DRUG REPURPOSING: ASTEMIZOLE-METHYLENE BLUE COMBINATION THERAPY HAS ANTIPLASMODIAL ACTIVITY AGAINST *PLASMODIUM FALCIPARUM* STRAINS *IN VITRO*

ABSTRACT

Plasmodium species are protozoa from the *Apicomplexa* phylum which cause malaria. In the tropics and sub-tropics, approximately 3.3 billion people are threatened by this disease. Artemisinin Combination Therapy, has been reported to have a possible emergence of resistance. Therefore, there is an urgent need for new drug formulations. Drug repurposing offers an appealing alternative to *de novo* drug development. Although astemizole and methylene blue have been reported to have anti-malarial properties, their efficacy when used in combination has not been studied. Five concentrations ranging from 7.81 µg/ml to 125 µg/ml were combined in various ratios and assessed against two *Plasmodium* strains *in vitro*. Parasite load (per µl of blood) was determined by microscopy. The results were represented as mean ± standard error. ANOVA analysis was used to determine differences in the treatment groups at $p<0.05$. Anti-plasmodial activity was observed in all drugs that were cultured with *P. falciparum* chloroquine sensitive *(3D7)* and chloroquine resistant *(W2)* strains. Strain dependent differences were observed in the efficacy scores of the tested drugs. Astemizole-methylene blue combinations of ratios 1:1, 3:1 and 1:3 interacted antagonistically. The least antagonistic interactions were 3:1 and 1:3 ratios at 31.25 µg/ml against the *Plasmodium* strains (FIC of 2.2 and 2.6 respectively). Astemizole antagonized methylene blue in the combinations. This study ~~provided information on the importance of astemizole-methylene blue combination therapy against malaria and~~ emphasized on the relevance of the drug repurposing in malaria. ~~This study~~ shows that the drugs work better as monotherapies and that combinations in these ratios have insignificant antiplasmodial activity.

Keywords: Drug repurposing, malaria, astemizole, methylene blue, Plasmodium falciparum

1. INTRODUCTION

Malaria is a parasitic disease caused by protozoa parasites of the genus *Plasmodium*. As of year 2018, 228 million cases of malaria were recorded globally [1] . At present, chemotherapy still remains one of the major approaches for both malaria prevention and remedy [2]. However, in some endemic areas, delayed clearance of the disease in patients to whom antimalarial drugs were administered remains a great concern. An interesting observation is that, the patients still record high levels of parasitemia despite having a considerable amount of anti-malarial drugs in their body systems [3]. Due to the widespread anti-malarial drug resistance, combination therapy has been advocated for by WHO rather than monotherapy which is becoming less effective [4]. The use of combination therapies with artemisinin was aimed at combating parasite resistance against monotherapies that was reported in laboratory settings [5]. *Plasmodium* resistance in addition to chloroquine has had a negative impact on malaria treatment [6]. Furthermore, the emergence of artemisinin resistant *Plasmodium falciparum* strains along the Thai-Cambodia border negatively impacted disease control efforts[7]. The traditional methods of drug development are not only time consuming but also costly. Furthermore, 90% of drug trials fail as early as the developmental stage [3]. Thus drug repurposing provides an alternative and favorable channel in faster development of future antimalarial drug candidates.

Astemizole (AST), with the common trade names Histmanol, Cilergil and Almizol, on the other hand, is an artificial piperidinyl-benzimidazol derivative that has anti-allergic properties [8]. This drug works by competing with histamine at the H_1-receptor sites in the uterus, gastro-intestinal tract and bronchial muscles [9]. Orally administered astemizole is metabolized into *O*-desmethylastemizole [10]. The O-desmethylastemizole concentration in serum rises higher than that of astemizole is eventually eliminated in 9 -13 days. It has been observed that during the intra-erythrocytic stage*, P. falciparum* crystallizes heme released from the disintegration of hemoglobin within the food vacuole to enhance their survival. Astemizole works by inhibiting this crystallization process thereby suppressing parasite survival [11]. Astemizole inhibits the growth of chloroquine sensitive and resistant *Plasmodium* parasites *in vitro* [12].

