Repurposing Dihydroartemisinin-Piperaquine-Doxycycline as an Antimalarial Drug: A Study in *Plasmodium berghei*-Infected Mice

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Abstract

Artemisinin-based combination (ACT) therapy is the mainstay for malaria treatment. However, *Plasmodium* parasite with decreased susceptibility to ACT has emerged. Hence, it is imperative to discover new drugs or explore new drug combinations that can decrease *Plasmodium* parasite resistance. This study assessed the antiplasmodial activity of dihydroartemisinin-piperaquine- doxycycline (D-P-DX) on mice infected with *Plasmodium berghei*. Swiss albino mice (25-30g) of both sexes inoculated with 1×10^7 *Plasmodium berghei* intraperitoneally were used. The mice were randomly grouped and orally treated with DX (2.2 mg/kg), D-P (1.71/13.7 mg/kg) and D-P-DX daily in curative, suppressive and prophylactic studies. The negative and the positive controls were treated daily with normal saline (0.2mL) and chloroquine (CQ) (10mg/kg), respectively. After treatment, blood samples were assessed for percentage parasitemia, hematological and lipid parameters. The mice were observed for mean survival time. D-P, DX, and D-P-DX produced significant decreases in percentage parasitemia at $p<0.05$, $p<0.01$ and $p<0.001$, respectively when compared to negative control. In the curative study, D-P, DX, and D-P-DX produced 64.9%, 71.1%, and 93.6% parasitemia inhibitions when compared to 75.0% inhibition produced by CQ. *Plasmodium berghei* -induced alterations in packed cell volume, white blood cells, red blood cells, hemoglobin, high-density lipoprotein cholesterol, total cholesterol, low-density lipoprotein cholesterol, and triglyceride levels were significantly restored by DX ($p<0.05$) and D-P ($p<0.01$) and D-P-DX ($p<0.001$) when compared to the negative control. D-P-DX showed significant antiplasmodial activity against *Plasmodium berghei*- infected mice. It may be clinically useful for the treatment of malaria.

Keywords; artemisinins; doxycycline; malaria; repurposing; resistance.

INTRODUCTION

World Health Organization (WHO) estimates that nearly half of the world's population lived in malaria endemic areas (WHO, 2016). Malaria, a *Plasmodium* parasite infection is one of the greatest health challenges in tropical regions, despite the availability of antimalarial drugs, mosquito repellents and insecticide-treated nets. Malaria chemotherapy remains a major focus of research, and new molecules are being discovered prior to the emergence of drug-resistant strains of *Plasmodium* parasite (Gaillard et al., 2015). The use of antimalarial drugs is faced with the development of resistance from *Plasmodium falciparum* in primarily endemic areas. Other challenges include financial costs, contraindications, and clinical tolerance (Gaillard et al., 2015).

Doxycycline (DX), a broad-spectrum bacteriostatic agent is synthetically obtained from naturally occurring tetracyclines produced by *Streptomyces* sp. (McEvoy et al., 2008). It acts by binding to several proteins in the 30S ribosomal small subunit and to different ribonucleic acids in the 16S ribosomal RNA. In addition to its antimicrobial activity, it is a partially efficacious prophylactic drug with activity against liver stage of *Plasmodium* and blood schizontocides. It is highly effective for the prevention of malaria. The U.S. Food and Drug Administration (FDA) approved the use of DX for prophylaxis of *Plasmodium falciparum* in short-term travelers to areas with chloroquine or pyrimethamine-sulfadoxine-resistant strains (Tan et al., 2011). DX can be used for the treatment of malaria in children less than 8 years old and non-pregnant adults in combination with quinine sulfate for uncomplicated and chloroquine-resistant *Plasmodium falciparum*. It is also used with primaquine and quinine sulfate for uncomplicated chloroquine-resistant *Plasmodium vivax* and with parenteral quinidine for severe malaria (Griffith et al., 2007).

