Potential Antimalarial Activity of Artemether/Lumefantrine/Doxycycline: A Study in Mice Infected with *Plasmodium berghei*

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Abstract

Antimalarial drug resistance is one of the greatest challenges towards eradicating malaria. Exploring new combination therapies can overcome resistance challenges. The present study examined the antiplasmodial effect of artemether/lumefantrine/doxycycline (A/L/D) on a mouse model infected with *Plasmodium berghei*. Adult Swiss albino mice (22-30g) intraperitoneally infected with blood containing 1×10^7 *Plasmodium berghei* were randomly grouped and orally treated daily with D (2.2 mg/kg), A/L (1.71/13.7 mg/kg) and A/L/D. The negative control was treated daily with normal saline (0.2ml) whereas the positive control was treated daily with chloroquine (CQ) (10mg/kg). After treatment, blood samples were assessed for percentage parasitemia and biochemical parameters. Mice were observed for mean survival time (MST). D, A/L and A/L/D produced significant decreases in percentage parasitemia levels at $p<0.05$; $p<0.01$ and $p<0.001$, respectively when compared to negative control. In the curative test, D, A/L and A/L/D produced 60.4%, 70.3%, and 90.0% parasitemia inhibitions, respectively whereas CQ produced 76.0% parasitemia inhibition. D, A/L, A/L/D and CQ produced 63.2 %, 80.1%, 92.3% and 83.6% parasitemia inhibitions, respectively in the suppressive test. D, A/L, and A/L/D prevented *Plasmodium berghei*-induced alterations in biochemical parameters by increasing packed cell volume, red blood cells, hemoglobin, and high-density lipoprotein and decreasing white blood cells, total cholesterol, low-density lipoprotein cholesterol, and triglyceride levels significantly at $p<0.05$ and $p<0.01$ and $p<0.001$, respectively when compared to the negative control. A/L/D produced significant antiplasmodial activity therefore, it may be used clinically for the treatment of malaria.

Keywords; Antiplasmodial; artemether; tetracycline; antimalarial; lumefantrine; *Plasmodium berghei*.

INTRODUCTION

Antimalarial drug resistance has been acknowledged to be one of the greatest challenges to the Roll Back Malaria programme. The situation is highly precarious due to the rising incidence of *Plasmodium* resistance to currently available antimalarial drugs (Yeung et al., 2004). Chloroquine (CQ) resistant *Plasmodium falciparum* now predominates in Southeast Asia, South America and Africa. Resistance to sulphadoxine-pyrimethamine is widespread in Asia and South America and is spreading in Africa and even quinine has become less effective over time (Khan et al., 2004). The use of combination therapy with artemisinins and partner drugs has been a rational approach to combating drug resistance. Drugs with different mechanisms of action may enhance their respective efficacies and extend their therapeutic life spans (White & Olliaro, 1996). Despite, the combination therapy approach, *Plasmodium* resistance is still a serious challenge (Yeung et al., 2004).

Doxycycline (D) is one of the most active antibiotics against *Plasmodium* parasites. It belongs to the tetracycline family. The tetracyclines, which have a very wide spectrum of activity are bacteriostatic and inhibit bacterial protein synthesis (Gaillard et al., 2015). Among the tetracyclines, D is widely used for malaria prophylaxis and is highly acceptable for long-term therapy, except in pregnant women and children (WHO, 2005). *In-vitro* and *in-vivo* investigations have shown that D may be an effective antimalarial drug against drug-resistant *Plasmodium* strains (Basco & Bras, 1993). It is used in combination with quinine as an effective standby emergency treatment of malaria associated with *Plasmodium falciparum* (WHO, 2005). Recently, it has shown antimalarial potential against *Plasmodium berghei-* induced cerebral malaria in experimental models by inhibiting brain inflammation, tumour necrosis factor and chemokines expressions (Schmidt et al., 2018).