The phenothiazinium salt, methylene blue (MB) is a synthesized textile dye. In 1891, it was approved for use as the initial synthetic antimalarial [5]. However, its use was discontinued when chloroquine was introduced into the market because chloroquine served as a better remedy against malaria [13]. Methylene blue inhibits *P. falciparum* glutathione reductase and reverses chloroquine resistance. In the trophozoite stage, *P. falciparum,* metabolizes glutathione at an intense rate to protect against oxidative stress [7]. Glutathionine helps in antioxidative protection and in its reduced state, it supports parasite growth by providing electrons for the deoxyribonucleotide (DNA) [7]. It also prevents the polymerization of heme into hemozoin [13]. Methylene blue specifically inhibits glutathione reductase and possibly reverses parasite resistance to chloroquine [13,14]. A study, 99% chemo suppression of methylene blue was demonstrated against rodent malaria at 45 mg/kg by day 5 post treatment [14].

There is an urgent need to develop new formulations [12]. Drug repurposing offers this option. This is the use of known drugs to treat diseases for which they were not primarily intended [15]. Although not primarily used to treat malaria, both methylene blue and astemizole have been reported to be potent against malaria [14,11]. Hence, there is a potential for both to be repurposed and used in malaria therapy. In this article, we explored the possibility and potential of astemizole-methylene blue combination *in vitro* against two laboratory maintained *P. falciparum* strains.

2. MATERIAL AND METHODS

2.1 Study area

The study was conducted at the Department of Tropical and Infectious Diseases (TID), Institute of Primate Research (IPR) in Karen, Nairobi County, Kenya.

2.2 Preparation of drugs and chemicals

Anhydrous methylene blue (sourced from Sigma-Aldrich,Germany), was weighed and dissolved in distilled water to produce a 1 mg/ml stock solution [14]. Astemizole (sourced from University of Cape Town – Department of Chemistry) was dissolved in absolute ethanol to produce a 1 mg/ml stock solution. The solutions were filter sterilized through a 0.22 µm membrane filter (Sartorius Stedim Biotech, USA, Ministart ®) and stored at 4^0C until use [8]. Astemizole-methylene blue combinations in ratios of 1:1, 1:3, 3:1 were prepared.

2.3 *In vitro* assay of *Plasmodium falciparum*

Chloroquine sensitive (*3D7)* and chloroquine resistant (*W2*) *Plasmodium falciparum* strains (acquired from Institute of Primate Research and Kenya Medical Research Institute bio-repository), respectively, were used. In this study, Sodium chloride (NaCl) gradient methodology was used to culture the parasites as described by Nzila [16]. Each strain was assessed *in vitro* in triplicate using five concentrations ranging from 7.81 µg/ml to 125 µg/ml of drugs: methylene blue, astemizole and astemizole-methylene blue combination ratios of 1:1, 1:3, and 3:1 as described by Fivelman [17]. *Plasmodium falciparum* parasites (*3D7* and *W2* strains) were inoculated at 1% parasitemia (>70% ring forms) and 1.5% hematocrit.

2.4 Dosing of culture plates with test drugs and incubation of treated parasites

Drug assays of astemizole-methylene blue combinations (1:1, 1:3, 3:1) were set up in 96 well plates for each parasite strain. Diluted pellets of *Plasmodium falciparum* parasites were incubated with the drugs for 48 hours at 36.8^0C. Thin blood smears were prepared and observed under the microscope (Zeiss standard 20, Germany) with x100 objective under oil immersion. Parasitemia was determined by counting the infected red blood cells relative to the total red blood cells computed:

$$\text{Parasite load per µl} = \frac{\text{Number of infected red blood cells}}{\text{Number of total red blood cells}} \times 5 \times 10^6 \quad (1)$$

Parasite suppression (%)=

$$\frac{\text{Parasitemia in negative control} - \text{parasitemia in drug treated groups}}{\text{Parasitemia in negative control}} \times 100 \quad (2)$$

2.5 Statistical analysis

Graph Pad Prism 7 (San Diego, CA) software, was used to determine the Inhibitory concentration (IC_{50}) of the drugs activity *in vitro*. Subsequently, the IC_{50} values were used to calculate Fractional Inhibitory Concentration (FIC) values which in turn, provided information on the drug interactions.

