Research Article

Antiplasmodial Assessment of Artemether-Lumefantrine/Ivermectin on Mice Infected with *Plasmodium berghei*

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ABSTRACT

Substantial progress made in the fight against malaria has been undermined by challenges including drug resistance instigating the search for new antimalarial drugs or the repurposing of already approved drugs. This study evaluated the antiplasmodial activity of ivermectin (IM) in combination with artemether/lumefantrine (A/L) in a mice model infected with *Plasmodium berghei.* Parasitized (*Plasmodium berghei*) adult mice weighing 23-28g were used. The mice were grouped and treated orally with 1M (0.17 mg/kg), A/L (2.3/13.7mg/kg) and A/L/IM daily in curative, suppressive and prophylactic tests. The negative control (NC) was treated orally with normal saline (0.2ml) whereas the positive control was treated orally with chloroquine (CQ) (10mg/kg). After drug treatment, blood samples were collected and evaluated for percentage parasitemia levels, percentage parasitemia inhibition, lipid and hematologic parameters. Mean survival time was also evaluated. The 4-day curative, suppressive and prophylactic test showed significant decreases in percentage parasitemia levels at IM (0.17 mg/kg) (p<0.01), A/L (2.3/13.7 mg/kg) (p<0.001) and A/L/IM (p<0.0001) when compared to negative control. IM (0.69 mg/kg), A/L (2.3/13.7mg/kg) and A/L/IM increased mean survival time significantly at p<0.05, p<0.01 and p<0.001 respectively when compared to negative control. Red blood cells, packed cell volume, hemoglobin, high density lipoprotein cholesterol levels were increased significantly whereas total cholesterol, white blood cell, low density lipoprotein cholesterol and triglyceride levels were decreased significantly at IM (0.17 mg/kg) (p<0.05), A/L (2.3/13.7mg/kg) (p<0.01) and A/L/IM (p<0.001) when compared to negative control. This study suggests the use of A/L/IM as a viable malaria treatment.

Key words: Artemether/lumefantrine, Ivermectin, Antimalaria, Mice, Repurposing.

INTRODUCTION

Malaria is an ancient disease that could be traced back to the very earliest human history.[1] It is a life threatening infection transmitted by mosquitoes. Five species of plasmodium are currently known to cause malaria infection in humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi.* The geographical distributions of plasmodium parasites differ with *P. vivax* and *P. falciparium* infections common in Asia and Africa respectively.[2] It is estimated that over 500 million people suffer from malaria infection annually, resulting in about 1-2 million deaths, of which 90% are children in sub-Saharan Africa.[3] Despite the best efforts to reduce global malaria impact, increased malaria infection and death in endemic regions is a nagging challenge which can be attributed to decreased malaria control programs and the prevalence of drug resistant strains of parasites.[4] Resistance to antimalarial drugs necessitates the search for new antimalarial drugs or unexplored drug combinations with propensity to reduce the menace of malaria infection and parasite resistance.[5]

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There has been little output in relation to the amount of effort towards drug development by pharmaceutical companies. Despite an enormous investment in novel technologies such as high-throughput screening, the number of approved drugs has been on the decline.[6] The emergence of an alternative approach; drug repurposing, which takes advantage of off-target effects of the existing drugs has contributed immensely to drug development. Drug repurposing is the reassessment of existing drugs for new therapeutic uses. It is an important, fast and cost effective modality, which overcomes the traditional challenges that characterized *de novo* drug discovery.[7] The repurposing of established drugs is less risky since they have already been tested and have established safety profile.[6,8] Drug repurposing has been employed as a strategy to identify novel antimalarial hits or as lead optimization in malaria drug discovery.[9]

Avermectins belong to the family of macrocyclic lactones that includes compounds with antiparasitic activity and strong insecticidal effect.[10] The impact of avermectins on vectors led to the suggestion of potential function in reducing the incidence and prevalence of vector-borne diseases.[11] Ivermectin (IM), a member of the avermectin family is used for the treatment of arthropod and nematode parasites in animals. It is also used for the treatment of tropical diseases such as lymphatic filariasis, onchocerciasis and strongyloidiasis in humans.[12] Recently, it has gained attention as a potential agent for malaria control.[12] It has effect as an endectocide, causing the demise of Anopheles mosquitoes that ingest sufficient doses in a blood meal.[13,14] Also, it causes mosquitoes mortality in clinical studies using direct-feeding and membrane methods.[15,16] Modeling based studies showed that IM has the potential to prevent or reduce malaria transmission by detrimentally impacting mosquito survival and fertility.[17,18] It has inhibitory effect on the blood stage and liver stages of *P. berghei* and *P. falciparum* infections.[12,19] In view of the aforementioned, this study assessed the potential antimalarial benefit of the co-administration of IM and artemether/lumefantrine in *P. berghei* infected mice.

