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Research Article

Enhanced in vivo antimalarial activity of artemether by clotrimazole against drug-sensitive and resistant *Plasmodium berghei*

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Abstract

The emergence of resistance parasites to currently approved artemisinin-based combination therapies (ACTs) highlight the need for regimens incorporating repurposed antimalarials. In this study, we investigated the *in vivo* performance of artemether/clotrimazole combination against chloroquine-sensitive and multidrug-resistant *Plasmodium berghei* (Pb) in a preclinical mouse model. The antimalarial activity of artemether, clotrimazole and combination of artemether (8 mg/kg) and clotrimazole (2 mg/kg) was investigated using standard protocols for uncomplicated malaria (UM) and severe malaria (SM) in mice infected with chloroquine-sensitive Pb (CPb) and Pb ANKA (PbA), respectively. Hematological parameters (white blood cells, red blood cells, packed cell volume and haemoglobin) and lethality of infected mice in comparison with controls, tested in parallel, were also monitored. The reduction in parasitemia caused by peroral (p.o.) administration of artemether/clotrimazole combotherapy in CPb-infected mice was significantly greater than artemether monotherapy (**p<0.01), clotrimazole monotherapy (****p<0.0001) and marketed chloroquine (*p<0.05) but less than that obtained with therapeutic dosage of marketed ACT (artemether-lumefantrine) (4mg/24mg/kg x 3 days). Similarly, the reduction in parasitaemia in mice infected with PbA by the combination administered intraperitoneally (i.p.) (12.14%) was significantly higher than monotherapies of artemether (**p<0.01) and clotrimazole (****p<0.0001) but less than commercial i.m. artemether (19.17%). Importantly, the combinations administered both p.o. and i.p. ameliorated Pb-induced alterations in hematological parameters of the malariogenic mice similar with conventional antimalarial regimens (controls). Therefore, artemether/clotrimazole combination would be potential therapeutic options for UM and SM. Our ongoing research would seek to investigate the effect of encapsulating artemether/clotrimazole combinatorial regimen in nanocarriers on the antimalarial activity.

Keywords: *Plasmodium berghei* malaria, Clotrimazole, Drug repurposing, Artemisinin-based combination therapy (ACT), *In vivo* antimalarial activity, Artemether.

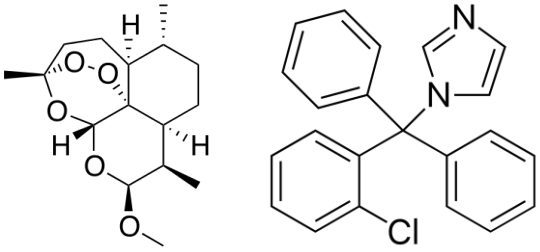
INTRODUCTION

Malaria is a deadly infectious disease caused by the *Plasmodium* parasite and continues to pose a huge threat to public health globally[1]. The World Health Organization (WHO) reported about 229 million cases and 409,000 deaths as a result of this disease in 2019[2,3]. The two most clinically important *Plasmodium* species are *Plasmodium falciparum* and *Plasmodium vivax,* as they are responsible for most of the malaria cases as well as the resulting deaths[3]. *P. falciparum* has been reported to be responsible for malaria mortality and morbidity in the African region, occurring in 99 % of the cases while *P. vivax* and *P. knowlesi* cause relapsing forms of malaria[4]. *P vivax* has been reported to cause 64% of malaria cases in the Americas and 34% in Southeast Asia[4,5].

The current lack of effective vaccines to prevent Plasmodium infection has made vector control, prophylaxis and chemotherapy the major strategies for tackling the disease[6]. Over the years, several scientific efforts have been invested into devising strategies for the prevention, treatment as well as eradication of malaria in some countries with the discovery of artemisinin-based therapies being the zenith of these scientific efforts[3]. However, more recently, reports from the Greater Mekong Subregion in Southeast Asia have reported *P. falciparum* resistance to multiple antimalarial drugs including the first-line treatment: artemisinin-based combination therapies (ACTs). This brought about increased morbidity and mortality in that region. The threat posed by the emergence and spread of ACT and

partner drug resistance in *P. falciparum* has created an imminent need to develop newer active entities or more effective treatment regimens for the treatment of malaria[7].

To date, artemisinin and its derivatives, including artemether (Figure 1A) are the most potent antimalarial agents available and as such are the first-line drugs for the treatment of uncomplicated malaria[7]. This is partly due to the fact that these agents are able to bring about a 10,000-fold reduction in *P. falciparum* burden within 48 hours[7]. Despite the potency of these agents, they have some features that have placed limitations on their use and encouraged the development of resistance to their use by the malaria parasites. One of these is that artemisinin and its derivatives have short half-lives (approximately 1 hour) thus necessitating the use of a longer regimen to achieve complete parasite elimination[7]. The short duration of action and consequent occurrence of recrudescent infections brought about the introduction of the use of ACTs in the treatment of uncomplicated multidrug-resistant *P. falciparum* malaria[8,9]. Artemisinin combination therapy (ACT) usually combines artemisinin derivatives due to their desirable pharmacokinetic properties and a less potent partner drug with a longer half-life. Artemether/lumefantrine, artesunate/amodiaquine, artesunate/mefloquine, artesunate/sulfadoxine pyrimethamine, and dihydroartemisinin-piperaquine are the major ACTs recommended by the WHO for the treatment of malaria.



A B

Figure 1: Chemical structures of (A) Artemether and (B) Clotrimazole. [31,32]

Recently, there are several reports of patients with severe *P. falciparum* malaria which have been unresponsive to treatment with intravenous artesunate[8,9]. There is therefore a need for the discovery and development of novel therapeutic entities for the treatment of malaria. However, the cost as well as the very long duration of time involved in the discovery of new pharmaceutical agents calls for urgent research into the development of alternative strategies to ensure a more effective treatment of malaria. One of these strategies includes several attempts to curb resistance to artemisinin derivatives by extending the length of treatment to six days in place of the conventional three-day regimen[7,9]. However, the challenge of poor patient compliance greatly impeded the actualization of this strategy. Also, several researchers have also begun exploring the development of novel and seemingly more effective combination therapies. This includes the recent combination regimen called the triple artemisinin-based combination therapy (TACTs). Several reports have also considered the use of TACTs which majorly involves the use of two instead of one partner drugs in combination with artemisinin[9]. Some reports have explored adding mefloquine to the ACT, dihydroartemisin/piperaquine while some have considered the addition of amodiaquine to artemether/lumefantrine[7,10]. An example of this was a study conducted in Cambodia, Thailand, Vietnam, which revealed an approximately 50% treatment failure with the use of dihydroartemisin/piperaquine; however, on addition of mefloquine to the treatment regimen, a 98% efficacy was recorded[10]. The study also reported that the treatment regimen was not only efficacious but also safe and well-tolerated by the patients, suggesting that the use of novel combination therapies such as the TACTs represents a safe as well as effective approach for the treatment of drug-resistant malaria. The principle guiding the use of TACTs explores the combination of different drugs with different targets and resistance mechanisms in order to reduce the emergence as well as spread of resistance to its individual drug components[10,11].