Differences between groups were compared and analyzed by ANOVA (Graph Pad Prism 7) and these were considered significant if the *P* values were less than 0.05 ($p< 0.05$). Additionally, the antagonistic interactions of the drug combinations were illustrated through isobolograms of the test drugs against the *Plasmodium falciparum* strains by using the fixed dose ratio method in Microsoft Excel 2013.

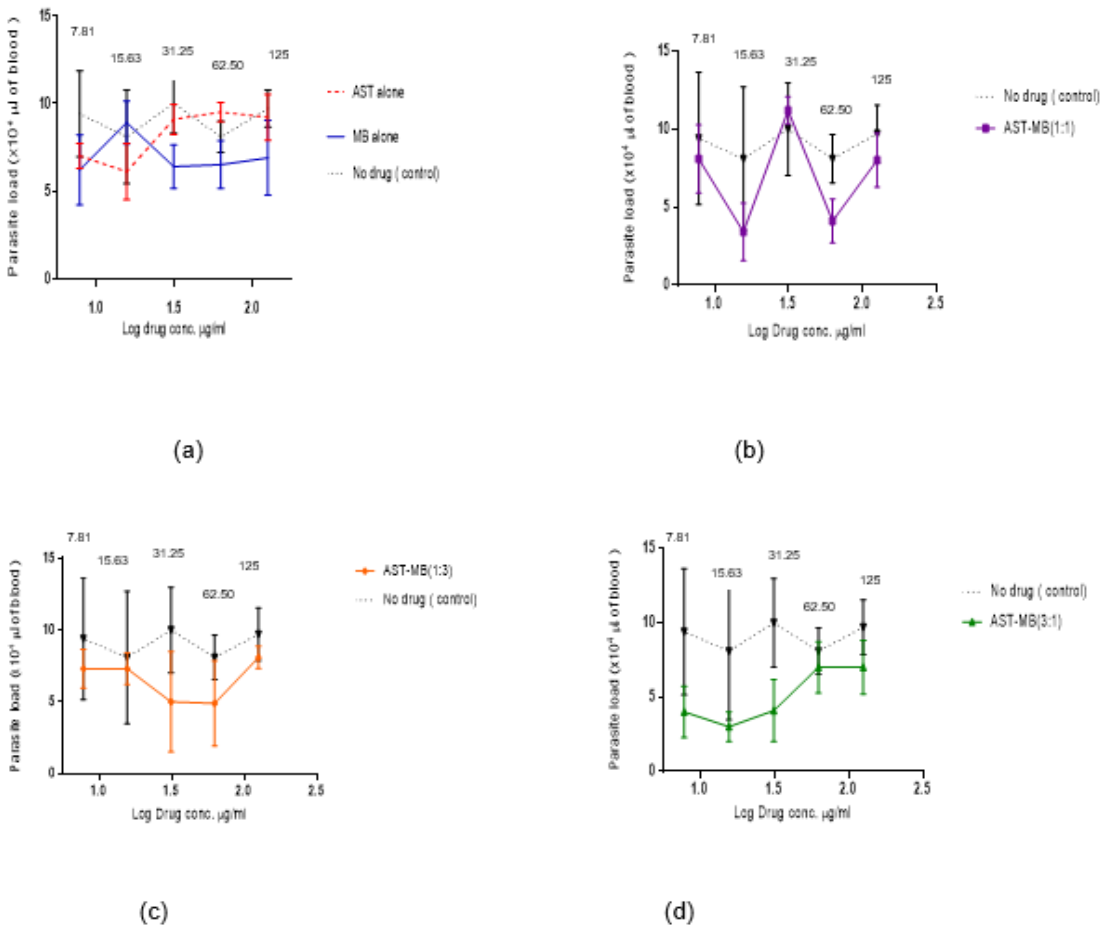
3. RESULTS AND DISCUSSION

3.1 Anti-plasmodial activity of drug combinations

In this study, antiplasmodial activity was observed in both mono and combination therapies. Astemizole (AST) and methylene blue (MB) monotherapies showed potency against both *Plasmodium* parasite strains and concurred with findings by Nzila [16] and Chong [11] (Figure 1 a). The parasite loads in methylene blue alone and astemizole alone were less than the load in the negative control against *Plasmodium falciparum* chloroquine sensitive *(3D7)*. According to the results, the monotherapies were more efficacious at low concentrations. Astemizole-methylene blue 1:1 performed better at both high and low concentrations (Figure 1b). Astemizole-methylene blue 1:3 and 3:1 had better efficacy as compared to the monotherapies at both low and high concentrations (Figure 1 c and d respectively).

