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Anti-plasmodial activity of rifampicin/ dihydro-artemisinin/piperaquine combination on *Plasmodium berghei* infected mice

Georgewill Udeme Owunari * and Dan-Jumbo Opuada Victor

Department of Pharmacology, Faculty of Basic Clinical Sciences, University of Port Harcourt, Nigeria.

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Abstract

Introduction: The huge cost of de-novo drug development and the problem of multidrug resistance by plasmodium parasites has made research on drugs with potential for repurposing attractive. The study evaluated the antiplasmodial properties of clinical doses of rifampicin in combination with dihydroartemisinic/piperaquine in *Plasmodium berghei*-infected mice.

Materials/Methods: Curative, suppressive, and prophylactic tests were carried out on adult mice (26 – 30g) infected with *P.berghei* parasite. They were grouped and treated per oral (p.o) with RIF (15mg/kg), DP (2.5/20 mg/kg) and RIF/DP (15/2.5/20 mg/kg) daily. The negative control (NC) and the positive control (PC) were treated daily p.o with normal saline (0.2mL) and chloroquine (CQ) (10 mg/kg). Normal saline and chloroquine were administered to mice in the first and second groups, respectively, for 3 consecutive days. Similarly, the third group of mice were treated with dihydroartemisinin/piperaquine combination for 3 consecutive days, while rifampicin was given to the fourth group for 7 consecutive days. The last group of mice were administered the dihydroartemisinic/piperaquine dose for 3days and the rifampicin dose for 7 consecutive days.

Results: There was significant reduction in parasitemia in the prophylactic, suppressive, and curative studies at RIF (15 mg/kg) ($p<0.01$), DP (2.5/20 mg/kg) ($p<0.001$) and RIF/DP ($p<0.0001$) when compared with to negative control. There was an approximately three-fold decrease in percentage parasitemia in the rifampicin/dihydroartemisinin/piperaquine treatment group when compared to the Chloroquine treatment group.

Conclusion: The combination of rifampicin with dihydroartemisinin/piperaquine represent potentially viable additions to the antimalarial arsenal currently available. RIF may be repurposed in combination with DP for malaria treatment.

Keywords: *Plasmodium berghei*; Rifampicin; Dihydroartemisinin; Piperaquine; Combination; Repurposing

1. Introduction

Malaria is a disease that continues to claim more than 400 000 lives each year even though recent concerted effort by a coalition of countries, global agencies, organizations, and philanthropic individuals have led to significant declines in the yearly statistics of the disease impact and burden. In 2018, there were an estimated 405 000 deaths from malaria globally, compared with 416 000 estimated deaths in 2017, and 585 000 in 2010. In the same year, an estimated 228 million cases of malaria occurred worldwide, compared with 231 million cases in 2017, and 251 million cases in 2010 [1]. As a response to the problem of multidrug resistance of malaria to currently used medications, the World Health Organization recommends the combination of artemisinin and its derivatives with other antimalarial drugs with

* Corresponding author: Georgewill Udeme Owunari
Department of Pharmacology, Faculty of Basic Clinical Sciences, University of Port Harcourt, Nigeria.

different mechanisms of action and longer half-lives to optimize efficacy and protect the potent artemisinins from the development of resistance [2].

Some of the solutions to the problem of malaria parasite drug resistance include the development of new artemisinin-based combinations; research into the development of novel medications with different mechanism of action; and repurposing of already existing drugs. The repositioning of drugs with good safety profiles will gain quick approval for newer indications using the same route of administration [3]. It is encouraging that drug regulatory associations in Europe and USA have launched drug repurposing programs to identify new uses for existing medications [4].

