Original Research Article

Insights into the *In-Vivo* Antiplasmodial Activity of Trisdimethylamino Pyrimidine Derivative in *Plasmodium berghei*-Infected Mouse Model

ABSTRACT

Aims: To evaluate the antiplasmodial activity of a trisdimethylamino pyrimidine derivative synthesized by indirect methylation of *p*-nitrophenylacetic acid via a substituted amine or amide derivative against *Plasmodium berghei* NK65 strain in an *in vivo Plasmodium berghei*-Infected mouse model

Methodology: *Plasmodium berghei*-parasitized suppressive model in Albino mice was adopted for the antiplasmodial activity evaluation. The schizontocidal activity of the compound was evaluated by a 4-day suppressive test. All the treatments (test sample, standard control and blank) were administered intraperitoneally to the mice. Parameters such as rectal temperature, packed cell volume, survival time and parasitemia were measured.

Results: The 10, 20 and 40 mg/kg doses of 2, 4, 6-trisdimethylamino-5-(4´-nitrophenyl) pyrimidine elicited a statistically significant reduction ($p < 0.01$) in parasitemia level in treated mice. The 40 mg/kg dose caused 97.19% suppression of parasitemia and a mean survival time of 22 days compared with 97.64% and 24 days respectively in the standard control group (artemether-lumefantrine combination, 5 mg/kg). All the treatments restored the packed cell volume to the baseline (~39.8%). A dose-dependent decrease in the rectal temperature was observed in all the treatment groups. However, only the 10 and 20 mg/kg doses of 2, 4, 6-trisdimethylamino-5-(4´-nitrophenyl) pyrimidine caused a significant decrease ($p < 0.01$) compared with the control.

Conclusion: The strong suppression of *Plasmodium berghei* in the mice treated with 2, 4, 6-trisdimethylamino-5-(4´-nitrophenyl) pyrimidine has further provided insights into the potential of the compound as a lead in the discovery of antimalarial agents.

Keywords: Antimalaria, Infected mouse model, *Plasmodium berghei, Pyrimidine derivative, Vilsmeier-Haack reaction*

1. INTRODUCTION

Apart from flora and fauna, organic synthesis is one the source of lead compounds in drug discovery, development and optimization. Heterocyclic rings provide a pharmacologically active nucleus for many drugs, and important biological and synthetic intermediates for some reactions [1]. Pyrimidine has remained an important heterocyclic pharmacophore because it is a constituent of living cells, it has a unique metabolic process, symmetrically positioned atoms about 2,5-axis of the ring, low resonance energy of 26 Kcal/mol and feasibility of its bench synthesis, antineoplastic, analgesic and anti-inflammatory, anti-hyperlipidemic, anti-microbial and diabetogenic activities [2].

Aside from isolation from natural sources, pyrimidine could be synthesized depending on the fundamental nature of the fragments, which combine to form the nucleus [3,4]. The first step is by cyclization involving a double bond condensation with the elimination of water, alcohol, or hydrogen halide between amino, carbonyl, carboxylic acid, carboxylic ester, acid chloride,

or enol ether; the second step, by condensation via the addition of amino to CN groups or polarized bonds without an elimination reaction and the third, by reactions involving the insertion of a single C-atom between the N-atoms of a 1,3-diamine to obtain hydrogenated pyrimidine via phosgene or aldehydes treatment [3,5]. A modification of the first method is regarded as Vilsmeier–Haack reaction (VHR) which involves the electrophilic substitution of a suitable carbon nucleophile (N, N-dimethylaniline, alkene derivative, or activated methyl/methylene compounds) with a halomethyleniminium salt to yield the corresponding iminium species (called Vilsmeier reagent) which hydrolyses to a corresponding ketone or aldehyde [6].

A VHR technique is a good synthetic tool and has been applied in cyclization, ring annulations, cyclohaloaddition and formylation [7]. The reaction has become very important in organic synthesis because it is capable of executing a large variety of synthetic transformations and the product, easily isolable, creates a situation whereby the chemist is in control of further reactions [8]. Pyrimidine derivatives are biologically very important heterocycles representing the most ubiquitous members of the diazine family. They have wide clinical and pharmacological applications; in addition to serving as a scaffold for drug synthesis of several antiprotozoal agents [9].