Astemizole-methylene blue 3:1 was the most efficacious test drug against *P. falciparum* chloroquine sensitive (*3D7)* (*p=0.0017*). These results revealed that for the combination to cause a high reduction in parasitemia, the amount of methylene blue must be less than that of astemizole against *Plasmodium falciparum* chloroquine sensitive (*3D7).* Astemizole has been shown to be effective against *Plasmodium* drug chloroquine sensitive strains [18]. A higher concentration of astemizole in the drug combination increased the maximal therapeutic activity against *P. falciparum* chloroquine sensitive (*3D7)* [19] .

169



170
171

172 **Figure 1. Parasitemia of drugs and the control against *Plasmodium falciparum 3D7*. (a)**
173 **astemizole alone and methylene blue alone, (b) astemizole-methylene blue drug**
174 **combination (1:1), (c) astemizole-methylene blue drug combination (1:3), (d)**
175 **astemizole-methylene blue drug combination (3:1)**

176
177

178 At low concentrations, both monotherapies and drug combinations showed antiplasmodial
179 activity (Figure 2). Astemizole-methylene blue 1:1 performed better at higher concentrations
180 (Figure 2 b). Astemizole-methylene 1:3 and 3:1 were more efficacious at all concentrations
181 and observed to be similar to those in the monotherapies (Figure 2 c and d). The results
182 showed that AST-MB 1:3 had more antiplasmodial activity against *P. falciparum* chloroquine
183 resistant (*W2)* strain. According to Meissner [13], methylene blue reverses chloroquine
184 sensitivity in *P. falciparum.* More methylene blue in comparison to astemizole therefore
185 increased the susceptibility of the parasite to the drug combination.
186

