International Journal of Health Research, June 2011; 4(2): 83-89

Available at http://www.ijhr.org

Original Research Article

Open Access
Online Journal

In-Vivo Evaluation of the Antiplasmodial Effect of Amodiaquine and Amodiaquine-Promethazine Combination in *Plasmodium berghei* Infected Mice

Abstract

Purpose: Antihistamine H1 receptor antagonists like promethazine (PR) are capable of reversing resistance of *Plasmodium falciparum* to some antimalarials drugs like amodiaquine (AQ). This work was carried out to evaluate the antiplasmodial activity of amodiaquine and amodiaquine-promethazine combination in *Plasmodium berghei* infected mice.

Methods: Groups of mice (112) infected with chloroquine resistant *Plasmodium berghei ANKA* strain were treated with 10mg/kg amodiaquine alone for three days or 10mg/kg AQ combined with graded doses (10, 20, 30, 40, 50 mg/kg) of PR twice daily over 7 days). Thin blood films were used to assess parasitemia for 60 days.

Results: Therapeutic effect of AQ combined with graded doses of PR was dose-dependent with the combination of AQ and the highest concentration of PR (50mg/kg) having the shortest parasite clearance time (PCT) (1.28± 0.49) days and longest recrudescence time (RT) of (17.33±11.86 days) compare to AQ alone. The mean PCT was significantly reduced as doses of PR increased up to 50mg/kg ($P<0.01$). The survival rates (93.8% and 50%) in the group of animals receiving 50mg/kg of PR plus AQ and AQ alone, respectively were significantly different ($P<0.01$).

Conclusion: Promethazine potentiates the therapeutic effects of amodiaquine against the chloroquine resistant *P. berghei* infection in male albino mice.

Keywords: Amodiaquine, Promethazine, Parasitemia, Plasmodium berghei.

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Introduction

Amodiaquine is a mannich base congener of chloroquine with marginally better efficacy [1]. As a result of widespread resistance, malaria therapy now consists of combination of two or more drugs that attack different biochemical processes in the Plasmodium species. Amodiaquine has been used in combination with artesunate or sulphadoxine –pyrimethamine with good result [2]. Though the combination of antimalarials in malaria therapy improved efficacy and reduced onset of resistance, it did not remove side effects like pruritus experienced with the administration of 4-aminoquinolines. This prompted the continuous search for a new class of resistance modulator drugs, which could be co-administered with amodiaquine, to effectively potentiate amodiaquine and reverse resistance without these side effects. In addition, poor sensitivity of *P. falciparum* to some antimalarial drugs have been reversed both *in vitro* and *in vivo* by the concomitant administration of antihistamine type 1 (H1) receptor notably chlorpheniramine and promethazine [3]. This resistance reversing capability of antihistamines remains to be fully elucidated. Nonetheless, resistance reversal provides yet another possible means to combating the malaria scourge.

Chlorpheniramine and promethazine are commonly used in Nigeria for the purpose of reducing or preventing chloroquine –induced pruritus and are safe and well tolerated [4]. The ability of some non-antimalarial drugs to reverse chloroquine and amodiaquine resistance in *Plasmodium falciparum* is a well-established phenomenon and represents a useful alternative strategy to restore and prolong the clinical utility of both chloroquine and amodiaquine [5, 6, 7]. Clinical application of chlorpheniramine to potentiate the antimalarial activity of chloroquine has been demonstrated in patients [7]. Both drugs are found to be safe and well-tolerated when administered in therapeutic doses [8]. Detectable reversing activities in human plasma, after an oral ingestion of promethazine in bioassay, designed to evaluate the potentiating effect of promethazine on chloroquine in chloroquine resistant parasites, have been reported [9] .These attributes of promethazine make it a potential candidate compound that could be useful for the clinical application of the reversal phenomenon. However, detailed studies on efficacy and safety in animals and also other phases of drug development are essential before clinical application may be recommended.

This study attempted to determine the efficacy and beneficial effect of amodiaquine, a 4-aminoquinoline antimalarial agent in combination with promethazine, an antihistamine of high potency against chloroquine - resistant *P. berghei* infection in male albino mice.

Materials and Methods

Parasites and Experimental animals

Chloroquine resistant *Plasmodium berghei ANKA* strain, obtained from Dr. Dennis Kyle of the Division of Experimental Therapeutics, Walter Reed Army Institute for Research, Washington DC were maintained in mice by serial passage in the animal house of the Malaria Research Laboratories, Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan, Ibadan, Nigeria. 10-12 week old in-bred male Swiss albino mice (24 – 26 g) free from *Eperythrozoon coccoides* were used for the studies. The mice were kept in cages at room temperature, fed with standard mouse cubes (Ladokun Feeds Nigeria Limited) and provided with access to clean drinking water *ad libitum*.