In 2019, a study by Jorge *et al* examined the safety and efficacy of artesunate/amodiaquine combined with gametocytocidal drugs - methylene blue, and primaquine in children with *P. falciparum* malaria. The data from this study suggest that the addition of methylene blue to the ACT could potentially reduce *P. falciparum* transmission intensity, increase treatment efficacy and also decrease the risk for the emergence of resistance against ACT in malaria parasites[12].

Another quick and effective strategy for devising novel and alternative treatments for malaria is the repurposing of active pharmaceutical entities employed in the treatment of other disease conditions[13]. This process which considers active entities already in use for other targeted diseases poses a lower risk of failure as the safety of the drugs has been previously established in humans[13,14]. As such, this process saves time as well as research and development costs for clinical research[15]. Over the years, various classes of antimicrobial agents have been evaluated for their antimalarial properties. For instance, antibiotics such as clindamycin have been used in the treatment of severe malaria[16,17]. Doxycycline has also been used for prophylaxis, especially among people traveling to malaria-endemic zones[6]. However, the success of these antibiotics as antimalarials has greatly been impeded by the slow onset of action against the malaria parasites leading to the delayed death effect. This necessitates the use of two cycles of antibiotics in order to exert reasonable action against the malaria parasites[15,17]. This drawback has greatly discouraged the use of these agents as monotherapies for the treatment of active *P. falciparum* infections. Other drugs such as Cotrimoxazole, an antibiotic comprising of trimethoprim and sulfamethoxazole, have also been reported to possess certain antimalarial properties. This was first observed among HIV patients on cotrimoxazole preventive therapy as several studies reported a reduction in clinical malaria incidence[11,18]. This broad-spectrum antibiotic was initially administered to HIV patients to prevent opportunistic infections; however,

several reports have suggested that in addition to the prevention of bacterial infection, it also possesses the ability to reduce the risk of *Plasmodium* infection, especially in endemic regions[18,19]. Thera *et al* also reported that the continued use of cotrimoxazole reduced parasite load and greatly suppressed malaria symptoms[20,21]. Davis et al conducted an observational cohort study in order to determine the impact of daily cotrimoxazole on clinical malaria and asymptomatic parasitemia in HIV-exposed non-infected infants. The result of the study suggested that cotrimoxazole appears to cause an overall reduction in malaria infection[22].

Reports have also shown that the antimycotic agent-clotrimazole (Figure 1B) effectively and rapidly inhibits the growth of various strains of *P. falciparum in vitro* irrespective of the organism's sensitivity to chloroquine[23,24]. The IC_{50} (concentration for 50% inhibition) was between 0.2 -1.1 µM. However, an increase to ≥ 2 µM resulted in 100% inhibition of the malaria parasite replication with a single intraerythrocytic cycle[24,25]. Despite the level of efficacy recorded *in vitro* with clotrimazole, its mechanism of action remains quite ambiguous. Some studies report that clotrimazole possesses a high affinity for heme, and thus inhibits reduced glutathione-dependent heme catabolism. This process then brings about heme-induced hemolysis [26,27]. Other reports show that clotrimazole can remove heme from the histidine-rich peptide-heme complex- a process that initiates heme-polymerization in malaria[26]. Report has shown that nanoemulsion improved the antimalarial activity of clotrimazole in experimental mice[24]. Interestingly, artemisinin derivatives in combination with imidazole antifungals have been reported to produce synergistic pharmacological effects. For instance, a study by Zhou *et al* revealed that artemether improved the antifungal efficacy of fluconazole against *C. albicans in vitro*[28]. In addition, ketoconazole and alpha, beta arteether have shown synergistic antimalarial activity in mice infected with multidrug-resistant *Plasmodium yogeli*[29]. Meanwhile, clotrimazole and artemisinin have been shown to produce synergistic antiplasmodial activity *in vitro*[23]. However, there are paucity of information in the literature on the *in vivo* evaluation of the synergistic effects of clotrimazole and artemether in the treatment of malaria, hence the need for this study. Interestingly, these two drugs are chemically distinct entities[30,31], with completely different proposed mechanisms of antimalarial activity[30,32]. Thus, this study aims to explore the possibility of improving the *in vivo* activity of artemether by concurrent administration of clotrimazole as a novel combinatorial tool for combating the development of resistance to ACTs. Specific objectives include to evaluate the antimalarial activity of each drug as well as combination of artemether and clotrimazole using standard protocols for uncomplicated malaria (UM) and severe malaria (SM) in mice infected with chloroquine-sensitive Pb (CPb) and Pb ANKA (PbA), respectively and then investigate the effect on hematological parameters of malariogenic mice and lethality.

METHODS

Chemicals

Pure artemether sample (May and Baker PLC, Lagos, Nigeria), pure clotrimazole sample (SKG-Pharma, Ikeja Lagos, Nigeria), Capryol® 90 (Gattefosse, St-Priest, France), Solutol® HS 15 (BASF, Ludwigshafen, Germany) and distilled water (Lion Water, UNN, Nigeria) were used as procured from their manufacturers without further purification. All other chemicals, reagents and solvents used were analytical grade or higher and obtained commercially.

Animals and parasites

The study used Swiss albino mice (weighing 16 – 22 g) of both sexes. The animals were obtained from the animal house of the University of Nigeria, Nsukka's Faculty of Veterinary Medicine. Two weeks were given to the animals to become used to their new surroundings before the study began. Throughout the study, the mice were housed in polypropylene cages at ambient temperature and humidity levels, with maintenance of a 12-hour light/dark cycle. They were fed normal rodent diet and had unlimited access to water.

Plasmodium berghei (NK 65 strain) and *Plasmodium berghei* (ANKA strain) malaria parasites utilized in the study were sourced from the Institute of Advanced Medical Research, College of Medicine, University of Ibadan (Ibadan, Nigeria) and the parasites were hosted by donor mice. These parasites [chloroquine-sensitive strain of *Plasmodium berghei* (CPb) and a resistant strain - *Plasmodium berghei* ANKA (PbA)] were used as a model to mimic *Plasmodium falciparum* that causes uncomplicated malaria (UM) and severe malaria (SM) or cerebral malaria (CM) in humans, respectively[33,34].

Preparation of Pb NK-65 and Pb ANKA Inoculums

In each case, the parasite was maintained in the Swiss mice through sequential passages of the blood of infected mice, obtained by ocular puncture[33]. Briefly, a stock solution of parasitized erythrocytes was obtained from infected mice, with a minimum peripheral parasitemia of 20 % through the retro-bulbar plexus of the median canthus of its eye. The blood was collected into an EDTA-coated tube. The percentage parasitaemia in each case was determined by counting the number of parasitized red blood cells against the total number of red blood cells. The cell concentration of the stock was determined and diluted with normal saline such that 0.2 ml of the final inoculums contained 1×10^7 parasistized red blood cells which are the standard inoculums for the infection of a single mouse.