MATERIALS AND METHODS

Drugs

Ivermectin (IM) (Merck and Co Inc), Chloroquine (CQ) (Evans Medical Nigeria Plc) and Artemether/Lumefantrine (A/L) (IPCA Laboratories Ltd) were used for this study. The following doses were used: IM (0.17 mg/kg,[20] CQ, (10mg/kg)[21] and A/L 1.1/4.6mg/kg.[22]

Malaria parasite

P. berghei strain sensitive to CQ was obtained from the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos. The parasite was maintained by blood passage from mouse to mouse. The donor mice were confirmed to have 20 - 30 % parasitemia. The blood obtained from the mice was diluted in 0.9% saline to make up a (0.2ml) solution containing 1×10^7 parasitized erythrocytes. Daily levels of parasitemia were monitored by microscopic examination of thin blood smears.

Experimental animals

Swiss albino mice (23 to 28 g) were used for this study. The mice were obtained from the animal house of the Department of Pharmacology, Faculty of Basic Medical Sciences, University of Port Harcourt, Rivers State, Nigeria. The mice were acclimatized for 2 weeks prior to the experiment with free access to food and water. The mice were kept in cages at 28°C and a 12 hr light/dark cycle. The directive (2010/63/EU) of the European Union Parliament and Council on care of experimental animals was used.

Determination of curative antiplasmodial activity

Curative antiplasmodial activity was determined using the method of Ryley and Peters (1970).[23] Thirty five mice were randomly assigned to 6 groups (n=5). Group 1 (Normal control) was not parasitized whereas groups 2-6 were parasitized with blood (0.2 ml/i.p) containing 1×10^7 of *P. berghei*. The mice were then left for 3 days post-infection. On day 4, drugs were administered orally as follows: Group 1 (Normal control) and group 1 (Negative control) were administered with normal saline (0.2 ml) for 4 days. Group 2 (Positive control) was administered with CQ (10mg/kg) for 4 days whereas group 3 was administered with IM (0.17 mg/kg) for 4 days. Group 4 was administered with A/L (1.1/4.6 mg/kg) whereas Group 5 was administered with A/L/IM (/1.1/4.6/0.17 mg/kg) for 4 days. On day 8, blood samples were collected, fixed with 70% ethanol and stained with 10% Giemsa. The blood samples were collected by snipping the tails of the mice and percentage parasitemia and inhibition determined.

$$\% \text{ parasitemia} = \frac{\text{Number of parasitized erythrocytes}}{\text{Total number of erthyrocytes}} \times 100$$

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

Determination of suppressive antiplasmodial activity

Suppressive activity was assessed according to Knight and Peters (1980).[24] Twenty five mice were randomly assigned to five groups (n=5). The mice were inoculated on day 1 with 0.2ml of parasitized blood from an infected mouse ip. After 2 hr, the mice were treated orally as follows: Group 1 (Negative control) was administered with normal saline (0.2ml) whereas group 2 (Positive control) was administered with CQ (10mg/kg) for 4 days. Group 3 was administered with IM (0.17mg/kg) whereas group 4 was administered with A/L (1.1/4.6 mg/kg) for 4 days. Group 5 was administered with A/L/IM (1.1/4.6/0.17 mg/kg) for 4 days. On day 5, blood samples were collected on microscope slides, fixed with 70% ethanol and stained with 10% Giemsa. The percentage parasitemia and inhibitions were determined as shown above.

Determination of prophylactic antiplasmodial activity

The prophylactic activity was determined according to Peters (1965).[25] Twenty five mice were assigned randomly to five groups (n = 5). The mice were orally pre-treated as follows: Group 1 (Negative control) was treated with normal saline (0.2ml). Group 2 (Positive control) was treated with CQ (10mg/kg) whereas group 3 was treated with IM (0.17 mg/kg). Group 4 was treated with A/L (1.1/4.6 mg/kg) whereas group 5 was treated with A/L/IM (1.1/4.6/0.17 mg/kg). This treatment continued once daily for 4 days. On day 4, the mice were inoculated with 0.2ml of blood containing 1×10^7 *P. berghei* infected erythrocytes i.p and treatment continued. On day 8, blood samples were collected from the tail on microscope slides, fixed with 70% ethanol and stained with 10% Giemsa. The percentage parasitemia and inhibitions were determined as shown above.