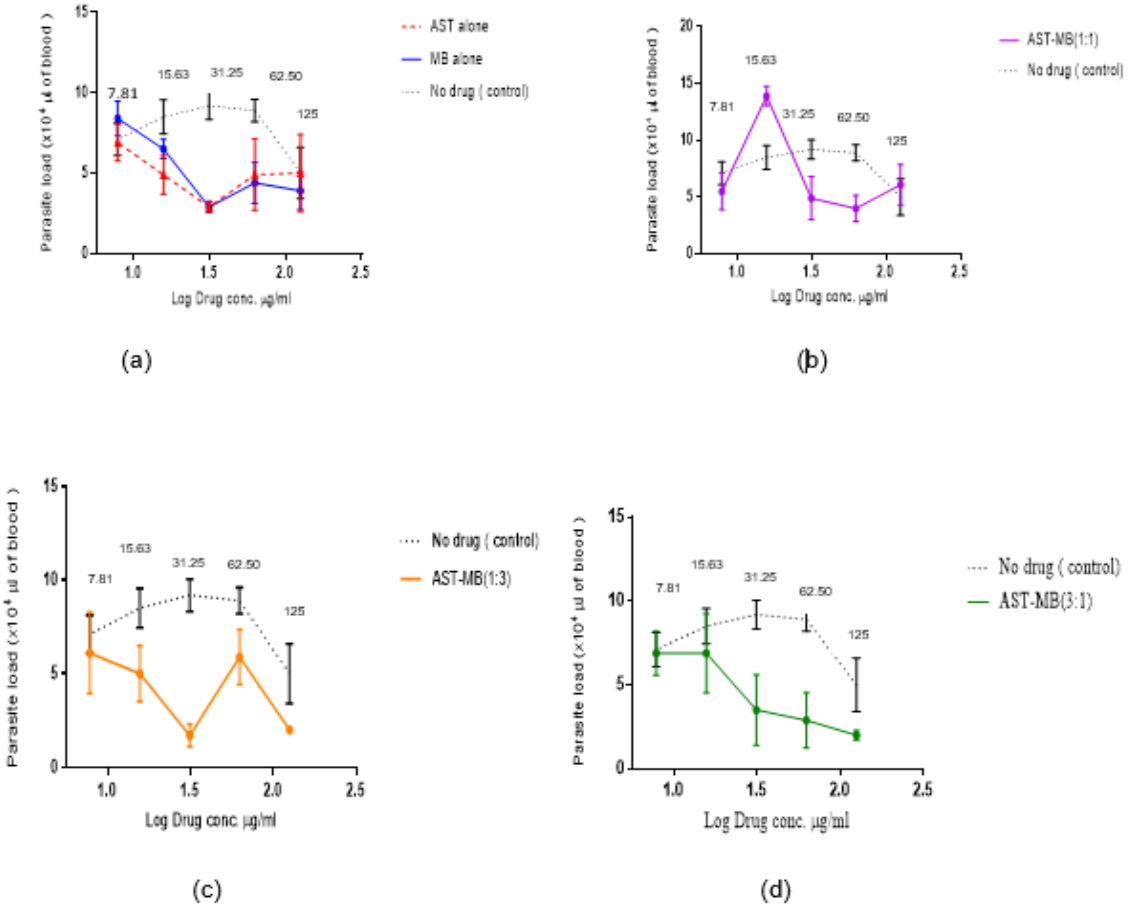


Figure 2. Parasitemia of drugs and the control against *Plasmodium falciparum W2*. (a) astemizole alone and methylene blue alone, (b) astemizole-methylene blue drug combination (1:1), (c) astemizole-methylene blue drug combination (1:3), (d) astemizole-methylene blue drug combination (3:1)

The drugs that caused the greatest reduction in parasitemia against chloroquine sensitive (*3D7*) and chloroquine resistant (*W2*) *Plasmodium falciparum* strains are shown in Table 1 and Table 2. However, only AST-MB 3:1 at 31.25 µg/ml caused significant parasite suppression (51%) (p= 0.0017) against chloroquine sensitive (*3D7)* and AST-MB 1:3 at 31.25 µg/ml caused significant parasite suppression(53%) (p=0.0035) against chloroquine resistant (*W2) Plasmodium* strains. An antimalarial drug with a patasite suppression of 30% is considered ideal [20]. In previous studies, astemizole alone and methylene blue each caused 80% parasite suppression against malaria parasites [21,22]. The parasite suppresion of the most potent astemizole-methylene blue combinations despite causing parasite suppression that is above the ideal percentage, was less than that of the inidividual drugs.

Table 1. Drug efficacy against *Plasmodium falciparum* chloroquine sensitive *(3D7)*

Concentration (µg/ml)	Most efficacious drug	Statistical significance (ANOVA)
125	MB alone	Insignificant
62.5	AST-MB 1:1	Insignificant
31.25	AST-MB 3:1	*****Significant (d=2, p=0.0017)
15.63	AST-MB 3:1	Insignificant
7.81	AST-MB 3:1	Insignificant

***** Caused 51% parasite suppression

Table 2. Drug efficacy against *Plasmodium falciparum* chloroquine resistant (*W2)*

Concentration (µg/ml)	Most efficacious drug	Statistical significance (ANOVA)
125	AST-MB 3:1 and 1:3	Insignificant
62.5	AST-MB 3:1	Insignificant
31.25	AST-MB 1:3	*Significant (d=2, p=0.0035)
15.63	AST	Insignificant
7.81	AST-MB 1:1	Insignificant

* Caused 53% parasite suppression

3.2 Interactions of drug combinations

In this study, inhibitory concentration 50 (IC_{50}) showed the efficacy of a drug and potency of antagonists in drug combinations [23]. In Table 3, methylene blue alone had the least IC_{50} value (17.96±0.22 µg/ml), seconded by AST-MB 3:1 (22.28 ±0.24 µg/ml) against *Plasmodium falciparum* chloroquine sensitive (*3D7*). Also, methylene blue alone had the least IC_{50} value (7.69±0.43 µg/ml), seconded by AST-MB 1:3 (15.07±0.60 µg/ml) against *Plasmodium falciparum* chloroquine resistant (*W2*). These results showed that astemizole alone had less potency as compared to methylene blue alone against both *Plasmodium falciparum* chloroquine sensitive *(3D7) and Plasmodium falciparum* chloroquine resistant (*W2)* strains.