Artemether/lumefantrine (A/L) is an artemisinin-based antimalarial drug approved by the US Food and

Drug Administration in 2009 for the treatment of *Plasmodium falciparum* malaria. The dual mechanisms of action of A/L provide fast and sustained *Plasmodium* clearance (Stover et al., 2012). It is the most widely used antimalarial drug combination in endemic regions. In 2017, A/L accounted for almost 75% of all purchased and clinically used artemisinin based combination therapies (ACTs) (Nsanzabana, 2019). Artemisinin derivatives rapidly clear parasites through a number of proposed mechanisms such as interference with plasmodial transport proteins, interference with plasmodial mitochondrial electron transport, and the production of free radicals (Stover et al., 2012). The precise mechanism for the antiplasmodial activity of lumefantrine is not well defined, but it is proposed to inhibit β-hematin formation, which is an important detoxification pathway for *Plasmodium* parasites (Stover et al., 2012). Despite the success achieved with the use of A/L in combating malaria scourge, the emergence of *Plasmodium* parasite resistance in some endemic countries has become a significant drawback for the fight against malaria (Nsanzabana, 2019). Due to the challenge posed by *Plasmodium* parasite resistance, there is a strong advocacy for the rational use of antimalarial drugs with antibiotics. This will afford the synergistic or additive killing of *Plasmodium* parasites, and thus prevent or reduce drug resistance (Miller et al., 2006; Alecrim et al., 2006). This study, therefore assessed the antiplasmodial effect of A/L/D on a mouse model infected with *Plasmodium berghei.*

MATERIALS AND METHODS

Drugs and Dose Selection

Artemether/lumefantrine (A/L) (IPAC Laboratory, India), Chloroquine (CQ) (Evans Medical Nigeria Plc), and Doxycycline (D) (Ranbaxy Laboratories Ltd, India) were used. The following doses were used: A/L (2.3/13.7 mg/kg) (Sirima et al., 2016), CQ (10mg/kg) (Somsak et al., 2018), and D (2.2 mg/kg) (Gaillard et al., 2015)

Animals

Adult Swiss albino mice (22–30 g) were used. The mice were obtained from the animal unit of the Department of Pharmacology, Faculty of Basic Clinical Sciences, College of Health Sciences, University of Port Harcourt, Rivers State. The mice were housed and fed throughout the study period as per recommended standards. The mice were allowed for 2 weeks to acclimatize to working environment and were handled according to the international animal care and welfare guidelines (ILAR, 2011).

Parasites Inoculation

CQ-sensitive *Plasmodium berghei* (*P. berghei*) (NK65 strain) was used for malaria induction in the experimental mice. *P. berghei* was obtained from Malaria Research Laboratory, Centre for Malaria Research and Phytomedicine, University of Port-Harcourt, Rivers State, Nigeria. Mice previously infected with *P. berghei* were used as donor mice and parasites were kept alive by continuous intraperitoneal (i.p) passage of blood from donor mice to uninfected mice weekly. Percentage parasitemia was determined using the formula below.

Protocol for Antiplasmodial Test
▪ *Protocol for curative test*
The curative test was performed as described by Ryley and Peters (1970). Thirty mice randomly grouped into 6 (A1-A6) of 5 mice each were used. Group A1 served as the normal control while A2-A6, which served as the experimental groups were inoculated with 1×10^7 *P. berghei*-infected blood (i.p). Three days later, the mice were treated orally as follows: Group A1 (Normal control) was treated with normal saline (0.2mL) daily for 4 days. Group A2 (Negative control) and group A3 (Positive control) were treated with normal saline (0.2mL) and CQ (10mg/kg) daily for 4 days, respectively. Groups A4-A6 were treated with D (2.2mg/kg), A/L (2.3/13.7 mg/kg) and A/L/D daily for 4 days, respectively. On day 5, tail blood samples were collected from the mice and thin smears were prepared on slides and stained with 10% Giemsa stain. The stained slides were examined microscopically with an oil immersion objective of 100× magnification power. The percentage parasitemia and inhibitions were calculated using the formula shown below.