Rifampicin is a macrocyclic antibiotic in the family of drugs known as rifamycins, which play a major role in the treatment of mycobacterial diseases [5]. It is a semisynthetic derivative of rifamycin, an antibiotic produced by Amycolatopsis rifamycinica, formerly named Streptomyces mediterranei. It is active in vitro against Gram-positive organisms, some Gram-negative organisms, such as Neisseria and Haemophilus species, mycobacteria, and chlamydiae. Susceptible organisms are inhibited by less than 1 mcg/mL. Rifampicin has been known to have activity in vitro against all erythrocytic stages of P. falciparum [6,7]. It is also active against the murine malarias Plasmodium chabaudi and Plasmodium berghei in vivo [8,7]. The mechanism by which rifampicin inhibits bacteria may be replicated in the plasmodium parasite since they contain an organellar circular DNA molecule that encodes the beta subunit of a prokaryocyte-like RNA polymerase [9]. This study evaluated the antiplasmodial activity of RIF in combination with dihydroartemisinin/piperaquine (DP) in P. berghei infected mice.

2. Material and methods

2.1. Experimental Animals

The animals used in this study were 8 weeks old Swiss Webster mice weighing 26 – 30 g. They were obtained from the animal house of the Department of Pharmacology, University of Port-Harcourt, Nigeria, and kept in cages to acclimatize for 2 weeks before the commencement of the study. They were fed with food and water ad libitum, and strict adherence to the National Research Council Guide for the care and use of laboratory animals (8th Edition), revised 2011, was observed throughout the study.

2.2. Malaria parasite (*Plasmodium berghei*) inoculation

P. berghei was obtained from the Malaria Research Laboratory, Centre for Malaria Research and Phytomedicine, University of Port-Harcourt, Nigeria, and used to prepare stock inoculum of 1 x 107 *P. berghei* infected erythrocytes in 0.2 mL by diluting portions of the blood infected with *P. berghei* with 0.9% normal saline. Blood samples obtained from the mice were screened to ascertain that they were parasite free before each mouse was inoculated via intra-peritoneal route of administration.

2.3. Drug Samples

Rifampicin (RIF), dihydroartemisinin/piperaquine, and chloroquine were provided by JOSIVIC MEDICALS LIMITED, Okuru-Abuloma road, Port Harcourt, Rivers State, Nigeria. Stock solutions of the compounds were prepared in distilled water and stored at −20 °C till required.

2.4. Anti-Malarial Test *in vivo*

The antiplasmodial effect of rifampicin on *P. berghei* was evaluated following standard procedures involving the inoculation of male Swiss Webster Mice with *P. berghei* and subjecting small groups of the inoculated mice to various treatments including rifampicin as monotherapy, and rifampicin/dihydroartemisinin combination. All compounds and combinations were tested in three independent experiments. The stock solutions of the drug samples were diluted to the desired final concentration with distilled water so that each animal received 200 µl (0.2 ml) at the time of administration of each drug.

2.4.1. Curative Test

Curative testing of the various drugs and combinations were performed using the Ryley and Peters method (1970). Thirty (30) mice were randomly assigned into groups of five, giving rise to 6 independent groups labelled groups I to VI. The first group was designated as normal control (non-parasitized), while the second group was designated as the negative control. All groups of mice, except for the first group (normal control), were inoculated with 1 x 10^7 *P. berghei* parasitized erythrocytes intraperitoneally (IP). Various treatments were then applied to mice in all the groups.

72 hours (3 days) after the inoculation event, the following treatments were applied to the various groups. Mice in Groups I and II were treated with normal saline (0.2 mL), while mice in Group III designated as the positive control, were treated with 10mg/kg body weight of chloroquine (CQ). Mice in Group IV, were treated with 2.5/20 mg/kg body weight of dihydroartemisinin/piperaquine combination. Similarly, mice in group V received 15 mg/kg body weight of Rifampicin. Groups VI received 15/2.5/20 mg/kg body weight of rifampicin/dihydroartemisinin/piperaquine combination.

Groups I, II, III and IV received the stated treatments for 3 consecutive days, while group V received treatment for 7 consecutive days while group VI received the dihydroartemisinin/piperaquine dose for 3days and the rifampicin dose for 7 consecutive days.