Malaria is a protozoal infection caused by the *Plasmodium* parasite and has remained a major public health challenge in Sub-saharan Africa [10]. There is a lack of effective antimalarial drugs with an increase in resistance of the plasmodium to existing antimalarial drugs [11]. There is also no effective prophylactic anti-sporozoite drug that is currently in use. It has been observed that our synthesized compound shares structural similarity with the components of a known antimalarial combo- trimethoprim and pyrimethamine. The importance of pyrimidine chemistry in drug development and the feasibility and versatility of the VHR prompted our interest to investigate 2, 4, 6-trisdimethylamino-5-(4´-nitrophenyl) pyrimidine (2, 4, 6-TMANP) for antiplasmodial activity against *Plasmodium berghei*.

2. MATERIAL AND METHODS

2.1 Materials

2.1.1 Test sample

The detailed synthesis of the intermediate products (4-nitrophenylacetyl chloride, N,N-dimethyl-4-nitrophenylacetamide, 1-chloro-1,3-bis(dimethylamino)-4-(4-nitrophenyl)-2-azabut-2-en-1-ylium, 1,1,3,5-tetrakis(dimethylamino)-6-tolyl-2,4-diazahexa-2,4-dien-1-ylium perchlorate) and 2, 4, 6-trisdimethylamino-5-(4´-nitrophenyl) pyrimidine has been described in a previous report and the purified and fully characterized 2, 4, 6-TMANP was used for this study [12].

2.1.2 Experimental mice

Albino mice (7 to 8 weeks and weight 18.2 - 21.8 g) of both sexes were procured from the animal house of the University of Nigeria Veterinary Teaching Hospital, Nsukka. The mice were allowed to acclimatize for 7 days at 25 ºC temperature, relative humidity of 40 – 50 % and 12 h light/ 12 h dark cycle. They were allowed access to feed, and water *ad libitum*.

2.1.3 *Plasmodium berghei* parasite

A strain of *Plasmodium berghei* NK65 was obtained from the Parasitology Unit, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The parasite was maintained by continuous intraperitoneal inoculation of the parasite into Albino mouse and passaging every 72 h.

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2.2 Methods

2.2.1 Preparation of *Plasmodium* inoculum

Blood from the donor mice at peak parasitemia was used. Blood was drawn from the donor mouse by heart puncture and collected into a heparinized syringe diluted serially in phosphate buffer to make a suspension strength of 1-2 × 10^6 infected red blood cells in every 0.2 ml suspension. A 0.2 ml appropriately diluted in phosphate buffer was used to infect each mouse intraperitoneally by serial passage to initiate infection [5].

2.2.2 Parasite inoculation

Plasmodium berghei parasitized erythrocytes were obtained from the tail of the donor mice and were diluted with 0.9 % normal saline. The mice were inoculated intraperitoneally with 0.2 ml blood suspension containing 1-2 × 10^6 parasitized erythrocytes on day 0 and were monitored for 3 h before treatment [13].

2.2.3 Experimental design

The mice were randomly divided into 6 groups of five (5) mice per group and treated for 4 consecutive days with a daily dose by the intraperitoneal route as follows: Group 1 mice were infected and treated with 10 mg/kg of 2, 4, 6-TMANP. The *P. berghei*-infected mice in group 2 received 20 mg/kg of 2, 4, 6-TMANP. Group 3 mice were infected and treated with 40 mg/kg of 2, 4, 6-TMANP. Group 4 mice were infected and treated with 5 mg/kg artemether/lumefantrine as standard control. Group 5 were infected but not treated while group 6 mice were neither infected nor treated.

2.2.4 *Plasmodium* suppressive test

Treatment was started 3 h post inoculation with *P. berghei* on day 0 and then continued daily for four days. After treatment was completed, blood obtained from the tail of each mouse was used to prepare a thick film to determine percentage suppression and parasitemia [13]. In addition, each mouse was observed daily to the determination survival time.

2.2.4.1 Determination of rectal temperature

The rectal temperatures of the mice were taken before infection; 4 h after infection, during treatment and after treatment had stopped for five days. The temperatures were measured using a digital thermometer before infection, 4 h after infection and then daily.

