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Original Article

Methylene blue inhibits lumefantrine-resistant *Plasmodium berghei*

Victor Irungu Mwangi[1,2], Ruth Mwende Mumo[1], Daniel Muthui Kiboi[3], Sabah Ahmed Omar[4], Zipporah Waithera Ng'ang'a[2], Hastings Suba Ozwara[1]

[1] *Department of Tropical and Infectious Diseases, Institute of Primate Research, Nairobi, Kenya*
[2] *Institute of Tropical Medicine and Infectious Diseases (ITROMID), Jomo Kenyatta University of Agriculture and Technology, Juja, Kenya*
[3] *Centre for Traditional Medicine and Drug Research (CTMDR), Kenya Medical Research Institute (KEMRI), Nairobi, Kenya*
[4] *Centre for Geographical Medicine Research Coast, Kenya Medical Research Institute, Kilifi, Kenya*

Abstract

Introduction: Chemotherapy still is the most effective way to control malaria, a major public health problem in sub-Saharan Africa. The large-scale use of the combination therapy artemether-lumefantrine for malaria treatment in Africa predisposes lumefantrine to emergence of resistance. There is need to identify drugs that can be used as substitutes to lumefantrine for use in combination therapy. Methylene blue, a synthetic anti-methemoglobinemia drug, has been shown to contain antimalarial properties, making it a candidate for drug repurposing. The present study sought to determine antiplasmodial effects of methylene blue against lumefantrine- and pyrimethamine-resistant strains of *P. berghei*.

Methodology: Activity of methylene blue was assessed using the classical four-day test on mice infected with lumefantrine-resistant and pyrimethamine-resistant *P. berghei*. A dose of 45 mg/kg/day was effective for testing ED90. Parasitemia and mice survival was determined.

Results: At 45 mg/kg/day, methylene blue sustained significant parasite inhibition, over 99%, for at least 6 days post-treatment against lumefantrine-resistant and pyrimethamine-resistant *P. berghei* ($p = 0.0086$ and $p = 0.0191$, respectively). No serious adverse effects were observed.

Conclusions: Our results indicate that methylene blue at a concentration of 45 mg/kg/day confers over 99% inhibition against lumefantrine- and pyrimethamine-resistant *P. berghei* for six days. This shows the potential use methylene blue in the development of antimalarials against lumefantrine- and pyrimethamine-resistant parasites.

Key words: methylene blue; lumefantrine; pyrimethamine; *Plasmodium berghei*; resistant; parasitaemia.

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Introduction

Malaria is a disease of great concern to Africa and other tropical regions of the world. About 90% of the estimated 584,000 worldwide malaria deaths in 2013 occurred in Africa [1], with children under five years of age accounting for 78% of all deaths. Chemotherapy is currently the most effective method to control malaria. Over time, monotherapy treatments have selected for resistant strains and consequently became ineffective. This has been witnessed with the use of chloroquine and sulfadoxine-pyrimethamine as first lines of treatments in Africa [2].

A major limitation of the existing antimalarial drugs is primarily resistance (and cross-resistance between closely related drugs) [3,4]. However, most of these drugs still have a place, and their lifespan could be prolonged if they were better deployed and used, and also rationally combined. Newer compounds are also being developed [5,6].

Current strategies for malaria treatment rely on the use of combinations of drugs. The combinational therapy rationale lowers the probability of the parasites having a resistant genetic profile, with mutations conferring resistance to both drugs surviving the treatment [7].

Lumefantrine (LM) is one of the current combinational therapy drugs with artemisinin, used as first-line treatment against malaria in Africa, including Kenya [8]. This artemether-lumefantrine therapy produces a > 95% cure rate [9-11]. However, the absorption and elimination half-life of LM is subject to very wide inter-individual variations, which produce chances of treatment failures [12]. There are reports that the use of LM in artemisinin combinational therapy

(ACT) selects for parasites that show increased tolerance to the combinational therapy. This selection, coupled with large-scale unsupervised usage and parasites having increased copy numbers of the *pfmdr1* gene associated with mefloquine (MFQ) resistance [13,14], is synonymous with emergence of LM resistance.