Preparation of extemporaneous injections and oral solutions

Here, we adopted reported procedures with slight modifications[24,35]. Injections containing artemether, clotrimazole or dual drug were successfully applied by intraperitoneal injections of each solubilized drug or rational combination of artemether and clotrimazole (4:1) in dimethylsulphoxide (DMSO) [35] at dosages stated under the experimental protocol in the subsequent

section. Similarly, oral extemporaneous preparations containing the dosages of artemether, clotrimazole or combination of artemether and clotrimazole (4:1) dissolved in a homogenous mixture containing Solutol® HS 15 and Capryol® 90 at 1:3 (w/w) [24] in distilled water, were administered perorally as stated under the experimental protocol in the subsequent section.

Experimental protocols

Seventy-two mice were divided into 12 groups of six mice each as shown in Table 1. The first six groups (A-F) were infected with chloroquine-sensitive strain of *Plasmodium berghei* (CPb), the next five groups (G-K) were infected with *Plasmodium berghei* ANKA (PbA) while the last group (L) was not infected (non-infected control, NC). Five and seven days after the inoculation of the mice with CPb and PbA, respectively, percentage parasitaemia was determined and, after the establishment of malaria, treatment was started on the same day (day 1) on the malariogenic mice and was repeated till day 3 and day 5 for CPb-infected and PbA-infected mice, respectively. Other details of the experimental treatments are shown in Table 1. Parasitemia was assessed from tail blood smears (Giemsa-stained) post treatment. From the tail, blood samples were collected from the mice; thin blood films were produced by fixing with methanol and stained with 10% Giemsa. Slides for the parasites were prepared for the groups of mice infected with CPb and PbA. The stained slide of the blood smear in each case was mounted on a binocular microscope and a drop of immersion oil applied to the slide[34]. Each slide field was evaluated microscopically (x1000 magnification) for parasitized and non-parasitized red blood cells (RBCs).

Table 1: Treatments administered to the mice

Group	Sample code	Treatment	Dosing	Route
A	Art-NK-65	Art monotherapy	8 mg/kg x 3 days	PO
B	CMZ-NK-65	CMZ monotherapy	10 mg/kg x 3 days	PO
C	Art-CMZ-NK-65	Art and CMZ 4:1 combo therapy	8mg/2mg/kg x 3 days	PO
D	AL-NK-65	Marketed ACT (Art and LMF combo therapy)	4 mg/24mg/kg x 3 days	PO
E	CQ-NK-65	Marketed CQ monotherapy	10 mg/kg on day 1, then 5 mg/kg on day 2 and 3	PO
F	DW-NK-65	Distilled water	2 ml/kg x 3 days	PO
G	Art-ANKA	Art monotherapy	8 mg/kg x 5 days	IP
H	CMZ-ANKA	CMZ monotherapy	10 mg/kg x 5 days	IP
I	Art-CMZ-ANKA	Art and CMZ 4:1 combo therapy	8mg/2mg/kg x 5 days	IP
J	Mktd Art-ANKA	Marketed Art injection	8 mg/kg x 5 days	IM
K	NS-ANKA	Normal saline	2 ml/kg x 5 days	IP
L	NC	Normal control (uninfected and untreated) but received distilled water	2 ml/kg x 5days	PO

Key: Art is artemether, CMZ is clotrimazole, AL is artemether plus lumefantrine marketed dosage regimen, LMF is lumefantrine, ACT is artemisinin-based combination therapy, CQ is chloroquine, NC is normal control, PO is peroral, IP is intraperitoneal, IM is intramuscular, Groups A to F were inoculated with chloroquine-sensitive strain of *Plasmodium berghei* (NK 65) while Groups G to K were inoculated with *Plasmodium berghei* ANKA.

In each field, the number of parasitized RBCs was counted, and the total number of RBCs was determined. The mean parasitemia (%) and percentage reduction in parasitemia were calculated using the formulas shown below[36].

$$Mean\ parasitemia\ (\%) = \frac{Number\ of\ infected\ red\ blood\ cells\ (RBCs)}{Total\ number\ of\ RBC\ count} x\ 100$$

$$Percentage\ reduction\ in\ parasitemia\ (\%) = \frac{Parasitemia\ of\ negative\ control\ (\%) - Parasitemia\ of\ treated\ group\ (\%)}{Parasitemia\ of\ negative\ control\ (\%)} x\ 100$$

Determination of hematological parameters

For both the Pb NK-65-infected and Pb ANKA-infected mice and normal control (uninfected and untreated), blood samples were collected from the tail of each mouse (post-treatment for the treatment groups) and evaluated with respect to red blood cells (RBCs), white blood cells (WBCs), packed cell volume (PCV) and hemoglobin (Hb) using an auto analyzer, adopting an established procedure[34].

Weight Determination

The various weights of the mice were determined before inoculation, after establishment of parasitemia and after treatment to assess the effect of the parasitemia and the treatments on the weights of the animals. The groups' mean body weights were calculated.

Assessment of CPb and PbA induced lethality in mice

The lethality of CPb and PbA infections was assessed by determining the number of infected mice that died in the untreated control and those treated with various samples[33]. At the end of the study, euthanasia was carried out on the rest of the animals still remaining alive by cervical dislocation.

Statistical analysis

The data was analyzed on GraphPad prism 10.3, using ordinary one-way or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Values were expressed as mean ± SEM (standard error of

means) of n = 6. A *P* value less than 0.05, 0.01, 0.001 and 0.001 was considered statistically significant and was flagged with one star (*), two stars (**), three stars (***), and four stars (****), respectively.

RESULTS

Antimalarial efficacy of artemether and clotrimazole against chloroquine sensitive P. berghei infection in mice

Figure 2 shows the mean parasitemia levels in the experimentally chloroquine-sensitive *Plasmodium berghei*-infected groups of mice with or without peroral treatment whereas percentage reduction in parasitemia of mice infected with NK-65 strain of *Plasmodium berghei* after three days of peroral treatment is depicted in Figure 3. The results showed that the mean parasitaemia count of the group treated with artemether alone reduced from 33.4 post-inoculation to 28 post-treatment; the mean parasitaemia count of the group treated with clotrimazole alone reduced from 46.5 post-inoculation to 44.1 post-treatment; the mean parasitaemia count of the group treated with artemether and clotrimazole combination reduced from 43.2 post-inoculation to 35 post-treatment; the mean parasitaemia count of the group treated with Coartem® (commercial artemisinin-based combination product containing artemether and lumefantrine) reduced from 32.8 post-inoculation to 20.2 post-treatment; while that of the group treated with marketed chloroquine phosphate reduced from 38.6 post-inoculation to 32.4 post-treatment. On the contrary, there was an increase in mean parasitemia count for the untreated group (i.e. DW-NK-65 that served as negative control) from 26 post-inoculation to 38 after treatment. Moreover, while commercial products (Coartem® and chloroquine phosphate) achieved percent reduction in parasitemia of 37.7 and 15.76%, respectively, post-treatment, the percent parasitemia reduction achieved on completion of the study (i.e. post-treatment) by artemether, clotrimazole and artemether/clotrimazole combination was respectively 14.88, 4.3, and 18.90%, respectively. From the results presented in Figures 2 and 3, it was obvious that the treatments were able to bring down the parasitemia level unlike what was observed in the untreated group (negative control), where there was an increase in parasitemia.