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

$$\% \, Inhibition \; = \; \frac{(\% \; Parasitemia \; of \; negative \; control - \% \; Parasitemia \; of \; treated \; group) \times 100}{\% \; Parasitemia \; of \; negative \; control}$$

▪ *Protocol for suppressive test*
The suppressive test was performed as described by Knight and Peters (1980). Twenty-five adult Swiss albino mice were inoculated with blood containing 1×10^7 *P. berghei* and randomized into 5 groups (B1-B5) of 5 mice each. The mice were treated after 3 hours of

inoculation as follows: Group B1 (Negative control) and group B2 (Positive control) were orally treated daily with normal saline (0.2mL) and CQ (10mg/kg), for 4 days, respectively. Groups B3-B5 were orally treated with D (2.2mg/kg), A/L (2.3/13.7 mg/kg) and A/L/D daily for 4 days, respectively. On day 5, tail blood samples were collected from the mice and thin smears were prepared on slides and stained with 10% Giemsa stain. The stained slides were examined microscopically with an oil immersion objective of 100×magnification power. The percentage parasitemia and inhibitions were calculated as explained above.

▪ *Protocol for prophylactic Test*
The prophylactic test was performed as described by Peters (1965). Twenty-five adult Swiss albino mice randomized into 5 groups (C1-C5) of 5 mice/group were orally treated as follows: Group C1 (Negative control) and group C2 (Positive control) were treated with normal saline (0.2 mL) and CQ (10 mg/kg) daily for 4 days, respectively. Groups C3 – C5 were treated with D (2.2 mg/kg), A/L (2.3/13.7 mg/kg), and A/L/D daily for 4 days, respectively. On day 5, the mice were inoculated with blood containing 1×10^7 *P. berghei*. After 2 days, tail blood samples were collected and percentage parasitemia and inhibitions were determined as explained above.

Determination of Mean Survival Time
From the time of inoculation with *P. berghei* until death, mortality of each mouse was monitored and recorded. Mean survival time (MST) was determined using the formula below.

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

Evaluation of Biochemical Parameters
In the curative study, blood samples were collected from the mice and evaluated for red blood cells (RBCs), hemoglobin (Hb), packed cell volume (PCV), white blood cells (WBCs), total cholesterol (CHOL), triglyceride (TG), low-density lipoprotein cholesterol and (LDL-C) and high-density lipoprotein cholesterol (HDL-C) using an auto analyzer.

Statistical Analysis
Values were expressed as mean ± SEM (Standard error of mean) of n=5. Values were analyzed using one-way ANOVA, followed by Tukey's *post hoc test*. *P* values less than 0.05, 0.01 and 0.001 were considered significant.

RESULTS

Curative Antiplasmodial Test
Treatment with D, A/L, and A/L/D produced significant decreases in percentage parasitemia at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively when compared to negative control (PU) **(Table 1)**. D, A/L and A/L/D produced parasitemia inhibitions of 60.4%, 70.3%, and 90.0%, respectively whereas CQ produced 76.0 % parasitemia inhibition. MST was significantly prolonged in mice treated with D, A/L, and A/L/D at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively when compared to PU **(Table 1)**.

Suppressive Antiplasmodial Test
Significant decreases in percentage parasitemia levels at $p < 0.5$, $p < 0.01$ and $p < 0.001$ were produced by D, A/L, and A/L/D, respectively when compared to PU **(Table 2)**. Parasitemia inhibitions, which represent 63.2 %, 80.1%, 92. 3% and 83. 6% were produced by D, A/L, A/L/D and CQ, respectively **(Table 2)**. Treatment with D, A/L and A/L/D significantly prolonged MST at $p < 0.5$, $p < 0.01$, and $p < 0.001$, respectively when compared to PU **(Table 2)**.