2.2.4.2 Determination of packed cell volume

Heparinized capillary tubes were used for the collection of blood from the eye of each mouse. The tubes were placed in a microhematocrit centrifuge with the sealed end outwards and centrifuged for 5 min at 11,000 rpm. The tubes were then taken out of the centrifuge and PCV was determined using a standard Micro-hematocrit Reader (Hawkey, Germany). The PCV was determined using equation 1 [14].

$$PCV\ (\%) = \frac{Packed\ red\ blood\ cells\ column\ height}{Total\ blood\ volume\ height}\ X\ 100 \quad \text{- Eq 1}$$

2.2.4.3 Parasitemia suppression

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On the 4th day, the hick smears of blood were made from the tail of each mouse. The smears were applied on microscopic slides (76 × 26 mm) (Menzel-Glaser, Germany), fixed with leishmann's stain (Bemac Scientific and Chemical, Nigeria) at 7.2 for 15 min. The stained slides were then washed gently using distilled water and air dried at room temperature. The stained slides for each mouse were examined under a microscope (Olympic, China) with an oil immersion nosepiece of magnification 100 × 1.25. Ten different fields on each slide were examined to calculate the mean parasitemia using equation 2 while the percentage suppression of *P. berghei* were compared with respect to the controls and percentage suppression was calculated using equation 3 [14].

$$Parasitemia\ (\%) = \frac{Number\ of\ parasitized\ red\ blood\ cells}{Total\ number\ of\ red\ blood\ cells}\ X\ 100 \text{ -} \qquad \text{Eq 2}$$

$$Suppression\ (\%) = \frac{Mean\ parasitemia\ of\ negative\ control - Mean\ parasitemia\ of\ treated\ group}{Mean\ parasitemia\ of\ negative\ control}\ X\ 100 \quad \text{Eq 3}$$

2.2.4.4 Determination of survival time

The survival time of the mice was calculated for 28 days, immediately after treatment as day 1 till the next 28 days. This is done to ascertain the survival rate of the mice after treatment is given. After the mean survival time was checked, the mice were sacrificed using chloroform anesthesia.

Analysis of data

All the chemical structures (in Figures 1 and 2) were built using ChemDraw Ultra 7.0 software. The data obtained were analyzed using GraphPad prism version 5.0 and expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Dunnet, post-hoc test was used to determine statistical significance for comparison of mean parasitemia, percentage parasitemia, percentage suppression, PCV, rectal temperature and survival time among groups. A $p < 0.01$ was considered statistically significant.

3. RESULTS AND DISCUSSION

Synthetic route of 2, 4, 6-TMANP

The reaction scheme (Figure 1) represents some critical steps in a typical Vilsmeier-Haack reaction scheme. Thionyl chloride reduces 4-nitrophenylacetic acid to the acetyl chloride derivative which upon hydrolysis with sodium hydroxide forms a nitrophenylacetamide which condenses with dimethylamine hydrochloride to form a disubstituted nitrophenyl acetamide. The disubstituted acetamide reacted with cyanide to form a perchlorate derivative which cyclizes in an alkaline medium to form a pyrimidine derivative.