Pyrimethamine (PYR) is used widely in combination with longer-acting sulphonamides such as sulfadoxine and sulfalene. It is used in malaria-endemic regions of Africa and the Western Pacific region for its important role in the prevention of malaria in pregnancy when used for intermittent presumptive treatment of malaria in pregnant women (IPTp) [15], and it has been included in several trials of intermittent presumptive treatment in infants (IPTi) [16]. Although no longer the official first-line anti-malarial in a number of countries, sulfadoxine-pyrimethamine (SP) remains available and is frequently used as a prophylaxis by travelers. The continued use of SP in the IPT provides drug pressure that selects for PYR-resistant parasites. Thus, PYR-resistant parasites are likely to remain in circulation.

The progressive increase in drug-resistant malaria, including delayed clearance of *Plasmodium falciparum* after treatment with artemisinin derivatives [17], highlights the need for novel effective antimalarial drugs. In this context, efforts have been encouraged to focus on research into novel and safe alternative compounds that have a low propensity to generate resistance [18,19]. One possible source is repositioning compounds that have been approved for other treatments but which have been found to have parasiticidal or disease-modifying effects in malaria infection, such as methylene blue (MB). Methylene blue already has other medicinal applications such as the treatment of methemoglobinemias, management of Alzheimer's, and applications in ifosfamide-induced encephalopathy and neurotoxicity.

In vivo evaluation of antimalarial compounds begins with the use of rodent malaria parasite and murine models. Although studies have been carried out on the potential of MB in the management of malaria, none has been done to assess its effect on LM-resistant *Plasmodium berghei*. Other studies demonstrated that MB is effective, with 50% inhibitory concentration at 3.62–3.90 nM, on 23 *P. falciparum* strains resistant to several standard antimalarials, including chloroquine (CQ) [20]. Dormoi *et al.* [21] went on to further demonstrate significant efficacy of MB in the treatment and survival of cerebral malaria in mice infected with non-resistant *P. berghei* ($p = 0.0011$).

This study aimed to assess MB antiplasmodial activity on stable LM-resistant ANKA lines [22] and PYR-resistant *P. berghei*, with a view of predicting its applicability and efficacy in LM- and PYR-resistant malaria. This is important since resistance to ACTs, the first-line anti-malarial regimen, has been reported in western Cambodia [23,24].

Methodology

Drugs and chemicals

Anhydrous MB from Sigma-Aldrich Laborchemikalien GmbH, (Seelze, Germany) was used in this study. LM was a kind donation from Daniel Kiboi, Kenya Medical Research Institute. Pure PYR was from Sigma Chemical Company, St. Louis, USA.

MB was dissolved in distilled water, PYR was weighed and dissolved in 5% (v/v) dimethyl sulfoxide, then vortexed gently to mix before topping up with 1X phosphate-buffered saline (PBS). PH adjusted to 4.0. LM was freshly prepared by dissolving in a vehicle consisting of 70% Tween-80 ($d = 1.08$ g/mL) and 30% ethanol ($d = 0.81$ g/mL) and subsequently diluted tenfold with double-distilled water.

Parasites and animals

To study the activity of MB on resistant rodent malaria parasites, resistant lines of *P. berghei* ANKA (MR4 catalog number MRA-868, MR4, ATCC Manassas, Virginia, USA) were used. The LM-resistant line (developed by passaging [22]) employed in the study was obtained from Kenya Medical Research Institute. The parasite was stable green fluorescent protein luciferase expressing and was subjected to selective LM pressure to the 68th passage. PYR-resistant line (developed by transfection technology as described previously [25,26]) and wild-type strains were also used for comparison purposes. The PYR-resistant and wild-type parasites were obtained from the Institute of Primate Research biobank.