Parasiticidal activity was assessed daily from day 3 post-infection till day 14. Tail blood smears were prepared, methanol-fixed, stained with Giemsa and microscopically examined to determine parasite density.

2.4.2. Suppressive test

Suppressive testing was similar to the curative test in terms of the treatment applied to the groups and was modelled after the method reported by Knight and Peters (1980). Twenty-five (25) mice were randomly assigned into groups of five, giving rise to five independent groups labelled groups I to V. The first group was designated as negative control (non-parasitized), while the second group was designated as the positive control. Mice in all groups were inoculated with 1 x 10^7 *P. berghei* parasitized erythrocytes intraperitoneally (IP). Three hours after the inoculation event, the following treatments were applied to the various groups. Mice in Group I were treated with normal saline (0.2 mL), while mice in Group II designated as the positive control, were treated with 10mg/kg body weight of chloroquine (CQ). Group III received 2.5/20 mg/kg body weight of dihydroartemisinin/piperaquine combination, while group IV were administered 15 mg/kg body weight of Rifampicin. Group V received 15/2.5/20 mg/kg body weight of rifampicin/dihydroartemisinin/piperaquine combination.

Groups I, II, and III received the stated treatments for 3 consecutive days, while group IV received treatment for 7 consecutive days. Group V received the dihydroartemisinin/piperaquine dose for 3 days and the rifampicin dose for 7 consecutive days. Parasiticidal activity was assessed daily from day 3 post-infection till day 14. Tail blood smears were prepared, methanol-fixed, stained with Giemsa and microscopically examined to determine parasite density.

The significant difference in the curative and suppressive testing lies in the time of administration of the treatments following inoculation of *P. berghei*. Treatments were commenced in all the groups, 3 hours after the inoculation. Evaluation of parasitemia afterwards was similar to the procedure in the curative testing.

2.4.3. Prophylactic test

Prophylactic testing was done by first randomly grouping the mice into five treatment groups similar to the suppressive testing previously described. However, treatment was commenced in all the groups, 4 days prior to the inoculation of the mice with *P. berghei*. On the day of inoculation (day 4), mice in all the treatment groups were injected intraperitoneally with *P. berghei* parasitized erythrocytes and treatment continued for 3 days in groups I – III, while group IV received treatment for 7 consecutive days' post-inoculation. Group V received the dihydroartemisinin/piperaquine dose for 3 days and the rifampicin dose for 7 consecutive days.

Percentage parasitemia was determined using the formula below:

$$\% \text{ parasitemia} = \frac{total\ number\ of\ parasitized\ RBC}{total\ number\ of\ RBC} \text{ x } 100$$

$$\% \text{ inhibition} = \frac{(mean\ parasitemia\ of\ negative\ control - mean\ parasitemia\ of\ treated\ groups)}{mean\ parasitemia\ of\ negative\ control} \text{ x } 100$$

2.5. Population means survival time

The average survival time in days, of the mice in each treatment group was determined by observing and documenting mortality in each group then applying the formula:

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

2.6. Hematological parameters

Blood samples of mice used for the curative test were collected and evaluated to determine their RBC, HB, PCV, WBC, and platelet counts.

2.7. Lipid profile parameters (Test Principles)

Cholesterol, triglyceride and HDL-cholesterol analyses were performed on a Hitachi 704 Analyzer.

2.8. Data analysis

Data analysis was done using IBM SPSS statistics (2017) software. Data was presented as mean ± standard error of the mean (SEM). Significant difference was evaluated through the one-way analysis of variance (ANOVA). Significance was considered at $p<0.05$; $p<0.01$ and $p<0.001$.

3. Results

3.1. Curative test

There was significant reduction in percentage parasitemia in mice treated with dihydroartemisinin/piperaquine in comparison to negative control, at $p<0.001$. More significant reduction in percentage parasitemia when compared to negative control was observed in the group treated with rifampicin and its combination with dihydroartemisinin/piperaquine, at $p<0.0001$. There was a statistically significant difference ($p<0.05$) in the decrease in parasitemia between the chloroquine and rifampicin/dihydroartemisinin/piperaquine treatment groups (table 1).