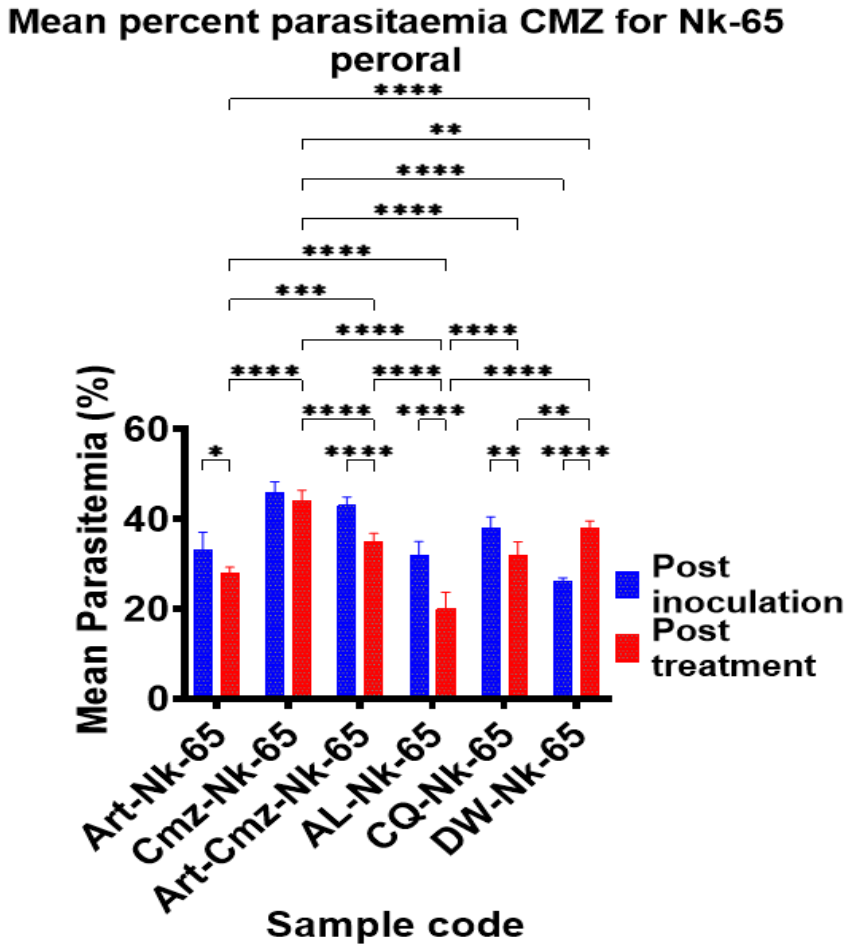


Figure 2: Mean parasitemia levels obtained post inoculation and post treatment with the samples (p.o.) in mice infected with NK-65 strain of *Plasmodium berghei*. Data were expressed as mean ± SEM (standard error of mean) n = 6, differences were considered significant for *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Key: Post treatment means with peroral treatment, Post inoculation means without peroral treatment, Art is pure artemether sample, CMZ is pure clotrimazole sample, AL is artemether plus lumefantrine marketed oral dosage regimen, DW is distilled water and CQ is conventional chloroquine phosphate oral dosage regimen.

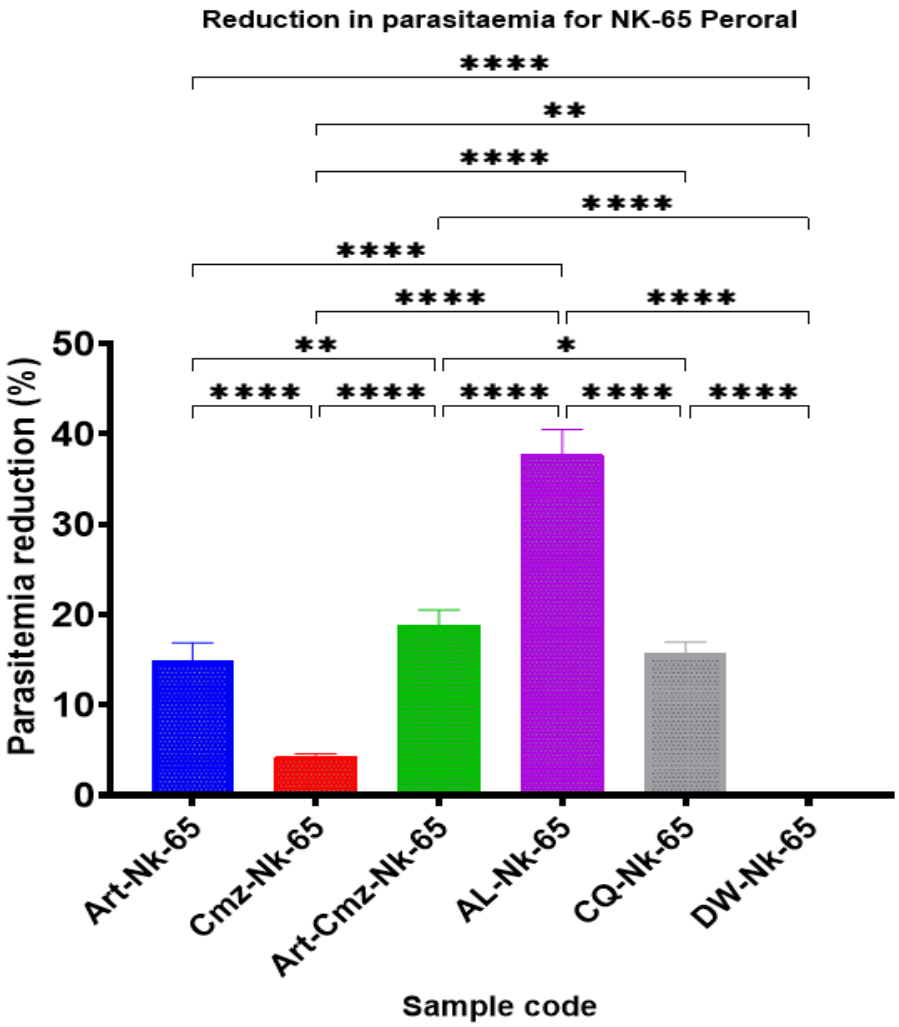


Figure 3: Percentage reduction in parasitemia of mice infected with NK-65 strain of *Plasmodium berghei* after three days of treatment with the samples (p.o). Data were expressed as mean ± SEM (standard error of mean) n = 6, differences were considered significant for *p<0.05, **p<0.01 and ****p<0.0001.

Key: Art is pure artemether sample, CMZ is pure clotrimazole sample, AL is artemether plus lumefantrine marketed oral dosage regimen, DW is distilled water and CQ is conventional chloroquine phosphate oral dosage regimen.