Adult BALB/c mice bred in the rodent facility of the Department of Animal Sciences, Institute of Primate Research, Kenya, were used. At the time of use, they were 7 to 10 weeks of age and weighed 20 ± 2 g. In the overall study, the female-to-male sex ratio of the animals used was 80:20. The animals were housed in an experimental room in standard Macrolon type II cages clearly labeled with experimental details at 22°C and 60%-70% relative humidity. They were fed a commercial rodent pellet diet obtained from Unga Farm Care (EA) Limited, Nairobi, and were given water *ad libitum*.

Parasite retrieval and infection

Cryopreserved stocks of *Plasmodium berghei* were revived by being thawed at 37°C in a waterbath before being intraperitoneally injected into donor mice. At average parasitemia of between 8% and 15%, the donor mice were sacrificed and cardiac puncture was performed on them to collect the infected blood. The heparinized blood collected from the donors was pooled and diluted in PBS. The parasite suspension was used to infect experimental mice intraperitoneally (10^6 infected erythrocytes per mouse). The inoculated experimental mice were randomly divided into groups of five. Control groups, each with five mice, were also included. Upon determination of successful infection, the mice were treated for four days with intraperitoneal injections of MB, while LM and PYR were administered orally also for four days. Parasitemia was assessed by microscopic examination of Giemsa-stained thin smears made from tail-vein blood.

Preliminary dose-determining experiments were earlier done on wild-type *P. berghei* at 5, 15, and 45 mg/kg/day of MB; 15 mg/kg had 67% activity, while 45 mg/kg had 100% activity. The effective dose (ED90) value was determined by its relative potency and so subsequent studies were done using 45 mg/kg/day of MB.

Drug efficacy was determined by overall percentage (%) parasitemia suppression after microscopic observation of Giemsa-stained thin blood smears. The mice survival rate (%) and drug curative effect to mice (%) relative to the untreated controls were also used as a measure of effect of these drugs on the infected mice.

Ethical approval

Approval for animal use was granted by the institutional research ethics committee of the Institute of Primate Research, Kenya that comprises the animal care and use committee. The animals were maintained according to institutional animal care and use policy.

Statistical analysis

Parasitemia and survivorship values are reported as mean ± standard error of mean. These were generated in Microsoft Excel. Statistical analysis of data to compare mean parasitemia of the appropriate parasite lines when treated with MB was carried out using student's two-tailed unpaired t-test while assuming 95% confidence limits. $P < 0.05$ was considered significant. The analysis of data obtained was done using the GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

This study aimed to determine how effective MB would be on resistant rodent parasites. Several doses of MB were tested to determine the effective dose value; 45 mg/kg/day was found to have the highest potency. This was similar to Garavito *et al.*'s [27] findings on MB efficacy on sensitive wild-type *P. berghei*. Based on this, subsequent tests were carried out using it. The dose had an inhibition activity that suppressed wild-type parasites to below detectable levels compared to 67% of 15 mg/kg and 43% of 5 mg/kg (Table 1).

Inhibition of parasite growth

Parasitemia patterns obtained after treatment with MB indicate MB inhibits parasite growth. It was observed to suppress parasites to below detectable levels for up to 11 days on the LM-resistant type of *P. berghei* (Figure 1). During MB administration, suppression was observed for the 4 days and for 7 days post-treatment (PT). Parasite recrudescence was detected on day eight PT.

Table 1. Effect of MB 45 mg/kg against CQr compared to LMr and PYRr parasites at day 5 post-treatment.