Artemisinin derivatives are highly potent with fast acting antiplasmodial activity. However, due to short half-life, and *Plasmodium* parasite resistance to artemisinin derivatives and older antimalarial drugs, artemisinin derivatives are often combined with partner drugs with longer half-life for fast clearance of malaria parasites (Nosten, 2007). This led to the development of

artemisinin-based combination therapies (ACTs), which have become the mainstay for the treatment of malaria especially in malaria endemic regions (Basco et al., 2017). However, *Plasmodium* parasites with decreased susceptibility to ACTs' have emerged due to both decreased susceptibility to artemisinins and partner drugs (Leang et al., 2013; Sanders et al., 2014). Also, dihydroartemisinin-piperaquine (D-P), one of the currently used ACTs, which is efficacious against malaria (Tayler et al., 2020) and is being considered for the prevention of malaria in pregnancy (Kakuru et al., 2016; Desai et al., 2016) has experienced *Plasmodium* resistance in malaria endemic regions. *Plasmodium* resistance was attributed to decreased susceptibility to both piperaquine and dihyroartemisinin (Amaratunga et al., 2016; Amato et al., 2017). Hence this study aimed to evaluate if the antiplasmodial effect of D-P can be augmented by DX in mice infected with *Plasmodium berghei*.

MATERIALS AND METHODS

Animals, Drugs and Parasites
Swiss albino mice of both sexes (25-30 g) used for this study were sourced from the animal husbandry of the Department of Pharmacology, Faculty of Basic Clinical Sciences, University of Port Harcourt, Rivers State. The mice were housed in cages at temperature 20°C with cycles of 12 h light/12 h darkness. The mice were acclimated for 2 weeks and fed with food pellets and given water *ad libitum*. CQ was manufactured by Alben Healthcare Ind Ltd, D-P was manufactured by Bliss GVS Pharma Ltd India whereas DX was manufactured by Ranbaxy Laboratories Ltd, India. Doses were selected based on previous studies: CQ (10mg/kg) (Somsak et al., 2018), DX (2.2 mg/kg) (Gaillard et al., 2015) and D-P (1.71/13.7 mg/kg) (Yavo et al., 2011). CQ-sensitive *Plasmodium berghei (P. berghei)* (NK65) was used. *P. berghei* was obtained from Nigerian Institute of Medical Research, Yaba, Lagos. Mice used were inoculated intraperitoneally (i.p.) with 0.2 mL of infected red blood cells (RBCs) containing *P. berghei* (1×10^7) obtained from the donor mice.

Evaluation of Antiplasmodial Activity
▪ *Evaluation of curative activity*
Curative activity was performed as reported by Ryley and Peters (1970). Twenty-five Swiss albino mice were inoculated i.p with blood containing 1×10^7. *P. berghei* and grouped into 5 of 5 mice each. The first two groups, AI (Negative control) and A2 (Positive control) were orally treated with normal saline (0.2mL) and CQ (10mg/kg) daily for 4 days, respectively. Groups A3-A5 were orally treated with DX (2.2 mg/kg), D-P (1.71/13.7 mg/kg) and D-P-DX daily for 4 days, respectively. On day 5, tail blood samples were collected from the mice; thin blood films were produced on slides and stained with Giemsa stain and viewed with the aid of a microscope. Evaluations for percentage parasitemia and inhibitions were performed using the formula below.

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

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

▪ *Evaluation of prophylactic activity*
Prophylactic test was performed using an established method described by Peters (1965). Twenty-five Swiss albino mice were assigned to 5 groups of 5 mice each. Group BI served as the negative control and group B2 served as the positive control and were orally treated with normal saline (0.2ml) and CQ (10mg/kg) daily for 4 days, respectively. Groups B3-B5 were orally treated with DX (2.2 mg/kg), D-P (1.71/13.7 mg/kg) and D-P-DX daily for 4 days, respectively. On day 5, the mice were inoculated i.p with 0.2 mL of infected blood containing $1 \times 10^7 P. berghei$. After, 3 days, tail blood samples were collected and percentage parasitemia and inhibitions were calculated using the formula above.

