Research Article

Repurposing dihydroartemisinin-piperaquine-doxycycline as an antimalarial drug: A study in *Plasmodium berghei* infected mice

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Received: 2 January 2021 Revised: 17 February 2021 Accepted: 21 February 2021

Abstract

Objective: Artemisinin-based combination (ACT) therapy is the mainstay for malaria treatment. However, parasites with decreased susceptibility to ACT have 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 - doxycyline (D-P-DX) on mice infected with *Plasmodium berghei* (*P. berghei*). **Materials and Methods:** Swiss albino mice (25-30g) of both sexes, inoculated with $1x10^7$ *P. 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 control and the positive control were treated with normal saline and chloroquine (CQ) (10mg/kg) daily for 4 days, respectively. After treatment, blood samples were assessed for percentage parasitemia, hematological and lipid profile. The mice were also observed for mean survival time. **Results and Conclusion:** DX, D-P 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, DX, D-P and D-P-DX produced 64.9%, 71.1%, and 93.6% parasitemia inhibitions when compared to 75.0%produced by CQ. *P. 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 negative control. **Conclusion:** D-P-DX showed antiplasmodial activity against *P. berghei* infected mice. It may be clinically useful for the treatment of malaria.

Keywords: Antiplasmodial, doxycycline, artemisinins, *Plasmodium berghei,* chloroquine, dihydroartemisinin

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 (Gilliard et al., 2015). The use of anti-malarial drugs is faced with resistance challenges from *Plasmodium falciparum* in primarily endemic areas. Other challenges include financial costs, contraindications, and clinical tolerance (Gilliard et al., 2015).

Doxycycline (DX), a broad-spectrum bacteriostatic agent was 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

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DOI: https://doi.org/10.31024/apj.2021.6.1.3

to areas with chloroquine (CQ) or pyrimethamine-sulfadoxine-resistant strains (Tan et al., 2011). DX can be used for the treatment of malaria in children less than 8 years and non-pregnant adults in combination with quinine sulfate for uncomplicated and chloroquine-resistant *P. falciparum*. It is also used with both 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 activities. However, due to short half-life, and parasite resistance to artemisinin derivatives and older antmalarial 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 treatment of malaria especially in malaria endemic regions (Basco et al., 2017). However, 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). There is an evolving *Plasmodium* resistance to dihydroartemisinin-piperaquine combinations (D-P) one of the presently used ACTs, which has shown tremendous efficacy against malaria parasites (Tayler et al., 202) and is being considered for the prevention of malaria in pregnancy (Kakuru et al., 2016; Desai et al., 2016). *Plasmodium* resistance to D/P is due to resistance to piperaquine (P) and decreased susceptibility to dihyroartemisinin, which is common in malaria endemic regions (Amaratunga et al., 2016; Amato et al., 2017). Hence this study aimed to evaluate if DX can increase the antimalarial activity of P-D in a mouse model infected with *P. 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. They were acclimated for 2 weeks and fed with food pellets and given water *ad libitum*. CQ used 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) (Gilliard et al.,2015) and D/P (1.71/13.7 mg/kg) (Yavo et al., 2011). CQ-sensitive *P. berghei* (ANKA strain) was used. *P. berghei* was obtained from Nigerian Institute of Medical Research, Yaba, Lagos. Each mouse used was inoculated intraperitoneally (i.p.) with 0.2 ml of infected red blood cells (RBCs) containing *P. berghei* (1×10^7) obtained from a donor mouse.

Protocol for curative test

Curative study was performed as reported by Ryley and Peters (1970).Thirty Swiss albino mice were used, but twenty-five mice were inoculated i.p with blood containing 1 × 107. *P. berghei* and grouped into 5 of 5 mice each. Group A1 (non-parasitized group) normal control was treated with normal saline (0.2ml) daily for 4 days. Groups, A2 (Positive control) and A3 (Negative control) were orally treated with normal saline (0.2ml) and CQ (10mg/kg) daily for 4 days, respectively. Groups A4-A6 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 percentage inhibitions were performed using the formula below:

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

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

Protocol for prophylactic test

Prophylactic test was performed using an established method described by Peters (1965). Twenty five Swiss albino mice were assigned to five groups of five mice each. Group AI served as positive control and group A2 served as negative control and 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, the mice were inoculated i.p with 0.2 ml of infected blood containing $1x10^7$ *P. berghei*. Thereafter, treatment continued for 4 days. On day 8, tail blood samples were collected and percentage parasitemia and inhibitions calculated using the formula above.

Protocol for suppressive test

Suppressive was carried out as described by Knight and Peters (1980). Twenty five mice were inoculated ip with blood containing $1x10^7$ *P. berghei*. After 72 h, the mice were randomized into five groups of five mice each. The first two groups, AI (Positive control) and A2 (Negative 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 and evaluated for percentage parasitemia and inhibition using the formula above.

Evaluation of biochemical parameters

Blood samples were collected from mice in the curative study, and evaluated for white blood cells (WBCs), hemoglobin (Hb), packed cell volume (PCV), red blood cells (RBCs), triglyceride (TG) , total cholesterol (CHOL), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C), with the aid of an auto analyzer using standard procedure specified by the manufacturer (Asanga et al., 2013).

Statistical analysis

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

Results

Curative study

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 negative control **(Table 1).** The observed parasitemia inhibitions produced by DX, D-P, 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 negative control (Table 1).