	Methylene blue chemosuppression		
	Parasitemia		
	Non treated	Treated	% suppression
LMr *P. berghei*	13.34	0	> 99.00
PYRr *P. berghei*	9.92	0	> 99.00
CQr (RC-clone) *P. berghei*	2.11	2.22 ± 0.59	0
P. yoelii 17X (CQ resistant)	0.22	1.68 ± 0.59	0
Wild-type *P. berghei*	26.21	0.21 ± 0.16	99.20
Wild-type *P. berghei* 15 mg/kg	26.21	8.73 ± 2.83	66.69
Wild-type *P. berghei* 5 mg/kg	26.21	14.88 ± 2.95	43.23

LMr: lumefantrine resistant; PYRr: pyrimethamine resistant; CQr: chloroquine resistant.

The stability of LM resistance in parasites used was proven by the steady parasitemia growth even after treatment with LM at 50 mg/kg. This shows that the resistance phenotype for the parasite had not been lost with dormancy.

The dose of 45 mg/kg of MB administered suppressed the bulk of parasitemia in the PYR-resistant *P. berghei* (Figure 2) for a total of 9 days. At day 5 post-treatment, suppression was maintained at 100% on the PYR-resistant parasites. MB-treated mice had undetectable parasitemia compared to 15% (1.5×10^6 parasites/μL of blood) of the PYR-treated group at day 3 post-treatment (Figure 2). However, recrudescence was observed to take place from day 6 PT. PYR at 1 mg/kg showed that the parasites (transfected with *tgdhfr*-bearing plasmids) were transformed since the parasitemia steadily increased even after exposure to PYR. Pyrimethamine exerts its antimalarial activity by interfering with folic acid synthesis by the enzyme, consequently interrupting the parasite's folate pathway. These observations suggest that MB has a different mechanism of action from PYR, thereby advocating its use in regions with reported PYR-resistant malaria.

The results of MB activity on other rodent malaria causing parasites are shown in Table 1. They reveal over 99% chemosuppression by MB at 45 mg/kg at day 5 PT on wild-type LM-resistant and PYR-resistant *P. berghei*. The same dose at the same time period did not achieve similar results on the chloroquine-resistant (RC) strain of *P. berghei* and non-lethal *P. yoelii* 17X (also CQ resistant), where suppression lasted 2 days. A low dose of MB (15 mg/kg) had a 67% suppressive effect on wild *P. berghei*.

Overall, the results show that MB inhibits LM-resistant and PYR-resistant *P. berghei* parasites. Against erythrocytic forms in rodent malaria parasites, MB at 45 mg/kg was more active on *P. berghei* than on *P. yoelii* after a 4-day treatment.

Mice survivorship

The results on mice survival in Figure 3A shows that the group treated with MB in the LM-resistant parasites category had 100% of mice surviving up to day 21 post-infection (PI) before one mouse died by day 24. The control group treated with 50 mg/kg LM had 60% of mice surviving at day 16 PI, and the last mouse died on day 18. The infected non-treated negative control group had only 40% of mice alive at day 18 PI, with the last one dying 3 days later (Figure 3A).

Mice infected with PYR-resistant parasites and treated with MB had a 100% survivorship at day 7 PI. By day 8 PI, one mouse died, reducing survivorship to 80%, which further dropped to 40% on day 16 PI (day 14 PT). In the artemether-treated control group, 60% of the mice survived from day 5 PI to day 9 PI, and 40% survived from day 7 to 12 PT (day 10–12 PI). The mice in the infected non-treated negative control had all died by day 10 PI. PYR (1 mg/kg)-treated groups had survivorship fall to 20% on day 10 PI from 80% on day 7 PI (Figure 3B).

Figure 1. Parasitemic profile of lumefantrine-resistant *P. berghei* 14 days post-treatment with methylene blue 45 mg/kg in 4-day curative test.