Prophylactic Antiplasmodial Test
Percentage parasitemia levels were significantly decreased in mice treated with D ($p < 0.05$), A/L ($p < 0.01$) and A/L/D ($p < 0.001$) when compared to PU **(Table 3)**. D, A/L and A/L/D produced parasitemia inhibitions of 65.1%, 82.8%, and 93.9%, respectively whereas CQ produced 86.7% parasitemia inhibition. Significant prolongations of MST occurred in mice treated with D, A/L, and A/L/D at $p < 0.5$, $p < 0.01$ and $p < 0.001$, respectively when compared to PU **(Table 3)**.

Hematological and Lipid Profile
P. berghei infected mice showed significant ($p < 0.001$) increases in serum TG, CHOL, LDL-C and WBCs levels with significant ($p < 0.001$) decreases in serum Hb, PCV, RBCs and HDL-C levels when compared to control **(Tables 4 and 5)**. On the other hand, treatment with D, A/L, and A/L/D significantly decreased serum TG, CHOL, LDL-C, WBCs levels and significantly increased serum Hb, PCV, RBCs and HDL-C levels at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively when compared to PU **(Tables 4 and 5)**.

Table 1. Curative effect of artemether/lumefantrine/doxycycline on *Plasmodium berghei* infected mice.

Treatment	%Parasitemia	%Inhibition	MST (Days)
PU	38.10±4.95	0.0	9.02±0.11
CQ	9.14 ±0.20[a]	76.0	25.30±2.37[a]
D	13.72±0.67[b]	60.4	16.21±1.57[b]
A/L	11.30±0.13[a]	70.3	24.82±3.48[a]
A/L/D	3.43±0.08[c]	90.0	31.63±3.21[c]

Data as mean ± SEM (Standard error of mean) n= 5, PU: Negative control, CQ: Chloroquine, D: Doxycycline, A/L: Artemether/lumefantrine, A/L/D: Artemether/lumefantrine/doxycycline, MST: Mean survival time. [a]$p<0.01$, [b] $p<0.05$, [c] $p<0.001$ Significant difference when compared to PU.

Table 2. Suppressive effect of artemether/lumefantrine/doxycycline on *Plasmodium berghei* infected mice.

Treatment	% Parasitemia	% Inhibition	MST (Days)
PU	20.30±1.03	0.00	9.66±0.45
CQ	3.33±0.32[a]	83.6	30.41±3.71[a]
D	7.47±0.11[b]	63.2	22.73±2.78[b]
A/L	4.04±0.05[a]	80.1	31.02±3.48[a]
A/L/D	1.56±0.02[c]	92.3	37.62±3.67[c]

Data as mean ± SEM (Standard error of mean) n= 5, PU: Negative control, CQ: Chloroquine, D: Doxycycline, A/L: Artemether/lumefantrine, A/L/D: Artemether/lumefantrine/doxycycline, MST: Mean survival time. [a]$p<0.01$, [b] $p<0.05$, [c] $p<0.001$ Significant difference when compared to PU.

Table 3. Prophylactic effect of artemether/lumefantrine/doxycycline on *Plasmodium berghei*-infected mice

Treatment	% parasitemia	% inhibition	MST (Days)
PU	18.50±1.57	0.00	9.66±0.61
CQ	2.46±0.01[a]	86.7	32.03±3.71[a]
D	6.46±0.35[b]	65.1	25.52±2.60[b]
A/L	2.44±0.17[a]	82.8	32.60±3.38[a]
A/L/D	1.29±0.03[c]	93.9	39.21±4.61[c]

Data as mean ± SEM (Standard error of mean) n= 5. PU: Negative control, CQ: Chloroquine, D: Doxycycline, A/L: Artemether/lumefantrine, A/L/D: Artemether/lumefantrine/doxycycline, MST: Mean survival time. [a]$p<0.01$, [b] $p<0.05$, [c] $p<0.001$ Significant difference when compared to PU.

Table 4. Effect of artemether/lumefantrine/doxycycline on hematologic parameters of *Plasmodium berghei*-infected mice.