Determination of mean survival time

The experimental animals were monitored daily. The numbers of days of survival were recorded post malarial infection. The mean survival time (MST) was calculated as follows:

$$\text{MST} = \frac{\text{Sum of survival times of all mice in a group}}{\text{Total number of mice in that group}}$$

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM) and was analyzed using GraphPad Prism (GraphPad Prism software, Inc., US). The means were compared using analysis of variance (ANOVA) followed by Tukey's *post hoc* test. P values < 0.05; < 0.01; < 0.001 and < 0.0001 were considered significant.

RESULTS

Curative study

The curative study, on day 7, showed significant reduction in % parasitemia levels in mice treated with IM, A/L and A/L/IM at p<0.05, p<0.01 and p<0.001 respectively when compared to NC. CQ produced significant reductions in % parasitemia levels at p<0.001 respectively when compared to NC (Table 1). Significant increases in MST at p<0.05 and p<0.01 were observed in mice treated with IM and A/L respectively when compared to NC. On the other hand, A/L/IM significantly increased MST at p<0.001 when compared to NC (Table 1).

Suppressive study

Treatment with IM and A/L significantly decreased % parasitemia levels at p<0.05 and p<0.01 respectively when compared to NC. However, treatment with A/L/IM significantly decreased % parasitemia level at p<0.001 when compared to NC. CQ produced significant reduction in % parasitemia levels at p<0.001 when compared to NC (Table 2). MST was significantly

Table 1: Curative activity of artemether/lumefantrine/ivermectin on *Plasmodium berghei*-infected mice.

Group	Parasitemia (%)	Inhibition (%)	MST
NC	45.8±1.04	-	9.30±0.85
CQ	6.73±0.18[a]	85.3	25.0±3.07[a]
IM	22.4±1.72[b]	51.1	15.2±4.29[b]
A/L	13.3±0.22[c]	70.8	20.0±3.05[c]
A/L/IM	4.16±0.68[a]	90.9	27.7±4.92[a]

NC: Negative Control; CQ: Chloroquine; IM: Ivermectin; A/L: Artemether / Lumefantrine; A/L/IM: Artemether/Lumefantrine/Ivermectin; MST: Mean Survival Time; n=5; Data as mean ± SEM; [a] p<0.001 when compared to NC; [b] p<0.05 when compared to NC; [c] p<0.01 when compared to NC.

Table 2: Suppressive activity of artemether/lumefantrine/ivermectin on *Plasmodium berghei*-infected mice.

Group	Parasitemia (%)	Inhibition (%)	MST
NC	20.9±0.88	-	9.65±0.85
CQ	3.91±0.15[a]	81.3	29.0±3.21[a]
IM	9.23±0.22[b]	55.8	20.2±4.29[b]
A/L	5.62±0.33[c]	73.1	25.0±4.33[c]
A/L/IM	1.46±0.93[a]	93.0	31.7±4.92[a]

NC: Negative Control; CQ: Chloroquine; IM: Ivermectin; A/L: Artemether / Lumefantrine; A/L/IM: Artemether/Lumefantrine/ Ivermectin; MST: Mean Survival Time; n=5; Data as mean± SEM, [a] p<0.001 when compared to NC; [b] p<0.05 when compared NC, [c] p<0.01 when compared to NC.

increased at $p<0.05$ and $p<0.01$ in mice treated with IM and A/L respectively when compared to NC. On the other hand, A/L/IM significantly increased MST at $p<0.001$ when compared to NC (Table 2).

Prophylactic study

Significant decreases in % parasitemia levels were observed in mice treated with IM ($p<0.05$) and A/L ($p<0.01$) when compared to NC (Table 3). Most decrease in % parasitemia level was observed in mice treated with A/L/IM ($p<0.001$) when compared to NC. Also, significant reduction in % parasitemia level was observed in mice treated with CQ at $p<0.001$ when compared to NC (Table 3). Treatment with IM and A/L significantly increased MST at $p<0.05$ and $p<0.01$ respectively when compared to NC. Treatment with A/L/IM significantly increased MST at $p<0.001$when compared to negative control (Table 3).