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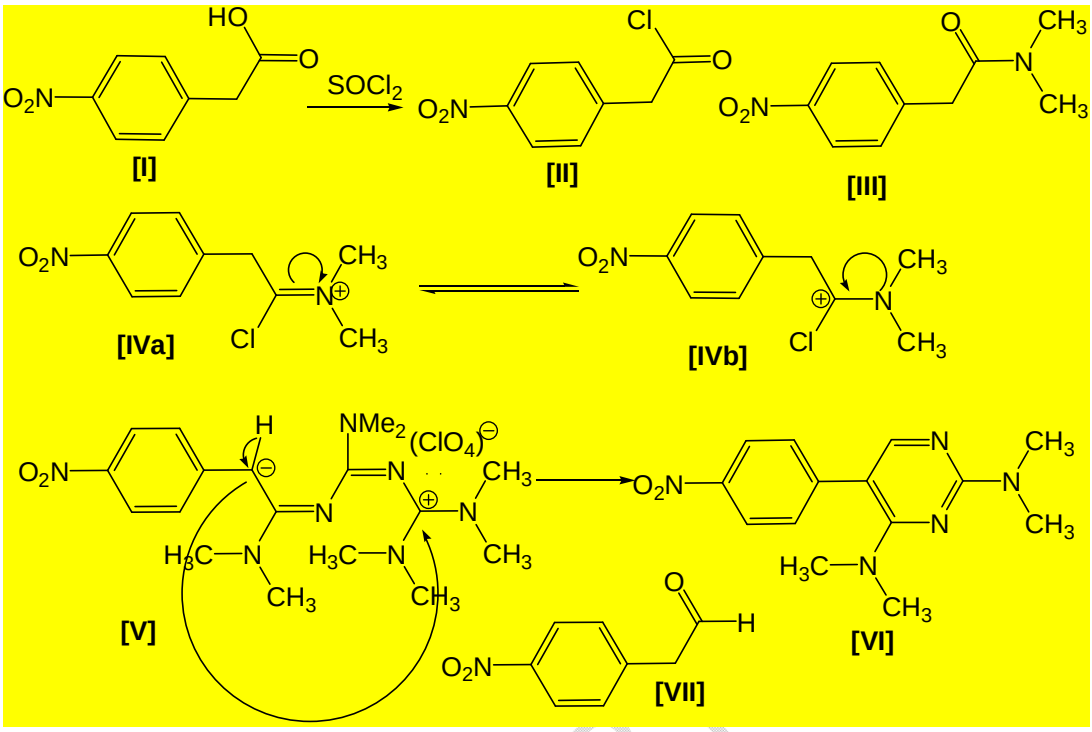

Figure 1. Some important steps in VHR synthesis of 2, 4, 6-TMANP

The reaction of *p*-nitrophenyl acetic acid [**I**] with thionyl chloride produced a more useful acid derivative [**II**] forming a substituted amide [**III**] with dimethylamine (Figure 1). The reaction of the substituted amide with phosphorous oxychloride produced a substituted electrophilic chloroiminium ion [**IVa**] called the Vilsmeier agent. The iminium ion rearranged to form a carbocation [**IVb**] which ordinarily combines with an electron-rich compound such as N, N-dimethylcyanamine, or aromatic moieties to produce aldehyde or ketone in a typical VHR [12]. However, the protocol was modified using a perchloric acid at very low temperature, to protect the cation from hydrolysis and cleavage of R_2NH group to form the traditional carbonyl compound [**VII**], which eventually lead to the formation of 2, 4, 6-TMANP via a 6→1-cyclization reactions of 1,1,3,5-tetrakis dimethylamino perchlorate derivative [**V**].

Antiplasmodial activity of 2, 4, 6-TMANP

The results *in vivo* antiplasmodial effects (parasitemia, plasmodium suppression and survival time of mice) of 2, 4, 6-TMANP are shown in Table 1. There was a dose-dependent and statistically significant ($p < 0.01$) suppression of parasitemia in all groups treated with 2, 4, 6-TMANP. The 20 and 40 mg/kg of 2, 4, 6-TMANP caused a reduction in parasitemia comparable to the standard (5 mg/kg). similar trends were also observed in the survival time of mice with the 40 mg/kg treated dose causing the highest MST of 22 days compared with 24 days in the standard group. The higher blood schizontocidal activity of 2, 4, 6-TMANP was evident from the chemo suppression of parasitemia during the four-day suppressive test.

The 2, 4, 6-TMANP is an important product of VHR and was generated via several reaction steps. The reaction of p-nitrophenyl acetic acid with thionyl chloride produced a more useful acid derivative forming a substituted amide with dimethylamine [6]. The reaction of the substituted amide with phosphorous oxychloride produced a substituted electrophilic chloroiminium ion called the Vilsmeier agent [9]. The iminium ion is rearranged to form a

carbocation that ordinarily combines with an electron-rich compound such as N, N-dimethylcyanamine, or aromatic moieties to produce aldehyde or ketone in a typical VHR [7,8,15]. However, the protocol was modified using a perchloric acid at a very low temperature, to protect the cation from hydrolysis and cleavage of R_2NH group to form the traditional carbonyl compound, which eventually lead to the formation of 2, 4, 6-TMANP via a 6→1-cyclization reactions of 1,1,3,5-tetrakis dimethylamino perchlorate derivative.