Antimalarial efficacy of artemether and clotrimazole against PbA infection in mice

Figure 4 shows the mean parasitemia levels in the experimentally *Plasmodium berghei* ANKA-infected groups of mice with or without intraperitoneal or intramuscular treatment whereas percentage reduction in parasitemia of mice infected with *Plasmodium berghei* ANKA after five days of intraperitoneal or intramuscular treatment is depicted in Figure 5. *In vivo* efficacy in PbA-infected mice showed that the mean parasitaemia count of the group treated with artemether alone reduced from 48 post-inoculation to 44 post-treatment; the mean parasitaemia count of the group treated with clotrimazole alone reduced from 61 post-inoculation to 59 post-treatment; the mean parasitaemia count of the group treated with artemether and clotrimazole combination reduced from 50 post-inoculation to 44 post-treatment; while that of the group treated with marketed artemether injection reduced from 41 post-inoculation to 33 post-treatment. On the contrary, there was an increase in mean parasitemia count for the untreated group (i.e. NS-ANKA that served as vehicle or negative control) from 32 post-inoculation to 40 after treatment. Besides, while commercial artemether injection achieved percent reduction in parasitemia of 19.17%, post-treatment, the percent parasitemia reduction achieved on completion of the study (i.e. day 5 post-treatment) by artemether, clotrimazole and artemether/clotrimazole combination was respectively 8.35, 3.28, and 12.14%. From the results presented in Figures 4 and 5, it was obvious that the treatments were able to bring down the parasitemia level unlike what was observed in the untreated group (negative control), where there was an increase in parasitemia.

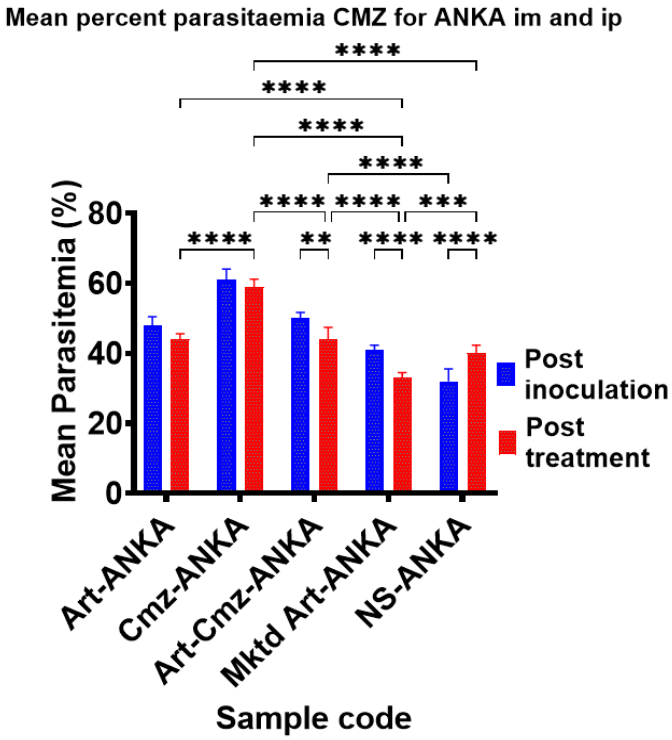


Figure 4: Mean parasitemia levels obtained post inoculation and post treatment with the sample (i.p. and i.m.) in mice infected with *Plasmodium berghei* ANKA. Data were expressed as mean ± SEM (standard error of mean) n = 6, differences were considered significant for **p<0.01, ***p<0.001 and ****p<0.0001.

Key: Post treatment means with parenteral treatment, Post inoculation means without parenteral treatment Art is pure artemether sample, CMZ is pure clotrimazole sample, Mktd Art is marketed artemether injection dosage regimen and NS is normal saline.

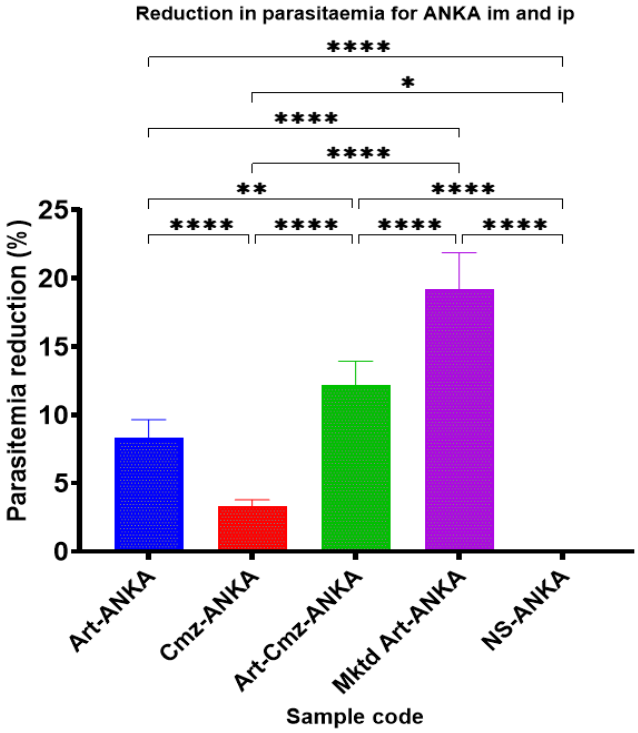


Figure 5: Percentage reduction in parasitemia of mice infected with *Plasmodium berghei* ANKA after five days of treatment with the samples (i.p. and i.m.). Data were expressed as mean ± SEM (standard error of mean) n = 6, differences were considered significant for *p<0.05, **p<0.01 and ****p<0.0001.

Key: Art is pure artemether sample, CMZ is pure clotrimazole sample, Mktd Art is marketed artemether injection dosage regimen and NS is normal saline.

Effects of artemether and clotrimazole on hematological parameters of mice infected with NK-65 P. berghei or P. berghei ANKA

The results of the hematological determinations are shown in Tables 2 and 3. From the result of the overall hematological studies carried out on the animals (Tables 2 and 3), it could be deciphered that there were variations in the hematological parameters due to the various effects of different treatments administered to the different animal groups. There was a significant increase in WBCs with a significant decrease in HB, PCV and RBCs in the mice infected with both Pb NK-65 and Pb ANKA (negative controls) when compared to normal control (uninfected and untreated) mice (Table 2 and 3). However, treatment with clotrimazole and artemether and combination of clotrimazole and artemether as well as with marketed artemether injection, chloroquine phosphate oral tablets and commercially available artemether-lumefantrine (AL) significantly decreased WBCs and significantly increased HB, PCV and RBCs, when compared to the negative control groups (DW for Pb NK-65 and NS for Pb ANKA) (Tables 2 and 3).

Effects of the drugs on weights of infected mice

Figure 6 shows the weights of the mice infected with NK-65 strain of *Plasmodium berghei* before and after three days of treatment with the samples while Figure 7 shows the weights of mice infected with *Plasmodium berghei* ANKA before and after five days of treatment with the samples.