Lipid and hematologic parameters

There were significant increases in TG, CHOL, LDL-C and WBC levels with significant decreases in LDL-C, RBC, PCV and Hb levels in NC when compared to normal control (MN) (Tables 4 and 5). However, significant decreases in TG, CHOL, LDL-C and WBC levels with significant increases in HDL-C, RBC, PCV and Hb levels were observed at $p<0.05$, $p<0.01$ and $p<0.001$ respectively in mice treated with IM, A/L, A/L/1M respectively when compared to NC (Tables 4 and 5).

DISCUSSION

Substantial progress has been made in fighting malaria infection since 2000. This progress is associated with the wide-scale deployment of malaria control interventions. Despite this remarkable progress, malaria infection continues to have a devastating impact on people's health and livelihoods. Increased side effects of conventional drugs and development of resistance by malaria parasites have become a global concern, which

Table 3: Prophylactic activity of artemether/ lumefantrine/ivermectin on *Plasmodium berghei*-infected mice.

Group	Parasitemia (%)	Inhibition (%)	MST
NC	13.5±1.74	-	9.75±1.47
CQ	1.61±0.15[a]	88.1	31.2±4.33[a]
IM	5.60±0.23[b]	58.5	23.6±3.91[b]
A/L	2.13±0.50[c]	84.2	27.0±3.56[c]
A/L/IM	1.20±0.47[a]	91.1	33.5±4.18[a]

NC: Negative Control; CQ: Chloroquine; IM: Ivermectin; A/L: Artemether/ Lumefantrine; A/L/IM: Artemether/Lumefantrine/ Ivermectin; MST: Mean Survival Time; $n=5$; Data as mean ± SEM, [a] $p<0.001$ when compared to NC; [b] $p<0.05$ when compared to NC; [c] $p<0.01$ when compared to NC

Table 4: Effect of artemether/lumefantrine/ivermectin on lipid profile of *Plasmodium berghei*-infected mice.

Treatment	TG (mg/dL)	CHOL (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)
MC	119.0±5.28	151.8±7.65	61.2±5.43	67.3±8.81
NC	298.9±8.55	380.2±17.7	28.5±3.42	292.0±12.0
CQ	191.7±6.73[a]	231.7±13.4[a]	55.6±3.92[a]	138.9±10.8[a]
IM	235.2±7.01[b]	277.1±15.9[b]	46.0±5.01[b]	184.6±10.6[b]
AL	208.1±11.0[a]	234.2±12.1[a]	53.3±4.80[a]	139.7±11.7[a]
A/L/IM	140.9±5.38[cd]	177.6±16.9[cd]	58.0±5.63[a]	91.1±9.88[cd]

MC: Normal control; NC: Negative control; CQ: Chloroquine; IM: Ivermectin; A/L: Artemether/lumefantrine; A/L/IM: Artemether/Lumefantrine/ Ivermectin; TG: Triglyceride; CHOL: Total cholesterol; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; $n=5$; Values are expressed as M±SEM, [a] $p<0.01$ when compared to NC; [b] $p<0.05$ when compared to NC; [c] $p<0.001$ when compared to NC; [d] $p<0.05$ when compared to CQ.

Table 5: Effect of artemether/lumefantrine/ivermectin on hematologic parameters of *Plasmodium berghei*-infected mice.

Treatment	RBC($\times10^6$/µl)	WBC($\times10^3$/µl)	PCV (%)	Hb (g/dl)
MC	6.05±0.31	5.83±0.21	67.2±3.90	17.9±2.89
NC	3.11±0.29[a]	13.1±0.48[a]	28.0±4.33	7.93±0.47
CQ	5.38±0.33[b]	7.15±0.31[b]	54.9±2.88[a]	15.0±2.81[a]
IM	4.42±0.30[c]	9.82±0.33[c]	43.7±4.05[b]	11.3±1.48[b]
A/L	5.42±0.37[a]	7.43±0.51[a]	55.1±2.99[a]	13.8±1.62[a]
A/L/IM	7.02±0.45[cd]	6.09±0.29[cd]	63.6±4.00[cd]	17.7±2.79[cd]

MC: Normal control; NC: Negative control; CQ: Chloroquine; IM: Ivermectin; A/L: Artemether/lumefantrine; A/L/IM: Artemether/Lumefantrine/ Ivermectin; RBC: Red blood cells; WBC: White blood cells; PCV: Packed Cell Volume; Hb: Hemoglobin; $n=5$; Values are expressed as M±SEM, [a] $p<0.01$ when compared to NC; [b] $p<0.05$ when compared to NC; [c] $p<0.001$ when compared to NC; [d] $p<0.05$ when compared to CQ.