▪ *Evaluation of suppressive activity*
Suppressive test was carried out as described by Knight and Peters (1980). Twenty-five mice were selected and inoculated i.p with blood containing 1×10^7 *P. berghei*. After 3 h, the mice were randomized into 5 groups of 5 mice each. The first two groups, CI (Negative control) and C2 (Positive control) were orally treated with normal saline (0.2mL) and CQ (10mg/kg) daily for 4 days, respectively. Groups C3-C5 were orally treated with DX (2.2 mg/kg), D-P (1.71/13.7 mg/kg) and D-P-DX daily for 4 days, respectively. On day 5, tail blood samples were collected and evaluated for percentage parasitemia and inhibitions using the formula above.

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

Statistical Analysis

Results as mean ± S EM (standard error of mean). Data was analyzed using one-way analysis of variance (ANOVA) and Tukey's test. Differences between means were considered significant at $p < 0.05$, 0.01 and 0.001, respectively.

RESULTS

Curative Activity

Treatment with DX, D-P, and D-P-DX significantly decreased percentage parasitemia levels at p<0.05, p<0.01 and p<0.001, respectively when compared to the negative control **(Table 1).** The observed parasitemia inhibitions produced by DX, D-P, and D-P-DX and CQ were 64.9%, 71.1%, 93.6%, and 75.0%, respectively **(Table 2).** MST was significantly prolonged in mice treated with DX, D-P, and D-P-DX at p<0.05, p<0.01, and p<0.001, respectively when compared to the negative control **(Table 1).**

Suppressive Activity

Percentage parasitemia levels were significantly decreased in mice treated with DX, D-P and D-P-DX at p<0.05, p<0.01, and p<0.001, respectively when compared to the negative control **(Table 2)**. Treatment with DX, D-P and D-P-DX produced 66.5%, 75.0%, and 95.1% parasitemia inhibitions, respectively whereas CQ produced 81.9% parasitemia inhibition **(Table 2).** Significant prolongation of MST in DX, D-P and D-P-DX-treated mice occurred at p<0.05, p<0.01 and p<0.001, respectively when compared to negative control **(Table 2)**.

Prophylactic Activity

The prophylactic test showed significant decreases in percentage parasitemia levels in mice treated with DX (p<0.05), D-P (p<0.01) and D-P-DX (p<0.001) when compared to the negative control **(Table 3).** The percentage parasitemia inhibitions produced by DX, D-P and D-P-DX were 65.1%, 86.8%, and 98.9%, respectively while CQ produced an inhibition of 85.7% **(Table 3)**. MST was prolonged in DX, D-P, and D-P-DX-treated mice. This occurred at p<0.05, p<0.01, and p<0.001, respectively when compared to the negative control **(Table 3)**.

Determination of Mean Survival Time

The mice in the control and the treated groups were observed for mortality and expressed in days. Mortality represented as mean survival time (MST) was calculated using the formula bellow.

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

Hematological and Lipid Parameters

In *P. berghei*-infected mice, TG, CHOL, LDL-C and WBCs increased whereas HB, PCV, RBCs and HDL-C decreased significantly (p<0.001) when compared to the normal control **(Tables 4 and 5)**. On the other hand, TG, CHOL, LDL-C WBCs levels were decreased whereas HB, PCV, RBC and HDL-C levels were increased significantly in mice treated with DX, D-P, and D-P-DX at p<0.05, p<0.01 and p<0.001, respectively when compared to the negative control **(Tables 4 and 5)**.

Table 1. Curative antiplasmodial effect of dihydroartemisinin-piperaquine-doxycycline on mice infected with *Plasmodium berghei*.

Treatment	% Parasitemia	% Inhibition	MST (Days)
PC	35.10±3.00	0.0	9.22±0.18
CQ	8.78±1.54[a]	75.0	27.62±2.39[a].
DX	12.30±1.27[b]	64.9	14.15±1.66[b]
D-P	10.11±1.11[a]	71.1	25.83±2.65[a]
D-P-DX	2.25±0.15[c]	93.6	36.96±3.48[c]

Data presented as ± SEM, n= 5, PC: Negative control, CQ: Chloroquine, DX: Doxycycline, D-P: Dihydroartemisinin-piperaquine, MST: Mean survival time. [a] p<0.01, [b] p<0.05, [c] p<0.001 Significant different when compared to PC, SEM: Standard error mean.