Effects of the drugs on lethality of infected mice

Table 4 shows the lethality of CPb and PbA infections on the mice in treated and untreated groups. For CPb-infected animals (groups A-F), mortality of mice in the infected group without treatment (i.e. DW-NK-65) was 66.67% (4/6) while mortality was 33.33% (2/6) in the infected groups treated with artemether and clotrimazole monotherapies (i.e. Art-NK-65 and CMZ-NK-65) and

Table 2: Effect of the samples on hematological parameters of mice infected with or without *Plasmodium berghei* NK-65

Sample code	Treatment	RBC ($x10^{12}$/L)	WBC (Cells/µL)	PCV (%)	Hb (g/dL)
NC	Normal control (uninfected and untreated)	5.51±0.29	7,250±13.96	56.7±3.71	15.8±0.16
Art-NK-65	Art monotherapy	3.92±0.12 (*p<0.05)	6,180±47.92 (*p<0.05)	36.8±4.49 (*p<0.05)	9.82±0.37 (*p<0.05)
CMZ-NK-65	CMZ monotherapy	3.50±0.21 (**p<0.01)	7,050±65.29 (**p<0.01)	29.8±2.95 (**p<0.01)	8.29±0.48 (**p<0.01)
Art-CMZ-NK-65	Art and CMZ 4:1 combo therapy	3.99±0.11 (*p<0.05)	5,670±83.45 (*p<0.05)	42.9±4.21 (*p<0.05)	10.5±0.29 (*p<0.05)
AL-NK-65	Marketed ACT (Art and LMF combo therapy)	5.14±0.17 (***p<0.001)	4,280±35.67 (***p<0.001)	53.6±3.54 (***p<0.001)	13.6±0.35 (***p<0.001)
CQ-NK-65	Marketed CQ monotherapy	4.16±0.09 (*p<0.05)	6,590±28.71 (*p<0.05)	38.4±3.17 (*p<0.05)	11.7±0.92 (*p<0.05)
DW-NK-65	Distilled water	2.87±0.07 (***p<0.001)	12,640±98.53 (***p<0.001)	24.8±4.94 (***p<0.001)	7.65±0.81 (***p<0.001)

Key: Art is pure artemether sample, CMZ is pure clotrimazole sample, AL is artemether plus lumefantrine marketed oral dosage regimen, DW is distilled water and CQ is conventional chloroquine phosphate oral dosage regimen. Data were expressed as mean SEM (standard error of mean) n = 6, differences were considered significant for ***p<0.001 when compared to normal control (NC), differences were considered significant for **p<0.01, *p<0.05, ***p<0.001 when compared to distilled water (negative control).

Table 3: Effect of the samples on hematological parameters of mice infected with or without *Plasmodium berghei* ANKA

Sample code	Treatment	RBC ($x10^{12}$/L)	WBC (Cells/µL)	PCV (%)	Hb (g/dL)
NC	Normal control	5.51±0.29	7,250±13.96	56.7±3.71	15.8±0.16
Art-ANKA	Art monotherapy	3.84±0.17 (*p<0.05)	6,290±46.78 (*p<0.05)	35.3±2.50 (*p<0.05)	9.14±0.45 (*p<0.05)
CMZ-ANKA	CMZ monotherapy	3.43±0.05 (**p<0.01)	7,180±65.29 (**p<0.01)	28.2±4.09 (**p<0.01)	7.72±0.28 (**p<0.01)
Art-CMZ-ANKA	Art and CMZ 4:1 combo therapy	3.78±0.13 (*p<0.05)	5,850±71.82 (*p<0.05)	41.5±3.21 (*p<0.05)	10.23±0.30 (*p<0.05)
Mktd Art-ANKA	Marketed Art injection	4.72±0.24 (***p<0.001)	4,370±32.64 (***p<0.001)	50.8±3.15 (***p<0.001)	14.61±0.15 (***p<0.001)
NS-ANKA	Normal saline	2.65±0.08 (***p<0.001)	13,700±89.35 (***p<0.001)	21.3±3.76 (***p<0.001)	6.91±0.54 (***p<0.001)

Key: Art is pure artemether sample, CMZ is pure clotrimazole sample, Mktd Art is marketed artemether injection dosage regimen and NS is normal saline. Data were expressed as mean SEM (standard error of mean) n = 6, differences were considered significant for p<0.001 when compared to normal control (NC), differences were considered significant for **p<0.01, *p<0.05 and ***p<0.001 when compared to normal saline (negative control).

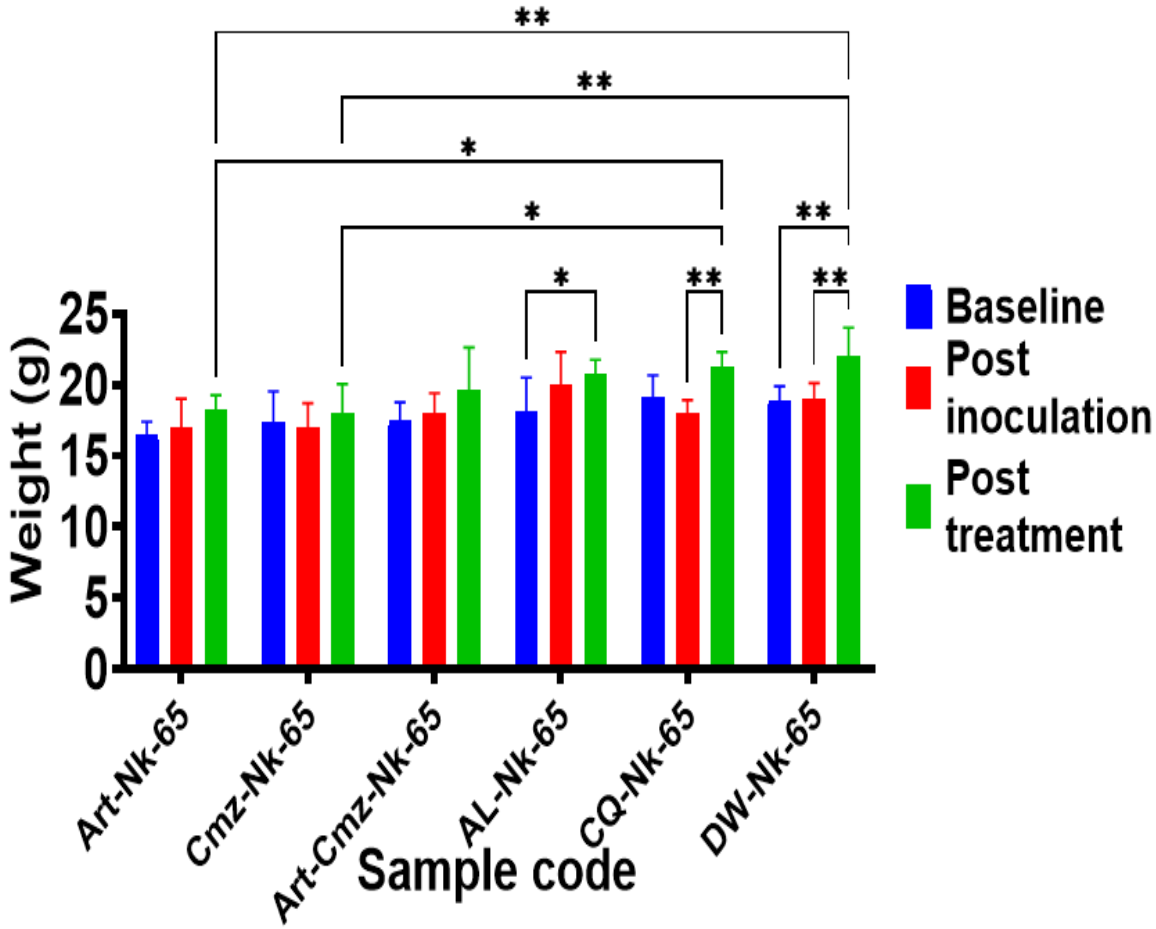


Figure 6: Weights of the mice infected with NK-65 strain of *Plasmodium berghei* before and after three days of treatment with the samples (p.o). Data were expressed as mean ± SEM (standard error of mean) n = 6, differences were considered significant for *p<0.05 and **p<0.01.