called for continued efforts to search for new antimalarial agents.[26] The search for antimalarial drugs involves the discovery of a new drug moiety or the repurposing of an already approved drug moiety. This study assessed whether IM could be repurposed as an antimalarial drug in combination with A/L in a mice model infected with *P. berghei*. Rodent malaria model in mice has been used extensively for the *in-vivo* evaluation of natural and synthetic anti-malarial drug candidates. This model can identify pro-drugs that are activated metabolically and the effect of screened agents on the immune system.

The 4-day suppressive test, which assesses the activity of antimalarial drug candidates on early malaria infection and curative test, which evaluates the curative effect of antimalarial drug candidates on established infection are two universally adopted methods for screening of agents with potential antimalarial activity. Also, prophylactic test has been adopted for the assessment of the prophylactic potential of new antimalarial agents.[27] All these tests, reliably and vividly determine the anti-malarial activities of test compounds on percentage parasitemia levels and percent parasitemia inhibitions.[23,28] In this study, A/L/IM decreased parasitemia levels and increased percentage parasitemia inhibition in the curative, suppressive and prophylactic tests and effects were best in comparison to individual doses of A/L and IM. The effectiveness of A/L/IM during early plasmodium infection (suppressive test) and in the established infection (Curative test) indicates it could be used for malaria treatment. The decreased percentage parasitemia levels and increased percent parasitemia inhibitions produced by A/L/IM were curative test (90.9 %), suppressive test (93.0%) and prophylactic test (91.3%). In the 4-day test, test compounds that showed ≥30% inhibition in parasitemia *in-vivo* following treatment are considered active.[29] This observation shows that A/L/IM has active schizonticidal activity. MST is an essential index used for evaluating the ability of an antimalarial drug candidate to prevent or reduce mortality. A test compound, which results in greater MST than that observed in the untreated group, is considered active.[30,31] In the curative, suppressive and prophylactic tests, A/L/IM increased MST in treated mice. The increases in MST produced by A/L/IM were best than individual doses of A/Land IM. One of the primary complications associated with malaria especially in children and pregnant women is the occurrence of death.[32] Malaria induced anemia is attributed to the clearance or destruction of infected RBCs, clearance of uninfected RBCs and the inhibition of erythropoiesis by plasmodium parasites.[33] This necessitates the assessment of the ability of antimalarial drug candidates to inhibit malaria induced anemia. In this study, RBC, HB and PCV decreased whereas WBC increased in parasitized mice, which indicates anemia. However, reduction in anemia was observed in A/L/IM treated mice characterized by increased serum RBC, HB and PCV with decreased WBC. Malaria parasite also affects host carbohydrate, lipid and protein metabolism.[34,35] The current study observed increased serum CH, TG and LDL-C with decreased HDL-C in the parasitized mice. But treatment with A/L/IM restored the serum levels of the aforementioned parameters characterized by decreased CH, TG and LDL-C with increased HDL-C. The effects produced by A/L/IM were most when compared to individual doses of IM and A/L. The mechanisms behind the antimalarial effect of IM is not well understood, but in arthropods, nematodes and insects it selectively binds to specific neurotransmitter receptors that function in the peripheral motor synapses of parasites causing paralysis by inhibiting the conduction of nervous impulses in the interneuronic (intermediary neurons) synapses of nematodes and the nerve-muscle synapses of the arthropods and insects. IM inhibits chemical transmission across the nerve synapses that use γ - aminobutyric acid- gated chloride or glutamate-gated anion channels.[36] The antimalarial activity of artemether is speculated to be mediated by free radicals and the alkylation of Plasmodium proteins. The active moiety of artemisinin derivatives "endoperoxide Bridge" is cleaved in the presence of ferrous iron, generating free radicals that kills plasmodium parasites.[37] A/L and IM have different mechanisms of action, thus they might have acted at different target points in *P. berghei* causing the highest parasite death compared to their individual doses.

CONCLUSION

The observation in the current study shows that A/L/IM may be employed for malaria treatment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

AL: Artemether-Lumefantrine; **IM:** Ivermectin; **CQ:** Chloroquine; **MST:** Mean Survival Time.

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