Suppressive test

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 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% inhibition (Table 2). Significant prolongation of MST in DX, D-P, 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 test

The prophylactic test showed significant decreases in percentage parasitemia levels in mice treated with DX

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

Treatment	% Parasitemia	% Inhibition	MST (Days)
PC	35.1±3.00	0.0	9.22±0.18
CQ	8.78±1.54[a]	75.0	27.6±2.39[a].
DX	12.3±1.27[b]	64.9	14.1±1.66[b]
D-P	10.1±1.11[a]	71.1	25.8±2.65[a]
D-P-DX	2.25±0.15[c]	93.6	36.9±3.48[c]

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

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

Treatment	% Parasitemia	% Inhibition	MST(Days)
PC	17.6±2.54	0.0%	9.40±0.33
CQ	3.19±0.01[a]	81.9%	31.6±2.72[b]
DX	5.90±0.08[b]	66.5%	20.4±2.67[c]
D-P	4.40±0.09[a]	75.0%	29.7±3.11[b]
D-P-DX	0.86±0.07[a]	95.1%	37.1±3,29[a]

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

($p<0.05$), D-P ($p<0.01$), and D-P-DX ($p<0.001$), respectively when compared to negative control (Table 3). The parasitemia inhibitions produced by treatment with DX, D-P, and D-P-DX were 65.1%, 80.8%, and 98.9%, respectively while CQ produced 83.7% inhibition (Table 3). MST was prolonged in DX, D-P, D-P-DX treated mice. This occurred at $p<0.05$, $p<0.01$, and $p<0.001$, respectively when compared to negative control (Table 3).

Hematological and lipid parameters

In *P. berghei* infected mice, TG, CHOL, LDL-C and WBCs were increased whereas Hb, PCV, RBCs and HDL-C were decreased significantly ($p<0.001$) when compared to normal control (Tables 4 and 5). On the other hand, TG, CHOL, LDL-C and WBCs levels were decreased whereas Hb, PCV, RBCs and HDL-C levels were increased significantly in mice treated with DX, D-P, and D-P-DX at $p<0.05$,$p<0.01$, $p<0.00$, respectively when compared to negative control (Tables 4 and 5).

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

Treatment	% Parasitemia	% Inhibition	MST (days)
PC	15.8±1.47	0.0%	9.46±0.22
CQ	2.58±0.06[a]	83.7%	33.4±2.53 [b]
DX	5.51±0.07[b]	65.1%	22.7±3.57 [c]
D-P	3.03±0.04[a]	80.8%	30.9±2.89 [b]
D-P-DX	0.17±0.08[a]	98.9%	38.0±3.86 [a]

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

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

Treatment	RBCs (x10^6)	WBCs (cells/L)	PCV (%)	Hb (g/dL)
NC	6.83±0.33	7.55±0.09	60.9±5.91	16.9±1.35
PC	3.21±0.43[a]	13.71±1.55[a]	26.7±3.45[a]	8.01±0.17[a]
CQ	5.44±0.28[b]	9.44±0.19[b]	44.2±4.33[b]	13.4±1.38[b]
DX	4.37±0.27[c]	10.00±0.37[c]	31.9±3.47[c]	11.7±0.81[c]
D-P	5.40±0.11[b]	9.57±0.16[b]	42.6±3.67[b]	13.0±1.11[b]
D-P-DX	6.61±0.32[d]	7.25±0.01[d]	55.9±4.41[d]	16.22±1.37[d]

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

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

Group	TG (mg/dL)	CHOL (mg/dL)	HDL-C (mg/dL)	LDL-C (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: Tryglycerides, TCHOL: Total cholesterol, HDL-C:High density lipoproteins cholesterol; LDL-C: Low density lipoprotein cholesterol. [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 of 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 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 DX can improve the antimalarial activity of D-P in mice infected with *P. berghei*. This study used *in-vivo* model, because it takes into cognizance the possible prodrug effect and involvement of the immune system in eradicating malaria infection. *Plasmodium berghei* (ANKA strain) has been used by studies in predicting experimental treatment outcomes and hence was appropriately used for the study (Satish et al., 2017). This study used a 4 day suppressive test which determines the activity of a candidate drug on early infection and Rane's test, which evaluates the curative activity of a candidate drug on established infection (Hiben et al., 2016; Mekonnen, 2015). In the present study, in the curative test, D-P-DX produced most decrease in percentage parasitemia level 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%, and 75.0% in DX, D-P, D-P-DX and CQ 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%, 95.1% and 81.9% parasitemia inhibitions were observed in DX, D-P, D-P-DX and CQ 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 prolongations of MST by D-P-DX were 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 fall of PCV, which occurred approximately 48 h post-infection (Nardos and Makonnen, 2017). In the present study, notable signs of anemia marked by low levels of Hb, PCV, WBCs and increased WBCs were observed in *P. berghei* infected mice. However, anemic signs were curtailed in D-P-DX treated mice and anti-anemic effects were most when compared to individual doses of DX, D-P, and CQ. Studies have reported that changes in serum lipids could be a characteristic feature of malaria infection (Visser et al., 2013). The present study observed elevated CHOL, TG, and LDL-C levels with decreased HDL-C levels in *P. berghei* infected mice. However, D-P-DX restored serum lipids characterized by decreased CHOL, 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 its constuents. Dihydroartemisinin act through the cleavage of 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 and hemoglobin which 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 showed that D-P-DX may be an effective antimalarial drug combination.

Acknowledgement

The authors appreciate the Laboratory staff of the Department of Pharmacology, Faculty of Basic Clinical Sciences, University of Port Harcourt, Nigeria.

Conflict of interest

The authors declare no conflicts of interest

Financial disclosure: None

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