Percentage parasitemia averaged 21.91 % on day 7 post-inoculation in the negative control group. There was an approximately three-fold decrease in percentage parasitemia in the rifampicin/dihydroartemisinin/piperaquine treatment group when compared to the chloroquine treatment group. By the 7th day post-inoculation, Percentage parasitemia in the rifampicin/dihydroartemisinin/piperaquine combination group had declined to 0.61%. This decline in percentage parasitemia translated to a percentage parasitemia inhibition of 97.22%.

Table 1 Curative activity of rifampicin & its combination with DP on *P. berghei*-infected mice

Treatment	Parasitemia %	MST (days)	Inhibition (%)
NC	21.91±0.51	7.60±3.50	-
CQ	1.70±0.21^{a}	28.8±2.50	92.24
DP	1.67±0.22^{a}	30.2±2.00	92.38
RIF	0.87±0.17^{b}	30.0±2.50	96.03
RIF/DP	0.61±0.22$^{c, d}$	34.6±3.00	97.22

KEY: Nc – Negative control; CQ – Chloroquine; DP – Dihydroartemisinin/piperaquine; RIF – Rifampicin; RIF/DP – Rifampicin/Dihydroartemisinin/piperaquine; MST – mean survival time.; Data is expressed as mean ± standard error of the mean. a $p<0.001$ when compared to NC; b$p<0.01$ when compared to NC; c$p<0.0001$ when compared to NC; d$p<0.05$ when compared to CQ.

3.2. Suppressive test

The results of the suppressive test resemble those of the curative test in terms of significant reduction in percentage parasitemia and percentage inhibition of the *Plasmodium berghei* parasite by the individual doses of the drugs used in the study (except the negative control) as well as the respective combinations with rifampicin (table 2).

By the 7th day post-inoculation, Percentage parasitemia in the negative control group stood at 22.77%. Similar to the findings in the curative test, there was a 3-fold decrease in percentage parasitemia in the rifampicin/dihydroartemisinin/piperaquine treatment group when compared to the chloroquine treatment group. Whereas parasitemia had peaked in the negative control group, 7 days' post inoculation, percentage parasitemia in the rifampicin/dihydroartemisinin/piperaquine combination group had declined to 0.45%. This decline in percentage parasitemia translated to a percentage parasitemia inhibition of 98.11%.

Table 2 Suppressive activity of rifampicin & its combination with DP on *P. berghei*-infected mice

Treatment	Parasitemia %	MST (days)	Inhibition (%)
NC	23.93±0.49	8.40±2.0	-
CQ	1.35±0.25[a]	29.40±3.00	94.36
DP	1.49±0.21[a]	31.00±2.50	93.77
RIF	0.56±0.18[b]	32.80±2.00	97.66
RIF/DP	0.45±0.21[c, d]	32.20±3.50	98.11

KEY: Nc – Negative control; CQ – Chloroquine; DP – Dihydroartemisinin/piperaquine; RIF – Rifampicin; RIF/DP – Rifampicin/Dihydroartemisinin/piperaquine; MST – mean survival time; Data is expressed as mean ± standard error of the mean. a $p<0.001$ when compared to NC; bp<0.01 when compared to NC; cp<0.0001 when compared to NC; dp<0.05 when compared to CQ.

3.3. Prophylactic test

All the treatments (except the negative control) led to significant reductions in parasitemia ($p<0.001$), similar to the observation in the curative and suppressive tests. The most profound reduction in parasitemia occurred in the group with rifampicin/dihydroartemisinin/piperaquine. This treatment group had the highest level of parasite inhibition at 98.67% (table 3). Parasitemia was also lower across all the treatment groups (excluding the negative control) than that observed in the curative and suppressive tests, right from the first day post-inoculation. There was close to six-fold reduction in percentage parasitemia in the rifampicin/dihydroartemisinin/piperaquine treatment group when compared to the positive control group (chloroquine treatment group). Seven days' post-inoculation, parasitemia had declined to 0.28% in the rifampicin/dihydroartemisinin/piperaquine combination group. The percentage inhibition of the parasites across the treatment groups ranged from 95.01% to 98.67%, with rifampicin/dihydroartemisinin/piperaquine treatment group having the highest parasite inhibition. Similar to the observation in the curative and suppressive tests, average survival time of the mice was greatest in the treatment group receiving rifampicin/dihydroartemisinin/piperaquine.