Inoculation of experimental animals

Parasitized red blood cells used for inoculation were obtained by cardiac puncture from an infected donor mouse. The infected blood was collected in an anticoagulant and diluted to the desired parasite density in 0.9% normal saline (Kendall McGraw Laboratories inc. Irvine CA, USA). Each mouse was inoculated intraperitoneally with 1.0 x 10^6 and 1.0x 10^7 parasitized red blood cells of *Plasmodium berghei* strain suspension in 0.2 ml of inoculum. The day of inoculation was defined as day zero

(D0) and subsequent days as D1, D2, and D3 up to D60.

Treatment of experimental animals

One hundred and twelve (112) male mice were randomly allocated into seven groups of sixteen animals each. Six groups were treated with standard doses of amodiaquine alone and with amodiaquine combined with graded doses of promethazine. The last group received phosphate buffered saline (PBS) and served as control. Animals in the treatment group received amodiaquine alone, once daily for 3 days. Those treated with the combination drug received amodiaquine (10mg/kg) daily for 3 days combined with 10, 20, 30, 40, 50mg/kg promethazine orally every 12 hr (twice daily) for 7 days.

Course of infection of *Plasmodium berghei* ANKA strain in male albino mice (untreated control)

Course of infection was monitored daily in each untreated mouse, starting from 72 hours (D3) after inoculation and until death. Thin blood smears were prepared from blood obtained from tail snip in each animal, fixed with methanol and stained with Giemsa. Red blood cells were classified into standard categories, based on differential stain uptake with Giemsa as (i) reticulocytes, which represent an immature, juvenile stage in the maturation of red blood cell between the normoblast and mature cell and (ii) mature red blood cells (erythrocytes), which stains pinkish under the microscope. The parasitized red blood cell (PRBCs) population therefore included both parasitized erythrocyte and parasitized reticulocytes. Number of parasitized reticulocytes was expressed as a percentage of total reticulocyte population. Mature RBC, both parasitized and non – parasitized were expressed as percentage of the total RBCs population of at least a 1000 RBCs counted and tabulated. Percentage parasitized cells were calculated and graphs were plotted from the raw data using the prism computer software.

Determination of responses of infection to treatment

Thin blood films were prepared every day till day 9 and thereafter every other day till day 60 in all surviving animals. Giemsa stained thin blood films were examined under a high power microscope (Olympus, Japan) objective (x100) to enumerate parasite density. Parasite density was determined by counting the number of parasitized erythrocytes among at least 1000 red blood cells.

Occurrence of clearance of parasites following treatment was determined in each animal as Parasite Clearance Time (PCT). The time taken for the parasites to reappear in the animals during the 60 days of follow up was defined as the recrudescence time (RT). The number of animals surviving till 60days after inoculation in each group was also determined.

Statistical analysis

Student t-test was used to compare the mean parasite density, parasite clearance time, recrudescence time and survival rate. Values were considered statistically different at $p<0.01$

Results

Course of infection of *P. berghei* (ANKA) in mice infected with 1.0×10^6 and 1.0×10^7parasitized red blood cells without treatment was evaluated. Infection in this group of animals became noticeable 3 days (72 hours) after inoculation and followed an acute course. Infection in the animals inoculated with 1.0×10^6 inoculum size terminated in death occurring on day 10 (Table 1) Infections in the animals reached highest parasite density on day 6 with 28.1% mean parasitaemia. This later reduced to 17.0% on day 8 and 16.8% on D9.

Similarly, infection in the animals inoculated with 1.0×10^7 also culminated into death at exactly day 7. Infections in this group of animals also became noticeable 3 days after inoculation and followed an acute course. Infections reached highest parasite density on day 5 with 38.5%

Table 1: Course of infection of chloroquine-resistant *Plasmodium berghei* in untreated male albino mice

Untreated	Mean Parasitaemia (%)						
Inoculum size	D3	D4	D5	D6	D7	D8	D9
$1.0X10^6$	0.3	7.0	12.8	28.1	17.4	17.0	16.8
$1.0X10^7$	2.2	12.7	38.5	34.6	30.8		

Table 2: Response of chloroquine resistant *Plasmodium berghei* infection to graded dosages of amodiaquine