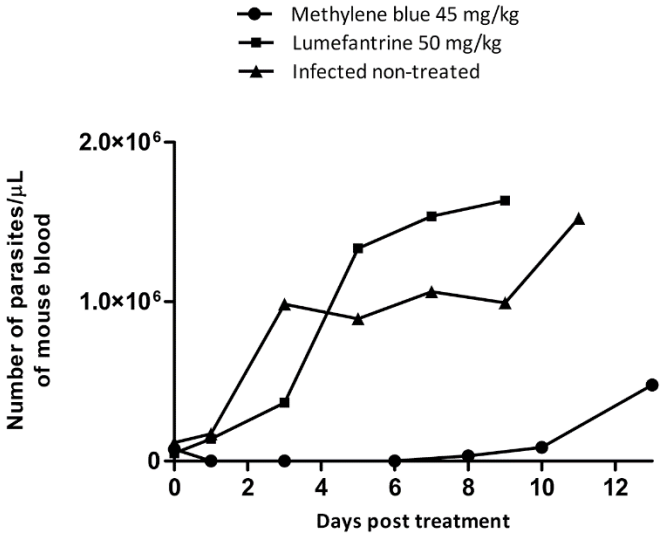


Figure 2. Parasitemic profile of pyrimethamine-resistant *P. berghei* 9 days post-treatment with methylene blue 45 mg/kg in 4-day curative test.

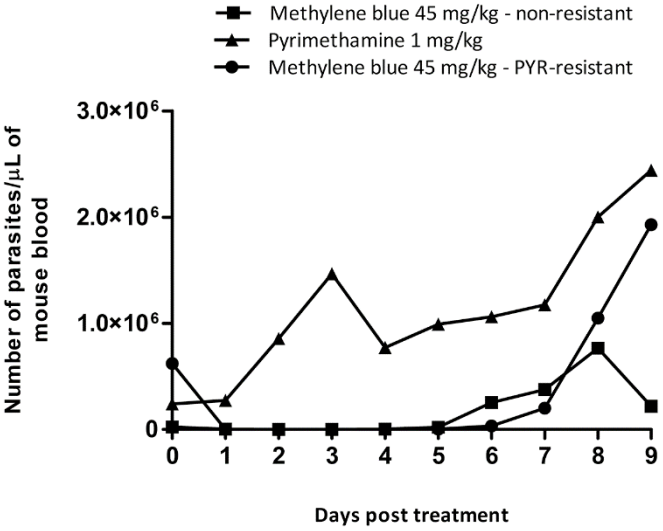
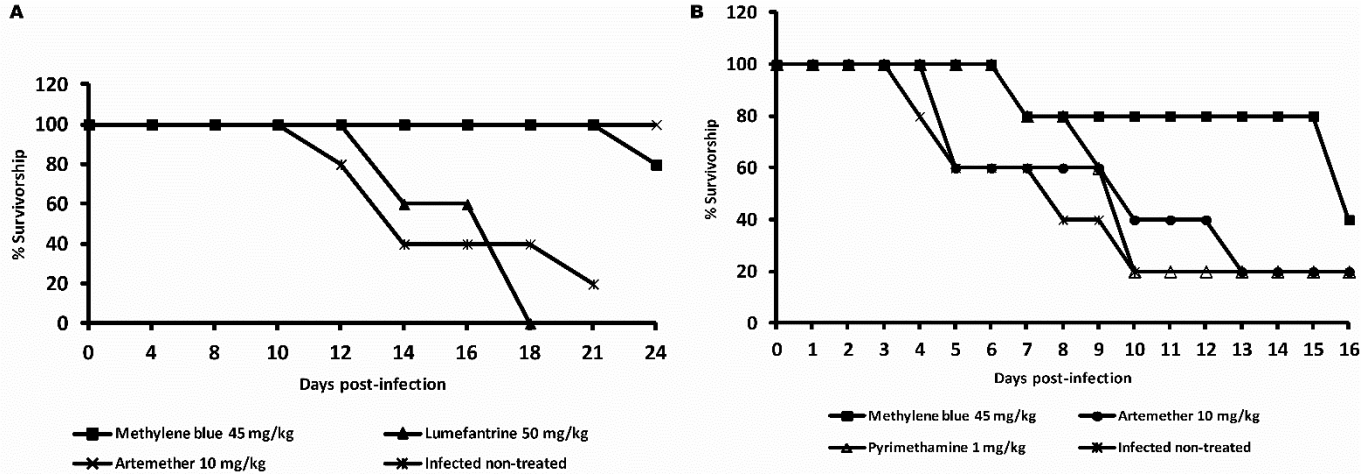