Table 2. Suppressive antiplasmodial effect of dihydroartemisinin-piperaquine-doxycycline on mice infected with *Plasmodium berghei*.

Treatment	% Parasitemia	% Inhibition	MST(Days)
PC	17.60±2.54	0.0	9.40±0.33
CQ	3.19±0.01[a]	81.9	31.61±2.72[a]
DX	5.90±0.08[b]	66.5	20.47±2.67[b]
D-P	4.40±0.09[a]	75.0	29.73±3.11[a]
D-P-DX	0.86±0.07[c]	95.1	37.12±3,29[c]

Data presented as ± SEM, n= 5, PC: Negative control, CQ: Chloroquine, DX: Doxycycline, D-P: Dihydroartemisinin-piperaquine, MST: Mean survival time. [a] p<0.01, [b] p<0.05, [c] p<0.001 Significant different when compared to PC, SEM: Standard error mean.

Table 3. Prophylactic antiplasmodial effect of dihydroartemisinin-piperaquine-doxycycline on mice infected with *Plasmodium berghei*.

Treatment	% Parasitemia	% Inhibition	MST (Days)
PC	15.80±1.47	0.0	9.79±0.22
CQ	2.58±0.06[a]	85.7	33.43±2.53 [a]
DX	5.51±0.07[b]	65.1	22.75±3.57 [b]
D-P	3.03±0.04[a]	86.8	30.91±2.89 [a]
D-P-DX	0.17±0.08[c]	98.9	38.02±3.86 [c]

Data presented as ± SEM, n= 5, PC: Negative control, CQ: Chloroquine, DX: Doxycycline, D-P: Dihydroartemisinin-piperaquine, MST: Mean survival time [a] p<0.01, [b] p<0.05, [c] p<0.001 Significant different when compared to PC, SEM: Standard error mean.

Table 4. Effect of dihydroartemisinin-piperaquine-doxycycline on hematologic parameters of mice infected *Plasmodium berghei*.

Treatment	RBC (x10⁶)	WBC (cells/L)	PCV (%)	Hb (g/dL)
NC	6.83±0.33	7.55±0.09	60.91±5.91	16.93±1.35
PC	3.21±0.43[a]	15.71±1.55[a]	26.72±3.45[a]	6.01±0.17[a]
CQ	5.44±0.28[b]	9.44±0.19[b]	44.21±4.33[b]	13.45±1.38[b]
DX	4.37±0.27[c]	11.00±0.37[c]	31.94±3.47[c]	10.73±0.81[c]
D-P	5.40±0.11[b]	9.57±0.16[b]	42.62±3.67[b]	13.07±1.11[b]
D-P-DX	6.61±0.32[d]	7.25±0.01[d]	55.9±4.41[d]	16.72±1.37[d]

Data presented as ± SEM, n= 5, NC: Normal control, PC: Negative control, CQ: Chloroquine, DX: Doxycycline, D-P: Dihydroartemisinin-piperaquine, MST: Mean survival time, RBCs: Red blood cell, WBCs: White blood cell, 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 PC, SEM: Standard error mean.

Table 5. Effect of dihydroartemisinin-piperaquine on lipid parameters of mice infected with *Plasmodium berghei*.

Group	TG (mg/dL)	TCHOL (mg/dL)	HDL-C (mg/dL)	LDL (mg/dL)
NC	80.8±7.03	110.8±11.4	50.4±4.00	44.2±3.11
PC	250.3±18.9[a]	273.4±18.0 [a]	22.2±1.41 [a]	201.1±18. [a]
CQ	150.1±5.87 [b]	181.6±22.4 [b]	39.7±4.63 [b]	111.9±11.6 [b]
DX	200.7±3.03 [c]	220.0±12.0 [c]	30.6±4.22 [c]	149.3±15.0 [c]
D-P	159.0±4.87[b]	170.7±13.5[b]	38.7±4.00[b]	100.2±12.5[b]
D-P-DX	97.4±8.88[d]	127.7±10.6 [d]	47.9±5.43[d]	60.3±10.1[d]

Data presented as mean ± SEM, n= 5, NC: Normal control, PC: Negative control, CQ: Chloroquine, DX: Doxycycline, D-P: dihydroartemisinin-piperaquine, TG: Tryglyceride, TCHOL: Total cholesterol, HDL: High density lipoproteins, LDL: Low density lipoprotein. [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 PC, SEM: Standard error mean.