Treatment	Response (Days)	
Treatment (mg/kg)	Parasite clearance Time (PCT)	Recrudescence Time (RT)
Amodiaquine (10mg/kg)	2.00 ± 0.00	5.75 ± 0.50
Amodiaquine (20mg/kg)	2.20 ± 0.55	9.80 ± 0.84
Amodiaquine (30mg/kg)	1.80 ± 0.45	11.25±1.89
Amodiaquine (40mg/kg)	1.75 ± 0.50	13.20±2.49
Amodiaquine (50mg/kg)	1.50 ± 0.71	24.25±6.90

Table 3: Response of infection with 10^6 Chloroquine-resistant *Plasmodium berghei* to standard dose of amodiaquine alone, and amodiaquine plus graded doses of promethazine

Treatment	Parasite Clearance time (PCT)	Recrudescence time (RT)
Amodiaquine (30mg/kg) AQ	1.88 ± 0.44	5.25 ± 3.37
AQ +Promethazine (10mg/kg)	2.00 ± 0.46	6.50 ± 4.55
AQ+ Promethazine (20mg/kg)	2.10 ± 0.57	8.60 ± 6.31
AQ+Promethazine (40mg/kg)	1.56 ± 0.53	9.44 ± 7.55
AQ+ Promethazine (50mg/kg)	1.28 ± 0.49	17.33± 11.86

mean parasitaemia, which later reduced to 30.8% on D7 before death (Table 1). In other words, for the 1 x 10^6 inoculum size, mean parasitemia increased readily from the day 3, peaked on day 6 and declined through to day 9. For the 1 x 10^7 inoculum size however, mean parasitemia increased from day 3, peaked on day 5 and declined from day 6 through day 7; the animals died on day 8.

Generally, as the dose of amodiaquine increased, the parasite clearance time reduced while the recrudescence time increased (Table 2).

Treatment with the combination of amodiaquine with selected doses of promethazine (10, 20, 30, 40, 50 mg/kg) resulted in a significant difference in PCT and RT compared with animals treated with amodiaquine alone ($p<0.01$). As the dose of PR combined with AQ increased, PCT decreased while RT increased. Treatment with the combination of amodiaquine with 50mg/kg promethazine produced the shortest parasite clearance time of 1.28 ± 0.49 days and the longest recrudescence time of 17.33± 11.86 days (Table 3).

Survival rate

None of the animals in the control group survived till day 60 (Table 4). The animals in the treatment groups (AQ alone and AQ +10, 20, 30, 40, 50 mg/kg PR) were retreated (between Day 9 and 10) 24 hours after noticing recrudescence parasites in the peripheral blood. Retreatment produced a longer curative effect and lengthened the survival period of the animals.

Retreatment with standard dosage of AQ plus 20, 30, 40 and 50 mg/kg concentration of promethazine produced survival rate between 75 to 94 % of the animals. Blood samples from the survived animals in each of the retreated groups failed to produce infection when sub-inoculated into other clean animals.

Table 4: Effects of treatment with amodiaquine alone or amodiaquine in combination with graded dosages of promethazine on the survival of male albino mice infected with chloroquine resistant *Plasmodium berghei* (ANKA)

Treatment	No of rats alive on day 60 (survival %)	No of rats alive on day 60 (survival %)
Saline (Control)	0	0
AQ (30mg/kg)	8	50
AQ+PR (10mg/kg)	8	50
AQ+PR (20mg/kg)	12	75
AQ+PR (30mg/kg)	13	81.25
AQ+PR (40mg/kg)	15	93.75
AQ+PR (50mg/kg)	15	93.75

Discussion

Plasmodium berghei has proved to be very convenient for the detection of antimalarial activity and it is generally considered that the infection with this parasite in the mouse is a valid model for the primary screening of conventional drugs for eventual use against human malaria [10]. At initiation of infection (Day3), *P. berghei* infects erythrocytes and towards the peak of infection (Day 6), invades predominantly more reticulocytes. At this stage, the animal becomes anaemic, with remarkable increase in the rate of destruction of red blood cell, which eventually triggers the erythroid tissue to start producing more reticulocytes to compensate for the destroyed erythrocytes [14]. It has been confirmed in previous reports that the ratio of reticulocytes to mature RBCs at the peak of infections was approximately 4:1 in male albino mice inoculated with either 1 X 10^6 parasitized red blood cells or with 1 X 10^7 parasitized red blood cells [11, 12, 13]. In the absence of enough reticulocytes, parasites invaded the available mature red blood cells, resulting in rapid death of animals between day 7 and 8 post inoculation. However, when reticulocytes were available in large number, the course of infection of *P.berghei* ANKA in male albino mice may be altered [14]. Infection of large number of reticulocytes resulted in increasing the survival time of infected animals. Infected mice with a large number of infected reticulocytes died between day 7 and day 10 post inoculation. Similarly it was also reported that invasion of all erythrocyte population or a sub-population (for example reticulocytes) can have a profound effect on malarial pathogenesis [14].