Figure 3. Survivorship curves of mice. **A)** infected with lumefantrine-resistant *P. berghei* and treated with methylene blue 45 mg/kg/day; **B)** treated with methylene blue 45 mg/kg/day after infection with pyrimethamine-resistant *P. berghei.*



Discussion

This study was designed to determine the effect of MB on *P. berghei* parasites resistant to LM and PYR. Results showed that MB is effective against the two resistant types of rodent parasites, suppressing parasitemia to below detectable levels for the duration under drug pressure. This potentiates MB into a candidate for drug repurposing against malaria.

MB demonstrated remarkable parasite suppression against the two resistant rodent malaria parasites at the onset of treatment. MB rapidly suppressed parasitemia to below detectable levels up to day 5 PT (Table 1) in relation to the negative controls. On the *P. berghei* parasites resistant to PYR and LM, MB demonstrated impressive chemosuppression for six and seven days post-treatment, respectively. This shows the potency of the 45 mg/kg dose of MB in treating LM-resistant malaria. The results obtained present the 45 mg/kg dose MB as a start in the development of alternative fast-acting treatment regimens, especially against non-CQ resistant parasites. The rapid antimalarial effect of MB, coupled with its short half-life, may promote slow development of resistance because exposure of parasites to sub-therapeutic levels of the drug would be very brief. It may next be possibly used as an alternative to artemisinin in combinational therapy. It is conceivable that MB can be considered for application in treatment of both uncomplicated and complicated malaria as well as for its potential for use in LM- and PYR-resistant malaria regions. This therapeutic potential is further highlighted in MB's capacity to efficiently manage cerebral malaria, a complication of untreated malaria [21].

Although the overall parasite burden was decreased from onset of treatment, response to treatment in this study can be categorized as clearance and recrudescence. Rapid parasite suppression was noted in both resistant and wild-type parasites before recrudescence was noted at least six days after the end of therapy. A factor that probably contributed to this observation is MB's short plasma half-life of 18 hours. The 72% bioavailability of the compound explains the fast parasite clearance *in vivo* [28]. It is also likely that inactive parasites are an omnipresent component of many malaria parasite infections, and act by introducing metabolic perturbations into active ones. Further studies need to be done to determine the reason for recrudescence and whether the parasite had possible resistance mechanisms to MB. More studies also need to determine a dose that would still be safe and completely cure the animals; this would substantially secure the need to have MB included in the armamentarium of malaria treatment. It would also be interesting to determine whether prolonged treatment at 45 mg/kg would result in increased recrudescence time or cure.

The findings indicate that MB was active on parasites resistant to the common antimalarials and displayed a different mode of action, which could be advantageous against the mechanisms of resistance displayed. Some of the common mechanisms of resistance are the result of epigenetic changes such as gene amplification, protein overexpression, and protein modifications.

The significant parasitemia suppression in mice treated with the MB dose was synonymous with longer survival time. Mice groups treated with MB had over

80% survivorship for at least 14 days in the two categories, which was indicative of better survival rates after treatment with MB. The improved survivorship could be attributed to the 6-day chemosuppression observed on parasites that, in turn, lessened the overall parasite burden on the mice. This possibly gave the immune systems of the mice time to recover and help control the parasite infection.