Table 1. Effect of 2, 4, 6-TMANP on parasitemia and mean survival time

Group	Parasitemia	Parasitemia (%)	Suppression (%)	MST (days)
1	7.25±0.85*	0.617*	94.75	16.00±1.96
2	4.15±0.70*	0.353*	97.00	18.20±1.96
3	3.88±1.30*	0.330*	97.19	22.00±1.52
4	3.25±0.75*	0.277*	97.64	24.00±1.33
5	76.80±2.41	11.75	-	15.00±1.87
6	-	-	-	29.00±0.95

*Data are expressed as Mean ± SEM (n = 5), Test samples: significant from group 5 control, *p < 0.01; Group 5 = Parasitized with Plasmodium berghei but not treated, Group 6 = Not parasitized and not treated. Mean survival time (MST).*

The 2, 4, 6-TMANP has the same basic structural unit as pyrimethamine, an antiprotozoal agent commonly used in the treatment and prevention of malaria and also used in combination with sulfadiazine antibiotic in the treatment of some infections in immunocompromised patients (Figure 2) [16]. Pyrimethamine interferes with tetrahydrofolic acid synthesis from folic acid by inhibiting the dihydrofolate reductase (DHFR) enzyme in protozoa thereby inhibiting DNA and RNA synthesis [17] while the compound 2,4,6-TMANP could have performed the same role in the present study. The close resemblances in the basic structural units of pyrimethamine, trimethoprim and 2,4,6-TMANP such as the pyrimidine ring (ring B), 2,4-disubstituted pyrimidine (ring B), and 4'-substituted phenyl (ring A) group (Figure 2) could also be responsible for strong antiprotozoal activity against *P. berghei* in the in-vivo suppressive model.

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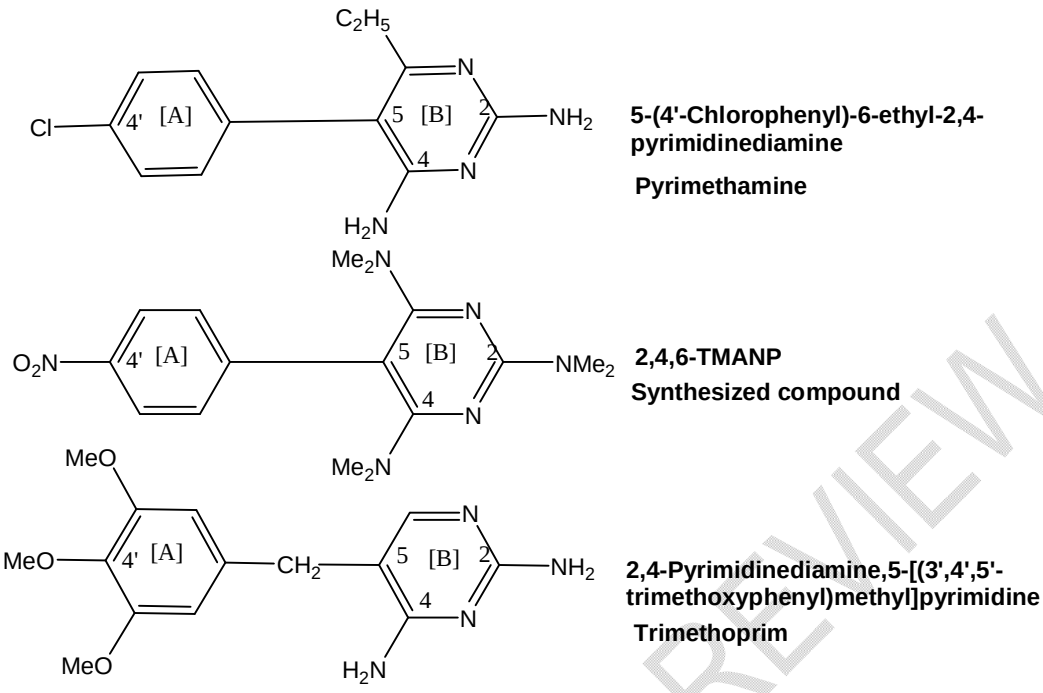

Figure 2. Structural relationship between 2,4,6-TMANP and pyrimethamine/trimethoprim.