Table 3 Prophylactic activity of rifampicin & its combination with DP on *P. berghei*-infected mice

Treatment	Parasitemia %	MST (days)	Inhibition (%)
NC	21.02±0.89	8.20±4.00	-
CQ	1.57±0.21[a]	26.40±2.00	92.53
DP	0.96±0.48[a]	26.80±2.50	95.43
RIF	0.60±0.44[b]	25.60±3.00	97.15
RIF/DP	0.28±0.55[c, d]	31.40±3.50	98.67

KEY: Nc – Negative control; CQ – Chloroquine; DP – Dihydroartemisinin/piperaquine; RIF – Rifampicin; RIF/DP – Rifampicin/Dihydroartemisinin/piperaquine; MST – mean survival time.; Data is expressed as mean ± standard error of the mean. a $p<0.001$ when compared to NC; bp<0.01 when compared to NC; cp<0.0001 when compared to NC; dp<0.05 when compared to CQ.

3.4. Effects of rifampicin on lipid profile and hematological parameters

The lipid profile of mice in the various treatment groups are displayed in table 4. Notably, the negative control group had the most modified values of all the parameters with high triglycerides (168.86±8.11 mg/dL), high total cholesterol (193.67±9.66 mg/dL), high HDL (82.11±2.67 mg/dL), and high LDL (125.03±6.10 mg/dL). In comparison, the normal control group had triglycerides (122.16±6.11 mg/dL), total cholesterol (106.13±9.21 mg/dL), HDL (55.21±2.91mg/dL), and LDL (87.32 ± 5.33 mg/dL). In the groups which received treatment, triglycerides were lower than in the untreated group (negative control), but higher than the normal control (the group without parasite inoculation). Similarly, total cholesterol was lower than in the negative control group, but higher than in the normal control group. HDL and LDL values were variable across all treatment groups.

There was a general decline in the hemoglobin levels and red cell counts of all the treatment groups, as well as an increase in the white cell counts of the various treatment groups when compared to the normal control (table 5). The packed cell volume, hemoglobin level, and white blood cell counts of the negative control and Chloroquine treatment groups were similar.

Table 4 Effect of rifampicin & its combination with DP on lipid profile of *P. berghei*-infected mice

Treatment	TG (mg/dL)	CHOL (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)
NMC	122.16±6.11	106.13±9.21	55.21±2.91	87.32 ± 5.33
Nc	168.86±8.11	193.67±9.66	82.11±2.67	125.03±6.10
CQ	163.70±10.28[a]	162.67±12.03[a]	61.23±1.56[a]	134.83±7.03[a]
DP	157.26±6.44[a]	120.75±11.17[a]	43.44±1.91[a]	137.67±7.03[a]
RIF	121.17±9.24[b]	108.94±10.41[b]	53.75±1.81[b]	88.94±7.03[b]
RIF/DP	132.45±7.87[c, d]	92.56±9.68[c, d]	58.39±2.22[a]	92.42±7.03[c, d]

KEY: Nmc – normal control; Nc – Negative control; CQ – Chloroquine; DP – Dihydroartemisinin/piperaquine; RIF – Rifampicin; RIF/DP – Rifampicin/Dihydroartemisinin/piperaquine; TG –triglyceride; CHOL – Total cholesterol; HDL-C – High density lipoprotein cholesterol; LDL-C – Low density lipoprotein cholesterol.; Data is expressed as mean ± standard error of the mean. a $p<0.001$ when compared to NC; b$p<0.01$ when compared to NC; c$p<0.0001$ when compared to NC; d$p<0.05$ when compared to CQ.