Recrudescence of infection documented on day 5 in animal treated with 30 mg/kg amodiaquine is a confirmation of the R1 resistance nature of *P.berghei ANKA* to amodiaquine. General reappearance of parasitaemia (recrudescence) following treatment with the standard dosages of amodiaquine alone or amodiaquine in combination with graded dosages of promethazine against chloroquine resistant *P. berghei* infection appeared to be an evidence of cross-resistance. Infection in thin blood smears from animals treated with 30 mg/kg amodiaquine was found mostly in the reticulocytes. The relevance of this observation reveals that the 50 % effective dose of amodiaquine within mature red blood cells inhibiting *P. berghei* is lower than that found in reticulocytes with decrease in amodiaquine sensitivity [15, 16].

Parasitaemia with lower grades of resistance disappear overtime from the peripheral blood below the level of microscopic detection but recur at a later time [16], this may be due to the longer retention time of amodiaquine acting synergistically with the host immune functions [17]. The potentiation appeared to be dose-dependent. Length of reduction of infection to subtherapeutic level was also dependent on the dosage of promethazine administered. Infections in animals treated with standard dose of amodiaquine and the highest dose of promethazine (50 mg/kg) remain sub patent (delayed recrudescence) for a longer period compared with those treated with lower dosage and also with amodiaquine alone. Survival of infected mice was significantly prolonged when retreated after recrudescence with the combination of amodiaquine and promethazine with animals surviving till 60 days.

It is imperative from the course of infection studies (Control group) to stress the fact that the effect of maintaining infection by serial passaging of parasites in the laboratory did not alter the resistance of the *P.berghei* ANKA to amodiaquine. This finding is contrary to the findings of Peters [11, 16] which stipulated that, chloroquine resistant lines of *P.berghei* were initially unstable, in the absence of drug pressure and may revert rapidly to sensitivity in the course of a few blood passages in the absence of drug pressure [18]. Data obtained in the study also indicated an enhanced efficacy of amodiaquine when combined with selected dosages of promethazine compared to amodiaquine alone against chloroquine resistant *P. berghei* malaria infections. Reversal of amodiaquine resistance infection in mice was dose dependent. The mechanism of amodiaquine resistance and reversal of resistance is not yet fully understood. The significant recrudescence of patent infections experienced with the combinations of amodiaquine with varying concentrations of promethazine may be explained by the fact that promethazine at moderate concentration induces its own metabolism [19].These authors further stated that, this auto-inductive metabolism of promethazine might be acting on the cytochrome P450 enzymatic complex of the liver, which is involved in the metabolism of various drugs.

Binding of drugs to plasma proteins especially albumin and acute phase proteins, is known to decrease antimalarial drugs efficacy [20]. Co-administration of amodiaquine and promethazine had been shown to increase the degree of bioavailability of amodiaquine and its metabolites in the plasma [19, 20]. This increase could be explained by the presence of promethazine, which may competitively displace amodiaquine or its metabolites (desethylamodiaquine) from their original binding sites on plasma proteins [21]. The clinical and therapeutic implication of this increase is that the antiparasitic effect of the amodiaquine is increased. In addition, it is well established that the effect of amodiaquine is dependent on the unbound concentration of the drug at the site of action [20, 21].

Conclusion

The result of this study indicates that promethazine potentiates the therapeutic effects of amodiaquine against the chloroquine resistant *P.berghei* infection in the male albino mice. The reversal of amodiaquine resistance with the combination of amodiaquine and promethazine achieved in this study may play an important role in efforts to control drug resistant malaria at economically feasible costs. The potential value of combination of promethazine with amodiaquine in the management of amodiaquine resistant infections in man would ultimately depend on thorough pharmacokinetics and toxicological evaluation for they remain important prerequisites to clinical application of the reversal phenomenon. Also, additional studies on pharmacokinetic interactions of the two drugs are necessary to optimize dosage regimens of the combination in the management of amodiaquine resistant malaria [21].

Acknowledgments

The staff of Malaria Research Laboratories at the Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan, Nigeria for their unflinching support and assistance.

The UNDP/World Bank/WHO Special Programme for Research & Training in Tropical Diseases (TDR) provided financial assistance in form of a research capability strengthening grant to the Malaria Research group.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Oluwasogo Olalubi designed and performs the experiments, collected the data and prepared the manuscript. Oluseyi Ogunlana analysed the data and assist in preparing the manuscript. Olukunle Fagbemi supervised the entire work. All authors mentioned in the article approved the manuscript.

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