Effects 2, 4, 6-TMANP on PCV and rectal temperature

The effects of 2, 4, 6-TMANP on the PCV and rectal temperature of treated mice are shown in Table 2. There was a dose=dependent restoration of the PCV to the baseline values. However, only the 20 and 40 mg/kg of 2, 4, 6-TMANP caused a statistically significant difference ($p < 0.01$) in the PCV compared with the control. Similarly, there was no statistically significant difference ($p > 0.05$) in the rectal temperature of mice treated with 40 mg/kg of 2, 4, 6-TMANP when compared with the control.

Table 2. Effects 2, 4, 6-TMANP on PCV and rectal temperature

Groups	PCV (%)		Rectal temperature (ºC)	
	Day 0	Day 4	Day 0	Day 4
1	41.20±0.66	34.00±0.19	36.28±0.15	35.25±0.28*
2	41.40±0.13	38.50±0.71*	35.36±0.41	35.35±0.30*
3	39.60±0.28	39.80±1.52*	35.06±0.47	36.67±1.18
4	40.70±0.31	40.30±0.05*	37.47±0.09	35.88±0.54*
5	39.20±0.26	27.75±1.04	37.20±0.20	38.20±0.24
6	41.10±0.25	40.25±0.38	37.08±0.36	37.28±0.50

*Data are expressed as Mean ± SEM (n = 5), Test samples: significant from group 5 control, *p < 0.01; Group 5 = Parasitized with Plasmodium berghei but not treated, Group 6 = Not parasitized and not treated.*

In the pathophysiology of malaria, the mechanisms contributing to anemia are categorized into two: increased destruction of parasitized and un-parasitized erythrocytes (immune-mediated lysis, phagocytosis, splenic sequestration) and decrease of red blood cells (RBC) production (dyserythropoietic and bone marrow suppression, inadequate reticulocyte

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production, effects of inflammatory cytokines, and effects of parasite factors) [18]. Each of these mechanisms has been implicated in both human and mouse malarial anemia. The target of the malaria parasite is RBC; so peripheral blood smear examination is the major diagnostic tool for the disease. The normal PCV value with a correspondingly low density of malaria parasite in mice treated with 10-40 mg/kg of 2, 4, 6-TMANP was suggested to be due to reduced destruction of red blood cells by the *P. berghei*. As observed in this study, it was established that more malaria parasite in the blood circulation causes more destruction of the RBC as demonstrated by the low PCV in the parasitized and untreated mice group. The significant reduction in PCV level indicates a relationship between the malaria parasite and anemia [19]. Antimalarial treatment with artemisinin or one of its derivatives is associated with a more rapid decline in parasitemia than with other antimalarial drugs. Artemisinin derivatives are most effective against the *Plasmodium* parasite as combination therapies consisting of artemisinins and other antimalarial drugs have been demonstrated to have better parasite clearance and efficacies. Artemisinins induce a decrease in parasitized RBC deformability. In the presence of heme Fe^{2+} these drugs generate carbon-centered free radicals that could damage the RBC membrane or cytoskeleton and thereby increase the rigidity of the infected RBC. Artemisinin by acting on young ring forms attenuated the reduction in deformability of parasites, prevented their development into more rigid mature trophozoites and thereby attenuated the reduction in deformability associated with continued parasite growth [20]. Rectal temperature determination shows the effect of the disease and possibly the medicinal effect of 2, 4, 6-TMANP on the treated mice. The relationship between the analgesic and antiprotozoal effects of known antimalarial compounds has been shown in previous studies [21]; the mechanism by which 2, 4, 6-TMANP inhibits an increase in temperature is not yet known, but a free radical scavenging activity could be hypothesized.

4. CONCLUSION

The study identified the schizontocidal potential of 2, 4, 6-TMANP, an important VHR product, in an *in vivo* suppressive mice model. A 40 mg/kg of 2, 4, 6-TMANP elicited a 97% *P. berghei* NK65 suppression, the survival time of 22 days and restoration of PCV and rectal temperature to baseline values in a short period comparable to the known artemether/lumefantrine combo. These promising effects provide a template for further development of 2, 4, 6-TMANP as an antiprotozoal agent.

ETHICAL APPROVAL

The research ethics committee of the university of Nigeria reviewed and approved the protocol involving the use of mice for this study (FRE/2020/11/00027). All authors hereby declare that Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee"

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