Table 3. Drug IC_{50} Values

Drug	Inhibitory concentration 50 (IC_{50}) (µg/ml)		
	Ratio	*P. falciparum 3D7*	*P. falciparum W2*
Methylene blue alone	-	17.96±0.22	7.69±0.43
Astemizole alone	-	23.12±0.30	23.55±0.26
Astemizole-methylene blue	1:1	23.25±0.66	29.23±0.84
Astemizole-methylene blue	1:3	34.16±0.37	15.07±0.60
Astemizole-methylene blue	3:1	22.28±0.24	26.14±0.46

This According to Te Dorshorst [24], the cut offs for drug interactions (x) are: if x <1 synergistic, 1≤ x <2 additive, 2≤ x<4 slightly antagonistic, 4 ≥ x antagonistic. The Fractional Inhibitory Concentrations of AST-MB combinations (1:1, 1:3, 3:1) against both *Plasmodium falciparum* chloroquine sensitive (*3D7)* and chloroquine resistant (*W2)* strains were all greater than 2 and therefore demonstrated antagonistic interactions [25] (Table 4). In this study, astemizole-methylene blue 1:1 demonstrated more antagonism than both astemizole-methylene blue 3:1 and astemizole-methylene blue 1:3. Drug interactions are highly dependent on the concentration of the drugs in a combination .Therefore, a drug can synergize another drug at one concentration (increase efficacy) or antagonize another drug at a different concentration [26].

Table 4. Fractional Inhibitory Concentrations (FIC) of the drugs

Drug	Fractional Inhibitory concentrations (FIC) (µg/ml)				
	Ratio	*P. falciparum* 3D7 FIC value	Interaction	*P. falciparum* W2 FIC value	Interaction
Astemizole-methylene blue	1:1	2.3	antagonistic	5.0	antagonistic
Astemizole-methylene blue	1:3	3.4	antagonistic	2.6	antagonistic
Astemizole-methylene blue	3:1	2.2	antagonistic	4.5	antagonistic

Isobolograms further illustrated drug interactions (Figure 3 and 4). All the drug FIC values were above the cut off line of methylene blue alone and astemizole alone, thus illustrating antagonistic interactions.