Key: Art is pure artemether sample, CMZ is pure clotrimazole sample, AL is artemether plus lumefantrine marketed oral dosage regimen, DW is distilled water and CQ is conventional chloroquine phosphate oral dosage regimen.

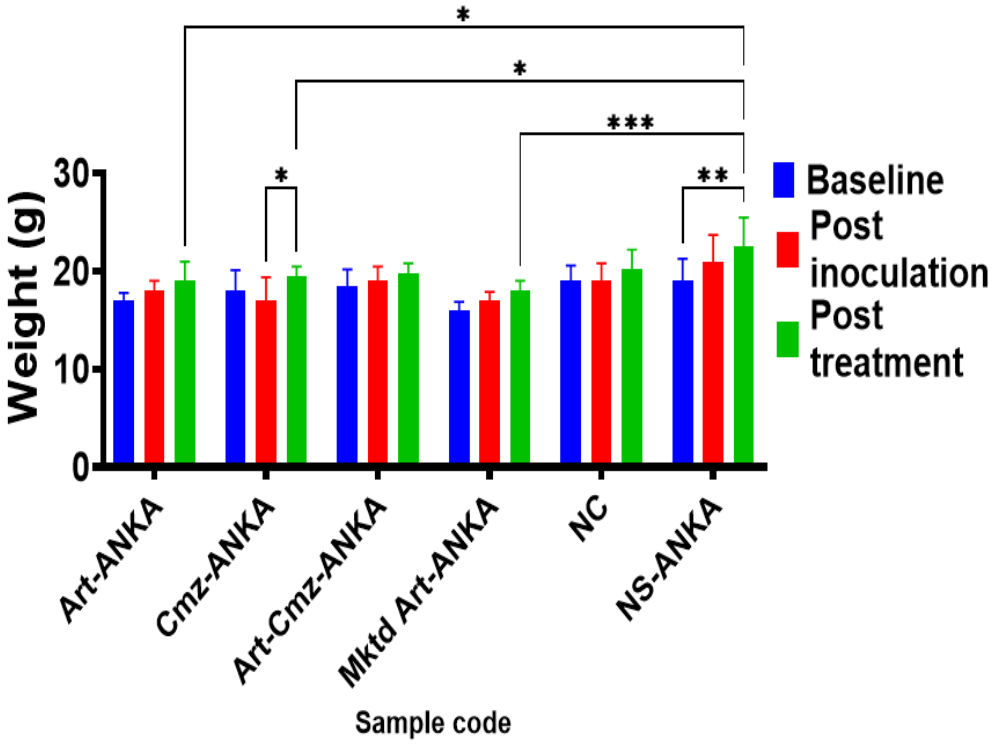


Figure 7: Weights of mice the infected with *Plasmodium berghei* ANKA before and after five days of treatment with the samples (i.p. and i.m.). Data were expressed as mean ± SEM (standard error of mean) n = 6, differences were considered significant for *p<0.05, **p<0.01 and ***p<0.001.

Key: Art is pure artemether sample, CMZ is pure clotrimazole sample, Mktd Art is marketed artemether injection dosage regimen and NS is normal saline.

Table 4: Number of surviving animals in the uninfected and infected (with and without treatment) groups of mice

Group	Sample code	Treatment	Number of surviving mice post inoculation (before treatment)	Number of surviving mice post treatment	Mortality (%)
A	Art-NK-65	Art monotherapy	6/6	4/6	33.33
B	CMZ-NK-65	CMZ monotherapy	6/6	4/6	33.33
C	Art-CMZ-NK-65	Art and CMZ 4:1 combo therapy	6/6	5/6	16.67
D	AL-NK-65	Marketed ACT	6/6	5/6	16.67
E	CQ-NK-65	Marketed CQ monotherapy	6/6	5/6	16.67
F	DW-NK-65	Distilled water	6/6	2/6	66.67
G	Art-ANKA	Art monotherapy	6/6	4/6	33.33
H	CMZ-ANKA	CMZ monotherapy	6/6	3/6	50.00
I	Art-CMZ-ANKA	Art and CMZ 4:1 combo therapy	6/6	4/6	33.33
J	Mktd Art-ANKA	Marketed Art injection	6/6	5/6	16.67
K	NS-ANKA	Normal saline	6/6	2/6	66.67
L	NC	Normal control (uninfected and untreated)	6/6	6/6	0.00

Key: Art is pure artemether sample, CMZ is pure clotrimazole sample, AL is artemether plus lumefantrine marketed oral dosage regimen, DW is distilled water, CQ is conventional chloroquine phosphate oral dosage regimen, Mktd Art is marketed artemether injection dosage regimen and NS is normal saline. Groups A to F were inoculated with chloroquine sensitive strain of *Plasmodium berghei* (NK 65) while Groups G to K were inoculated with *Plasmodium berghei* ANKA.

16.67% (1/6) in the infected groups treated with artemether and clotrimazole combo therapy (i.e. Art-CMZ-NK-65), marketed artemether/lumefantrine combo therapy (i.e. AL-NK-65) and marketed chloroquine monotherapy (i.e. CQ-NK-65). Then for PbA-infected animals (groups G-K), mortality of mice in the infected group without treatment (i.e. NS-ANKA) was 66.67% (4/6) while mortality was 33.33% (2/6) in the infected group treated with artemether monotherapy (i.e. Art-ANKA) as well as infected group treated with artemether and clotrimazole combo therapy (i.e. Art-CMZ-ANKA), 50% (3/6) in the infected groups treated with clotrimazole monotherapy (i.e. CMZ-ANKA) and 16.67% in the infected group treated with marketed artemether injectable monotherapy (i.e. Mktd ANKA). There was no mortality of mice in uninfected and untreated group that served as normal control (NC).

DISCUSSION

The development of multidrug resistance in malarial parasite has sabotaged majority of the eradicating efforts by restricting the inhibitory profile of the front-line antimalarial drugs (artemisinin derivatives), thus necessitating the development of medicines with superior potency against the drug-resistant and drug-sensitive *Plasmodium* parasite[37]. Interestingly, recent scientific reports have described antimalarial potential of clotrimazole and synergistic *in vitro* antimalarial activity of clotrimazole and artemisinin at therapeutically safe concentrations against drug-sensitive and resistant *P. falciparum*[23]. In this study, we adopted drug repurposing as a novel strategy to enhance malaria therapy and we investigated the *in vivo* performance of artemether/clotrimazole combination against chloroquine-sensitive and multidrug-resistant *Plasmodium berghei* (Pb) in a preclinical mouse model. Concomitant p.o. and i.p. administration of the drugs gave significantly greater antimalarial activity than p.o. and i.p. artemether monotherapy. Meanwhile, artemether is predominantly metabolized by CYP 3A4 enzyme [38]. Besides, imidazole antifungals are cytochrome P_{450} inhibitors and have been reported to potentiate the antimalarial action of artemisinin derivatives[29]. Additionally, Solutol® HS 15 used as surfactant for the extemporaneous oral preparation inhibits CYP 3A4[39,40].

