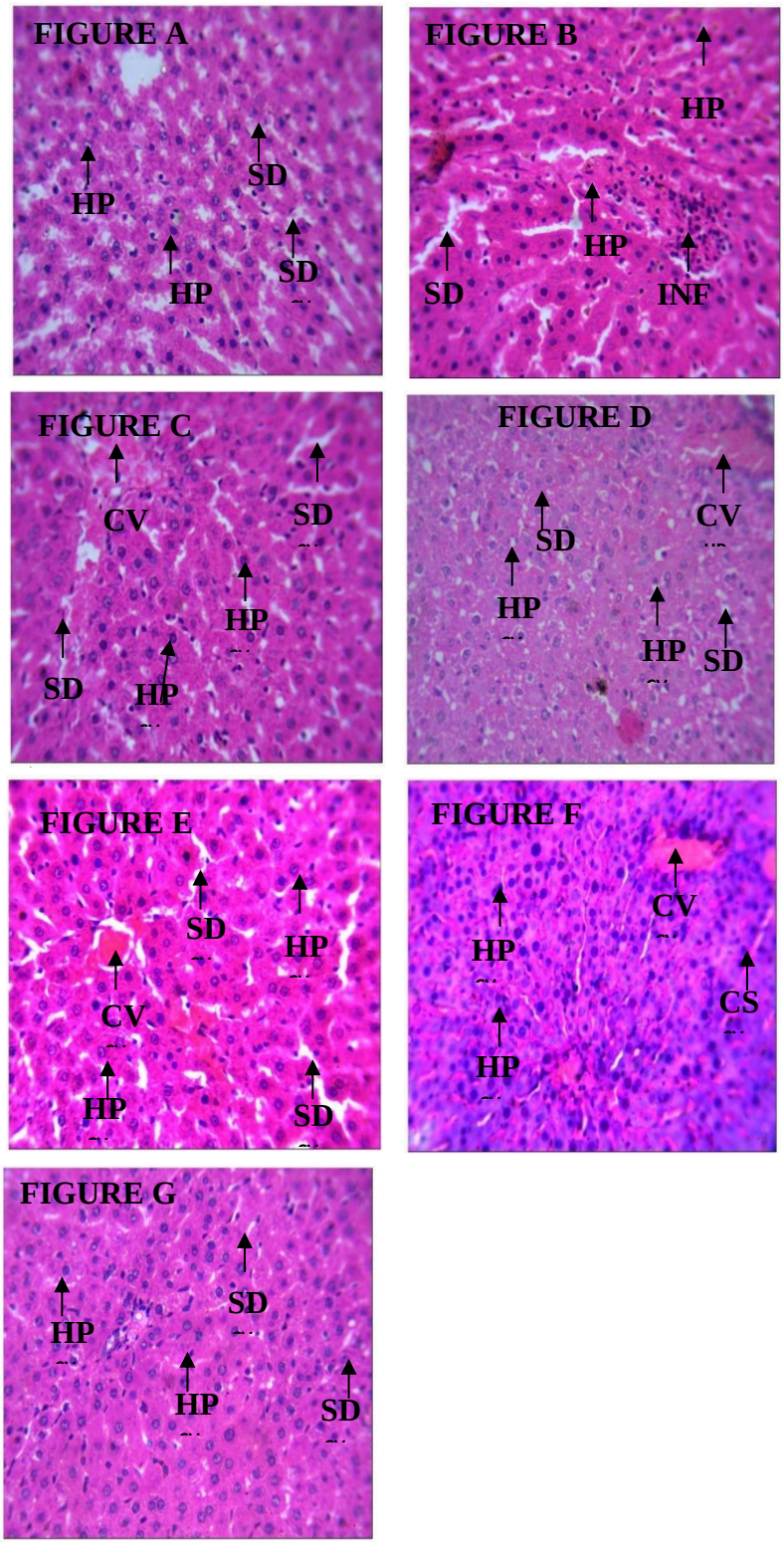
Treatment	% Parasitamia	% Inhibition	MST(Days)
NC	22.10±3.31	0.0	9.42±0.73
CQ	1.08±0.12[a]	90.51	36.38±2.13[a]
AZ	4.13±0.54[b]	81.31	26.07±3.96[b]
AQ	1.19±0.71[c]	94.62	31.04±3.36[c]
AQ-AZ	0.23±0.01[d]	98.96	49.56±3.52[d]

NC: Negative control, CQ: Chloroquine, AZ: Azithromycin, AQ: Amodiaquine, MST: Mean survival time, n= 5, Data as mean ± SEM (Standard error of mean). Values with different superscripts down the column significantly differ at p<0.05 (ANOVA).

Table 4: Effect of amodiaquine-azithromycin on hematological indices of *Plasmodium berghei*-infected mice

Treatment	RBCs ($x10^6$)	WBCs (cells/L)	PCV (%)	HB (g/dL)
C	5.58±0.86	4.12±0.64	56.63±5.12	16.05±1.26
NC	2.49±0.14[a]	13.00±0.58[a]	20.04±2.17[a]	6.14±0.38[a]
CQ	4.19±0.38[b]	6.15±0.13[b]	40.55±3.86[b]	12.58±1.44[b]
AZ	3.50±0.62[c]	8.06±0.52[c]	31.01±3.11[c]	9.66±1.50[c]
AQ	4.88±0.69[b]	5.99±0.52[b]	39.59±4.33[b]	12.86±1.07[b]
AQ-AZ	5.40±0.98[d]	4.23±0.88[d]	54.28±4.83[d]	15.98±1.01[d]

C: Normal control, NC: Negative control, CQ: Chloroquine (Positive control), AZ: Azithromycin, AQ: Amodiaquine. RBCs: Red blood cells, WBCs: White blood cells, PCV: Packed cell volume, HB: Hemoglobin, n= 5, Data as mean ± SEM (Standard error of mean). Values with different superscripts down the column significantly differ at p<0.05 (ANOVA).