Treatment	RBC (x10^6)	WBC (cells/L)	PCV (%)	HB (g/dL)
NC	5.11±0.24	6.61±0.11	54.63±5.00	16.31±0.08
PU	2.41±0.29[a]	13.91±1.06[a]	20.72±3.17[a]	6.63±0.37[a]
CQ	4.00±0.11[b]	9.11±0.09[b]	38.80±3.54[b]	11.93±0.15[b]
D	3.10±0.17[c]	10.27±0.17[c]	26.54±3.76[c]	9.65±0.45[c]
A/L	3.99±0.12[b]	9.42±0.09[b]	38.43±4.49[b]	10.04±0.80[b]
A/L/D	4.91±0.09[d]	6.33±0.21[d]	50.25±4.21[d]	13.95±0.05[d]

Data as mean ± SEM (Standard error of mean) n= 5, NC: Normal control PU: Negative control CQ: Chloroquine, D: Doxycycline, A/L: Artemether/lumefantrine, A/L/D: Artemether/lumefantrine/doxycycline, MST: Mean survival time, RBCs: Red blood count, WBCs: White blood count, PCV: Packed cell volume Hb: Haemoglobin, [a] $p<0.001$ Significant difference when compared to NC, [b] $p<0.01$, [c] $p<0.05$, [d] $p<0.001$ Significant difference when compared to PU.

Table 5. Effect of artemether/lumefantrine/doxycycline on lipid parameters of *Plasmodium berghei*-infected mice.

Treatment	TG mg/dL	CHOL mg/dL	HDL-C mg/dL	LDL mg/dL
NC	76.8±8.03	100.4±14.0	55.9±5.66	29.1±3.11
PU	266.3±15.0[a]	298.4±14.1 [a]	24.6±2.32 [a]	220.1±18.1[a]
CQ	169.1±10.6 [b]	190.8±16.4 [b]	39.1±5.72 [b]	117.8±11.6[b]
D	210.7±11.6 [c]	247.5±13.7 [c]	30.0±3.19 [c]	175.4±15.0[c]
A/L	179.0±14.0 [b]	199.6±14.3[b]	37.3±3.67[b]	126.5±12.5[b]
A/L/D	80.9±7.49[d]	117.8±12.5 [d]	49.5±4.39[d]	41.3±10.1[d]

Data as mean ± SEM (Standard error of mean) n= 5, NC: Normal control PU: Negative control CQ: Chloroquine, D: Doxycycline, A/L: Artemether/lumefanrine, A/L/D: Artemether/lumefantrine/doxycycline, TG: Tryglyceride, CHOL:Total cholesterol HDL: High Density Lipoproteins, LDL: Low Density Lipoprotein, VLDL: Very Low Density Lipoprotein. [a] $p<0.001$ when compared to NC, [b] $p<0.01$, [c] $p<0.05$, [d] $p<0.001$ when compared to PU.

DISCUSSION

Artemisinin based combination therapies (ACTs) are used as treatment for uncomplicated malaria (WHO, 2015). Unfortunately, the emergence of *Plasmodium* parasite resistant to ACTs has been reported in endemic regions (Cui et al., 2015; WHO, 2016). Antimalarial drug resistance poses one of the greatest threats to malaria control. In Africa, the efficacy of affordable antimalarial drugs is rapidly declining, and efficacious