Table 5 Effect of rifampicin & its combination with DP on hematological profile of *P. berghei*-infected mice

Treatment	PCV (%)	Hb (g/dl)	RBC ($\times10^6$/µl)	WBC ($\times10^3$/µl)	Platelet ($\times10^3$/µl)
NMC	52.4±2.14	16.71±0.89	5.45±0.22	5.66±0.15	257.00±12.61
NC	26.0±2.09	8.67±0.54	4.00±0.15	6.33±0.37	249.00±14.12
CQ	26.0±2.16[a]	8.67±0.77[a]	3.90±0.20[a]	8.03±0.17[a]	235.00±9.16[a]
DP	24.0±3.11[a]	8.00±0.79[a]	3.63±0.19[a]	9.50±0.18[a]	258.00±11.62[a]
RIF	29.0±2.97[b]	9.67±0.76[b]	4.20±0.19[b]	5.50±0.21[b]	244.00±8.54[b]
RIF/DP	26.8±3.09[c, d]	8.90±0.88[c, d]	3.98±0.21[c, d]	9.65±0.19[c, d]	233.75±9.11[c, d]

KEY: Nmc – normal control; Nc – Negative control; CQ – Chloroquine; DP – Dihydroartemisinin/piperaquine; RIF – Rifampicin; RIF/DP – Rifampicin/Dihydroartemisinin/piperaquine; PCV –packed cell volume; Hb – hemoglobin; RBC – red blood cell; WBC – white blood cell.; Data is expressed as mean ± standard error of the mean. a $p<0.001$ when compared to NC; b$p<0.01$ when compared to NC; c$p<0.0001$ when compared to NC; d$p<0.05$ when compared to CQ.

4. Discussion

The observed sustained decline in parasitemia in the rifampicin treatment group up to 7days post-inoculation was in agreement with the findings of Strath, Scott-Finnigan, Gardner, Williamson, & Wilson [7]. This observed difference in P. berghei response to rifampicin may be attributable to their use of chloroquine resistant ANKA strain of the parasite, which may suggest a possible relationship between chloroquine resistance and modified response to rifampicin.

Erhart et al. [12] ascribed observed hematological changes in malaria infection to several factors including level of malaria endemicity, background hemoglobinopathy, nutritional status, malaria immunity, and socio-demographic factors. Such hematological changes include anemia, thrombocytopenia, and either leucopoenia or leukocytosis. Emerging studies suggest that routine laboratory measurement of lipids could be a good and reliable adjunct in the early diagnosis of malaria especially in malaria endemic areas [13].

Though not currently regarded as a major antiplasmodial antibiotic, rifampicin has been shown to be effective against P. chabaudi and P. berghei in rodents [7,14,15]. This study showed significant efficacy of rifampicin and its combinations in the clearance of plasmodium parasitemia without adversely modifying the hematological and lipid profiles of the mice used for the study. Theoretically it would be expected that rifampicin would adversely alter the efficacy of dihydroartemisinin/piperaquine combination since it is a potent inducer of CYP3A4, the enzyme responsible for the metabolism of dihydroartemisinin/piperaquine. This was however, not the case in this study as the combination of rifampicin with dihydroartemisinin/piperaquine led to better parasite inhibition than rifampicin used as monotherapy

or dihydroartemisinin/piperaquine monotherapy. This finding was contrary to that observed in the study by Lamorde et al [16] which reported unfavourable pharmacokinetic interaction between rifampicin and dihydroartemisinin.

5. Conclusion

In this study, the antiplasmodial activity of rifampicin/dihydroartemisinin/piperaquine combination was significant, resulting in rapid and sustained clearance of the parasite, without the associated high recrudescence.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors hereby declare that no conflict of interest exists.

Statement of ethical approval

Ethical approval was sought before commencement of the research from the centre for research ethics and management of the university of Port Harcourt and approval was given to conduct the research.

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