Liver of the control and experimental mice are shown in figures A-G. Control (Figure A), mice parasitized with *Plasmodium berghei* (Figures B and C), treatment with chloroquine (10mg/kg) (Figure D), treatment with amodiaquine (10mg/kg) (Figure E), treatment with azithromycin (10mg/kg) (Figure F), treatment with amodiaquine-azithromycin (10mg/kg) (Figure G). CV: Central vein congestion, INF: Inflammatory cells, HP: Normal Hepatocytes, S: Sinusoids, CS: Congested sinusoids. H and E X 400

4. Discussion

The prevalence of parasite resistance to drug presently threatens the efficacy of antimalarial drugs in sub-Sahara Africa [23]. Notwithstanding, to overcome this threat there are efforts on the assessment of new combinations of antimalarial drugs [7,8]. This requires the discovery of combination therapies that can prevent post treatment transmission of antimalarial drug resistant parasite [24]. The current study examine whether AZ can be repurposed as an antimalarial drug in combination with AQ using a mouse model infected with *P.berghei.* Over the years, mouse model has been used extensively to provide insight into the mechanism of underlying diseases, and to explore the efficacies of candidate drugs and to predict patient response [25]. *P. berghei* is used as a model organism for the investigation of human malaria because of its similarity to *Plasmodium* species which causes human malaria. It has a similar life cycle and it causes disease in mice which has signs similar to those seen in human malaria [26]. This study used a 4 day suppressive test which determines the activity of a test compound on early infection and Rane's test, which evaluates the antiplasmodial activity of a test compound on established infection [27]. In this study, in the curative, suppressive and prophylactic tests, the parasitized mice showed elevated percentage parasitamia which is in agreement with previous studies [28]. But treatment with AQ-AZ decreased percentage parasitamia in treated mice. In antiplasmodial studies, test compounds are assessed for their abilities to decreased mortality caused by *Plasmodium* infection through the measurements of MST [28]. In the curative, suppressive and prophylactic tests, AQ-AZ prolonged MST in treated mice. Hematological abnormalities are influenced by diseases including endemic infections such as malaria [29]. Abnormalities such as severe anemia, coagulation disturbance, leukocytes numerical functional changes are constant hallmark of malaria. Malaria associated anemia has been associated with red blood cell lysis, intravascular haemolysis and decreased erythropoiesis [30]. In the current study, anemic signs marked by decreased RBCs, PCV and HB and increased WBCs were conspicuous in *P.berghei*-infected mice. The observation supports earlier reports [28]. But decreased anemia characterized by elevated RBCs, PCV and HB levels and decreased WBCs were visible in AQ-AZ treated mice. Liver involvement in severe *Plasmodium* infection is of significant challenge. The liver is an important organ involved during the hepatic stage of the malaria parasite's life cycle, where malaria sporozoites develop into merozoites. The merozoites are then released into circulation and enter the erythrocytic stage [31]. Liver pathology caused by malaria may be characterized by steatosis, hyperplastic Kupffer cells, portal tract inflammation, bile duct proliferation, sinusoidal congestion and haemozoin deposition [31]. In the liver of parasitized mice, the current study observed inflammatory cell infiltrations, and central vein congestion. Interestingly, the aforementioned liver pathological changes observed in *P. berghei*-infected mice were absent in AQ-AZ treated mice. The current study observed that in the curative, suppressive and prophylactic tests, the antiplasmodial activity of AQ-AZ was higher than the standard (CQ). Based on the observation in the current study, it can be deduced that the clinical use of AQ-AZ may eradicate liver and blood stages of malarial infection. The antiplasmodial activity of AQ-AZ observed in this study is a function of the differences in the mechanisms of action of the drugs. AQ is a 4-aminoquinoline, similar to CQ that has been used widely to treat and prevent malaria. It was used extensively as monotherapy, but currently in combination with artemisinins for the treatment of uncomplicated falciparum malaria [32] and with sulfadoxine-pyrimethamine for seasonal malaria chemoprevention [33]. Its mechanism of action is similar to CQ, but retains antimalarial activity against many CQ-resistant parasites. CQ accumulates in *Plasmodium* food vacuoles and forms a complex with haem, which leads to the accumulation of toxic haem product causing *Plasmodium* death [28]. AZ is a broad-spectrum macrolide antibiotic with bacteriostatic activity against many Gram-positive and Gram-negative bacteria [34]. Its antiplasmodial mechanism of action is not known, but it prevents bacterial protein synthesis by inhibiting the transpeptidation/ translocation step in protein synthesis. Also, it inhibits the assembly of 50s ribosomal subunit and the growth of nascent polypeptide chain [35].In conclusion: AZ potentiates the antiplasmodial

activity of AQ on *P. berghei*-infected mice. AZ can be repurposed in combination with AQ for the treatment of malaria.

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