antimalarial drugs tend to be too expensive. Cost-effective methods are needed to fight against antimalarial drug resistance. One of the primary solutions to challenges associated with *Plasmodium* parasites resistance to antimalarial drugs is to explore new combination therapies. In combination therapy, the possibility of the *Plasmodium* parasites developing resistance simultaneously to two or more combined drugs with different mechanisms of action is extremely low (WHO, 2001). The current study examined the antiplasmodial activity of A/L/D on *P. berghei*-infected mice. *P. berghei* is used in predicting treatment outcomes of antimalarial drug candidates, due to its sensitivity, making it an important parasite for antiplasmodial studies (Unekwuojo et al., 2011). Studies have shown that suppressive and curative tests are effective in the antiplasmodial evaluation of candidate drugs on early and established infections, respectively. Importantly, suppressive and curative tests give vital information on percentage parasitemia, and parasitemia inhibitions (Bobasa et al., 2018). In the current study, treatment with A/L/D produced the best curative and suppressive antiplasmodial effects in relation to individual doses of A/L, D and CQ. In the curative study, treatment with D, A/L, and A/L/D produced 60.4%, 70.3%, and 90.0% inhibitions, respectively. In the suppressive study, treatment with D, A/L and A/L/D produced 63.2 %, 80.1% and 92. 3% inhibitions, respectively. The prophylactic study showed best decrease in parasitemia level in A/L/D-treated mice when compared to individual doses of A/L, D and CQ. The observed parasitemia inhibitions in the prophylactic study were 65.1%, 82.8%, and 93.9%, and 86.7% in mice treated with D, A/L, A/L/D and CQ, respectively. In addition to percentage parasitemia and inhibition, this study determined the MST of the mice used for the curative, suppressive and prophylactic tests (Georgewill et al., 2021) to further buttress the antiplasmodial activity of A/L/D. Studies have shown that candidate drugs that can appreciably prolong the MST of parasitized animals may be active against malaria (Oliveira et al., 2009). In the current study, curative, suppressive and prophylactic tests showed best prolongation of MST in mice treated with A/L/D than D, A/L, and CQ alone. In the curative study, D, A/L and A/L/D prolonged MST to 16.21±1.57, 24.82±3.48 and 31.63±3.21 Days, respectively. Studies have shown that *Plasmodium* depend on host hemoglobin as a nutrient-source for growth and multiplication. It consumes more than 75% of haemoglobin during its intra-erythrocytic phase and metabolizes heme into hemozoin (Inbaneson and Sundaram 2012). *P. berghei*-infected mice are prone to anemia due to erythrocyte destruction, as a consequence of parasite multiplication or by spleen reticuloendotelial cell action causing the production of phagocytes by the spleen due to abnormal erythrocytes (Nardos and Makonnen, 2017). In the current study, anemia was conspicuous in *P. berghei*-infected mice

characterized by decreased PCV, Hb, and RBCs with increased WBCs levels. However, *P. berghei*–induced anemia was vividly reduced in mice treated with A/L/D which was characterized by increased PCV, HB, and RBCs levels and decreased WBCs levels. Interestingly, the anti-anemic activity of A/L/D was best when compared to individual doses of A/L, D and CQ.

Changes in serum lipid profile related to malaria infection have been reported in some studies. The underlying biological mechanisms for malaria related lipid changes remain unclear, but may be host related, parasite-related or a combination of the two factors (Visser et al., 2013). The present study observed significant alterations in lipid profile in parasitized mice marked by increased CHOL, TG, LDL-C and decreased HDL-C levels. The altered lipid parameters were best restored in A/L/D-treated mice marked by elevated HDL-C and decreased CHOL, TG, LDL-C levels. The observed antiplasmodial effect of A/L/D may be due to the independent mechanisms of action of the constituent drugs. The antiplasmodial mechanisms of D are not well defined, but D can inhibit mitochondrial protein synthesis and also decrease the activity of mitochondrial enzyme (dihydroorotate dehydrogenase) involved in pyrimidine synthesis (Prapunwattana et al., 1998). Also, D can inhibit the syntheses of nucleotides and deoxynucleotides in *Plasmodium* (Yeo et al., 1997). The artemisinins are speculated to interfere with plasmodial mitochondrial electron transport, transport proteins, and the production of free radicals (Stover et al., 2012). Lumefantrine is thought to inhibit β-hematin formation, an important detoxification pathway in *Plasmodium* parasites (Stover et al., 2012).

CONCLUSION

Results of the study concluded that A/L/D significantly decreased percentage parasitemia, increased percentage inhibition and prolonged MST in *P. berghei*-infected mice. The results of this study therefore showed prospect for the use of A/L/D for malaria treatment.

Conflict of interest: The authors declare no conflicts of interest.

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