In this research, the reduction in parasitemia caused by artemether/clotrimazole combo therapy in CPb-infected mice was significantly greater than artemether monotherapy (**p<0.01), clotrimazole monotherapy (****p<0.0001) as well as marketed chloroquine (*p<0.05) via peroral administration (Figure 3). Thus, the preclinical antiplasmodial activity results obtained from the CPA-infected mice further predict clotrimazole as a partner drug candidate to artemether for possible treatment of uncomplicated malaria. Similarly, the parasitemia reduction caused by artemether/clotrimazole combo therapy in PbA-infected mice was significantly greater than artemether monotherapy (**p<0.01) and clotrimazole monotherapy (****p<0.0001) via parenteral administration (Figure 5). Hence, the preclinical antimalarial activity results obtained from PbA-infected mice further predict clotrimazole as a partner drug candidate to artemether for possible treatment of severe or cerebral malaria. Besides, the variations in the hematological parameters of the negative control groups and the resultant untoward effects must have contributed to the death of many animals in these particular groups, which is in line with studies reported elsewhere[41,42]. *Plasmodium berghei*-infected mice are prone to anemia due to erythrocyte destruction, as a consequence of parasite multiplication or by spleen reticuloendothelial cell action causing the production of phagocytes by the spleen due to abnormal erythrocytes[38,43]. In the current study, anemia was conspicuous in *P. berghei* treated mice characterized by decreased PCV, Hb, and RBCs with increased WBCs levels. However, Pb–induced anemia was vividly reduced in mice treated with artemether and clotrimazole monotherapies, artemether/clotrimazole combo therapy, marketed chloroquine phosphate, commercial artemether-lumefantrine and conventional artemether injection which were characterized by increased PCV, Hb, and RBCs with decreased WBCs levels. The decreased WBCs can be attributed to the fact that antimalarial drugs can modulate immune system[36]. Interestingly, it could be clearly seen from Tables 2 and 3 that for both CPb-infected mice and PbA-infected mice, the anti-anemic activity of artemether/clotrimazole combo therapy was best when compared to artemether and clotrimazole monotherapies (i.e. individual doses of artemether and clotrimazole). However, marketed artemisinin-based combination therapy (AL) and marketed artemether injection gave the overall best anti-anemic activity when compared to other treatments among the CPb-infected mice and PbA-infected mice, respectively, which confirms the established antimalarial property of artemether-lumefantrine and artemether injection, especially against uncomplicated malaria and severe or cerebral malaria, respectively[44,45].

With respect to weight variation, all groups of the animals (except the animals dosed with artemether/clotrimazole combo therapy) showed significant changes in weight (*p<0.05 and **p<0.01) [for CPb-infected mice] (Figure 6). Similarly, for PbA-infected mice (with the exception of the animals dosed with artemether/clotrimazole combo therapy and negative control) all other animals showed significant changes in weight (*p<0.05, **p<0.01 and ***p<0.001). These results indicate the ability of the treatment to handle the worsening state of malaria in the mice that manifests as an increase in the weight (due to increase in the size of spleen, liver, and possibly other blood-forming tissues) [41,42]. It could be seen from Figures 6 and 7 that even though weight of animals in all groups increased generally, it was at a lower rate in the treatment groups in comparison with the negative control group, consistent with our recent report on nanosized artemisinin-based antimalarial combo therapy[42].

In the lethality study, the lethality of CPb and PbA infections in infected and untreated mice confirms these parasites to be virulent [33,34]. These parasites (Pb NK-65 and Pb ANKA strains) are laboratory models that are often used to mimic *Plasmodium falciparum* infections

which cause uncomplicated malaria and severe or cerebral malaria, respectively, in humans[34,46]. Lethality can be used as a measure of antimalarial activity of a drug[33,34]. In this study, clotrimazole reduced the mortality in CPb-infected mice and PbA-infected mice by 50% and 25%, respectively, implying that clotrimazole is more effective against uncomplicated malaria than severe malaria in Pb murine malaria model. In comparison, artemether reduced mortality of both CPb-infected mice and PbA-infected mice by 50%, which confirms the established antimalarial property of artemether, especially against severe or cerebral malaria[45] vis-à-vis clotrimazole (a repurposed antimalarial agent). Interestingly, the reduction in mortality of PbA-infected mice achieved with artemether/clotrimazole combo therapy was equal to that of artemether, an indication that clotrimazole did not suppress the inherent antimalarial activity of artemether; this further confirms that the antimalarial activity of artemether/clotrimazole was comparable to the antimalarial property of artemether. However, the marketed artemether injection achieved the best mortality reduction of PbA-infected mice (75%), thus adjudging it as better than the combo therapy against PbA. Nonetheless, the reduction in mortality of CPb-infected mice achieved with artemether/clotrimazole combo therapy was equal to that of marketed chloroquine and artemether/lumefantrine combo therapy. This not only confirms the established antimalarial activity of chloroquine and artemether/lumefantrine combination, especially against uncomplicated malaria[47,50] but also implies that the use of artemether/clotrimazole combo therapy in uncomplicated malaria should be initiated.

CONCLUSIONS

This study has demonstrated the antimalarial activity of clotrimazole in an *in vivo* mice experiment against drug-sensitive and resistant strains of Pb. In rational combination with a front-line antimalarial drug (artemether), this antimycotic drug decreased the percentage parasitemia and significantly (**$p<0.01$) increased percentage reduction in parasitemia in NK-56 and ANKA *P. berghei*-infected mice, thus projecting the use of artemether/clotrimazole for malaria treatment.

Ethical approval: The Ethical Guidelines of Animal Care and Use were followed during the conduct of this investigation and was approved by the Faculty of Pharmaceutical Sciences Research Ethics Committee of the University of Nigeria, Nsukka (approval no. FPSRE/UNN/20/00064).

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Availability of data and materials: All data generated or analyzed during this study are included in this published article.

Consent for publication: Not applicable. This work does not contain data from any individual person.

Competing interests: The authors declare that they have no competing interests.

Authors' Contributions: FCK designed and supervised the study, performed funding acquisitions, writing, and editing the manuscript. JCO participated in antimalarial evaluation and data analysis. CCA participated in drug treatment experiments. SCN drafted the manuscript and contributed substantially in revising the manuscript. BAO prepared drug stock solutions and participated in antimalarial evaluation. TO performed analysis and prepared drug stock solutions. MUO performed drug treatment experiments and analysis. LCN participated in weight determination. EFN prepared drug stock solutions and participated in lethality studies. JEO was involved in drug treatment experiments. JIN participated in weight determination. DON performed data and statistical analysis. WIU performed the hematological parameters determinations, antimalarial evaluations and funding acquisitions. MAM performed funding acquisitions and was a major contributor in writing and revising the manuscript. AAA provided the chemicals, performed funding acquisitions, reviewed and edited the manuscript. All authors read, reviewed and approved the final manuscript.

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