DISCUSSION

Malaria is a major health challenge in developing countries of sub-Saharan Africa and South East Asia. The emergence of widespread resistance of *Plasmodium* species to most antimalarial drugs, the increasing insecticide resistance by mosquitoes, and the lack of vaccines have made the fight against malaria seriously tasking (Beeson et al., 2016; Joseph et al., 2020). Hence there is an urgent need to discover alternative drugs with novel modes of action or a combination of currently existing antimalarial drugs to overcome these challenges. The present study, assessed whether antimalarial activity of D-P can be augmented by DX in mice infected with *P. berghei*. This study used *in-vivo* model, because it takes into cognizance the possible prodrug effect and the involvement of the immune system in eradicating malaria infection. *P. berghei* has been used in antiplasmodial studies in predicting experimental treatment outcomes and hence was appropriately used for the study (Satish et al., 2017). This study used suppressive test which determines the activity of a drug candidate on early infection and curative test, which evaluates the curative activity of a drug candidate on established infection (Mekonnen, 2015; Hiben et al., 2016). In the present study, in the curative test, D-P-DX decreased percentage parasitemia levels most when compared to individual doses of D-P, DX and CQ. The observed parasitemia inhibitions in the curative test were 64.9%, 71.1%, 93.6% in DX, D-P, and D-P-DX-treated mice, respectively. Also, in the suppressive and prophylactic tests, best decreases in percentage parasitemia levels occurred in D-P-DX-treated mice in comparison to individual doses of DX, D-P, and CQ. In the suppressive test, 66.5%, 75.0%, and 95.1%, parasitemia inhibitions were observed in DX, D-P, and D-P-DX treated mice respectively. In view of the antiplasmodial activity of D-P-DX observed in the

present study, the ability of D-P-DX to prolong MST in mice was also evaluated. Treatment with D-P-DX prolonged MST in the curative, prophylactic and suppressive tests. The prolongation of MST by D-P-DX was best when compared to individual doses of DX, D-P, and CQ. Hematological abnormalities like anemia caused by erythrocyte destruction are common characteristics of *P. berghei*-infected mice. Rodent malaria causes parasite-induced decrease in PCV, which occurs approximately 48 h post-infection (Nardos and Makonnen, 2017). In the present study, notable signs of anemia marked by low levels of HB, PCV RBCs and increased WBCs levels were observed in *P. berghei*-infected mice. However, P. berghei- induced anemia was curtailed in D-P-DX treated mice when compared to individual doses of DX, D-P, and CQ. Studies have reported that changes in serum lipids could be possible features of malaria (Visser et al., 2013). The present study observed elevated TCHOL, TG, and LDL-C and decreased HDL-C levels in *P. berghei*-infected mice. However, D-P-DX restored serum lipid characterized by decreased TCHOL, TG, LDL-C and increased HDL-C levels. The observed antiplasmodial effect of D-P-DX may be due to different modes of antiplasmodial activity of the partner drugs. Dihydroartemisinin acts through the cleavage of the endoperoxide bridge and the production of free radicals (Meshnick, 1994). Piperaquine is suggested to have similar mode of action as CQ (Meshnick, 1994). In parasite food vacuole, concentrated CQ binds free hematin forming CQ-hematin complex. This interferes with enzymatic processes in the parasite causing parasite death (Tärning et al., 2007). The antiplasmodial mode of action of DX is not clear, but studies suggested the inhibition of mitochondrial protein, nucleotides and deoxynucleotides syntheses in *Plasmodium* (Yeo et al., 1997; Prapunwattana et al., 1998).

CONCLUSION

This study showed that D-P-DX produced the best antiplasmodial activity in *P. berghei*-infected mice when compared to individual doses of D-P, DX and CQ. Also, alterations in lipid profile and hematological parameters were best restored by D-P-DX when compared to individual doses of D-P, DX and CQ. This shows that D-P-DX may be an effective antimalarial drug combination.

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