Recently, *PfMDR* N86, the chloroquine-susceptible allele, has been proposed as a molecular marker for LM resistance. Artemether-lumefantrine treatment selects for N86 in recurrent infections [29]. In Mozambique, there has been an increase in the prevalence of molecular markers associated with LM resistance since initial use of artemether-lumefantrine (AL), suggesting the need for continued surveillance for the emergence of resistance to the drug [30]. An increase in the gene copy number has been associated with increased risk of treatment failure with mefloquine, artesunate-mefloquine, or artemether-lumefantrine [31]. This further pushes the need to find antimalarials that would counter the foreseen problem of LM resistance.

Two main modes of action of MB have been suggested. One is its accumulation and probable concentration inside in the food vacuole where it inhibits the formation of hemozoin, just as the 4-aminoquinolines do. The other is inhibition of parasite glutathione reductase, which jeopardizes glutathione functionality, resulting in glutathione depletion, which sensitizes the parasite for chloroquine action [32,33].

Other than exhibiting inhibitor properties, MB has been reported to be a substrate that is reduced by flavoenzymes that produce reactive oxygen species [34] to generate oxidative stress. MB achieves generation of oxidative stress by inducing indirect structural changes in the interdimeric region of glutathione reductase. It targets the crucial redox machinery of *Plasmodium*, most effectively against the late ring/early trophozoite stages [28]. This further suggests the prospects of including MB in managing resistant malaria.

The over 99% suppression on the two resistant strains and remarkable survivorship observed in MB-treated mice demonstrates the high antimalarial potency of MB on LM- and PYR-resistant malaria. The results also present MB as safe to use since over 80% survivorship was observed at day 15 and 24 PI, respectively, in the mice groups infected with PYR- and LM-resistant parasites and treated with MB compared to the controls. It can thus be implied that the treatments with MB improve the survivorship of infected mice

Findings from this study have shown evidence that the thiazine dye is active against PYR- and LM-resistant parasites. This suggests that MB has the potential to be used in LM- and PYR-resistant malaria regions, having displayed suppression below detectable levels for 9 and 11 days, respectively, since the onset of treatment on the resistant *P. berghei*. Repositioning MB in the fight against malaria presents a different chemotherapeutic approach to resistant parasites that would, in turn, provide more options in terms of combinational therapy. Meissner *et al.* showed that MB is safe for use in the treatment of children presenting with uncomplicated *P. falciparum* malaria, with overall clinical and parasitological failure rates by day 14 of 10% and 24%, respectively [35].

Conclusions

This study has determined that MB could be a potential drug candidate in drug repurposing in the chemotherapeutic management of LM- and PYR-resistant malaria. It can, in the future, be considered as a replacement for LM in combination therapies. An in-depth study of its application against a wide range of resistant strains, including artemisinin-resistant *P. falciparum* and field isolates, and longer treatment periods would be warranted to determine its true utility and any possibilities of cross-resistance.

These results from resistant mutations obtained in a laboratory *in vivo* system may not be a clear representative of the natural populations of *P. falciparum*; therefore, further studies of MB on LM-resistant *P. falciparum* would be highly recommended. However, if these results obtained are general across parasite genotypes and species, they have significance especially for mutant parasites resulting from continued population-wide exposure to drugs.

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Authors' contributions

VIM, HSO, and SAO were involved in the study conception and design. VIM developed the original concept of the study. DMK provided the lumefantrine drug and lumefantrine-resistant parasites for the study. VIM executed the laboratory work, produced the graphs, and wrote the drafts. RMM assisted in conducting the experiments. VIM and HSO analyzed and interpreted the data, reviewed the manuscript, and had primary responsibility for final content. ZWN reviewed the study design and reviewed the manuscript.

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Corresponding author

Hastings Ozwara, PhD
Senior Scientist, Malaria Programme,
Department of Tropical and Infectious Diseases
Institute of Primate Research.
P.O. Box 24481-00502, Karen, Nairobi, Kenya.
Phone: +254 02 2606235, +254 721 483 326
Fax: +254 02 2606231
E-mail: ozwara@primateresearch.org,
kamonya2002@yahoo.co.uk

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