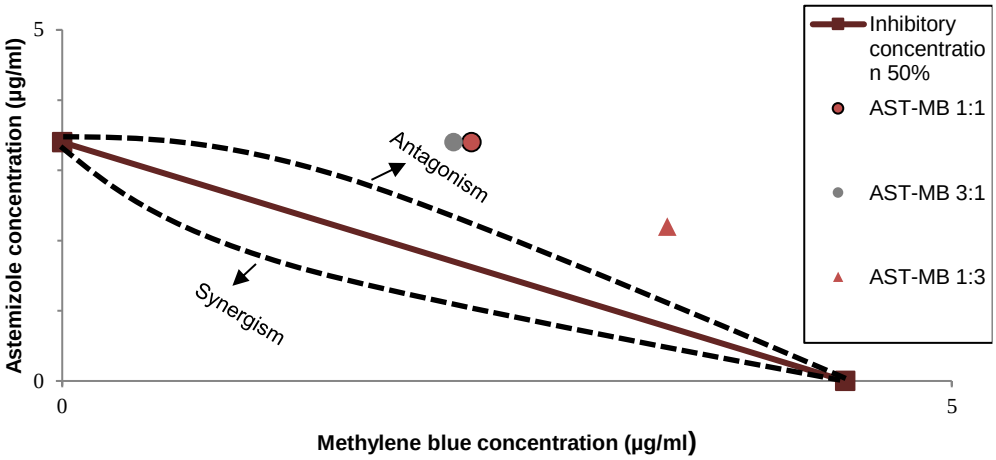


Figure 3. Isobologram of the drugs against *Plasmodium falciparum 3D7*

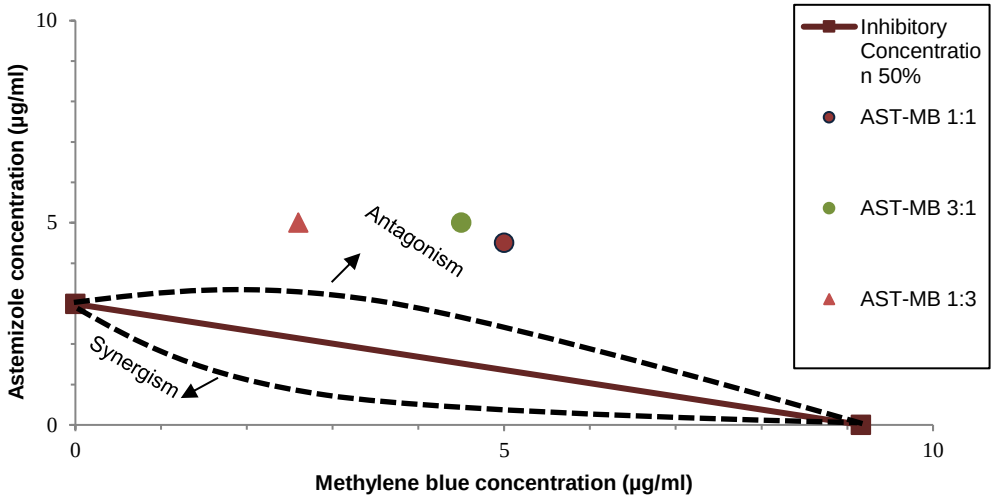


Figure 4: Isobologram of the drugs against *Plasmodium falciparum W2*

4. CONCLUSION

All the test drugs (astemizole alone, methylene blue alone, astemizole-methylene blue (1:1, 1:3 and 3:1) showed anti-plasmodial activity against both *Plasmodium falciparum* chloroquine

sensitive (*3D7)* and chloroquine resistant *(W2)* strains *in vitro*. Furthermore, astemizole alone-methylene blue 3:1 caused the highest reduction in parasite load against *Plasmodium falciparum* chloroquine sensitive (*3D7)* strain at 31.25 µg/ml (51% parasite suppression) whereas astemizole alone-methylene blue 1:3 drug combination caused the highest reduction in parasite load against *Plasmodium falciparum* chloroquine resistant (*W2)* strain at 31.25 µg/ml (53% parasite suppression). Despite the drug combinations offering parasite load reductions that are ideal (> 30%), the values were below those of astemizole alone and methylene blue alone (80%).

This study also showed that astemizole and methylene blue drug combinations interacted antagonistically. Therefore, astemizole-methylene blue drug combinations in the ratios: 1:1, 1:3 and 3:1, would not be effective when treating malaria caused by *Plasmodium falciparum*. However, the parasite load suppression of the drug combinations was above 30% less than the individual drug parasite load suppression of 80 %. This study therefore showed that astemizole -methylene blue combination in the ratios used reduces the potency of the drugs. This study therefore recommends repurposing astemizole and methylene blue drug combinations in other ratios.

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

Ethical clearance was sought from the Institutional Scientific Ethical Review Committee (ISERC) at Institute of Primate Research (ISERC/09/2017), which reviews all research protocols carried out in the institute. This committee is mandated by the National government through the National Commission of Science and Technology (NACOSTI).

REFERENCES

[1] "WHO | World malaria report 2019," *WHO*, 2020, Accessed: Jul. 12, 2020. [Online]. Available: http://www.who.int/malaria/publications/world-malaria-report-2019/en/.

[2] P. Schneider, B. H. Chan, S. E. Reece, and A. F. Read, "Does the drug sensitivity of malaria parasites depend on their virulence?," *Malar. J.*, vol. 7, no. 1, p. 257, Dec. 2008, doi: 10.1186/1475-2875-7-257.

[3] H. Matthews, M. Usman-Idris, F. Khan, M. Read, and N. Nirmalan, "Drug repositioning as a route to anti-malarial drug discovery: Preliminary investigation of the in vitro anti-malarial efficacy of emetine dihydrochloride hydrate," *Malar. J.*, vol. 12, no. 1, p. 359, Oct. 2013, doi: 10.1186/1475-2875-12-359.

[4] T. E. Wellems and C. V. Plowe, "Chloroquine-Resistant Malaria," *J. Infect. Dis.*, vol. 184, no. 6, pp. 770–776, Sep. 2001, doi: 10.1086/322858.

[5] R. Suwanarusk *et al.*, "Methylene blue inhibits the asexual development of vivax malaria parasites from a region of increasing chloroquine resistance," doi: 10.1093/jac/dku326.

[6] "WHO | World Malaria Report 2010," *WHO*, 2014.

[7] K. Becker, L. Tilley, J. L. Vennerstrom, D. Roberts, S. Rogerson, and H. Ginsburg, "Oxidative stress in malaria parasite-infected erythrocytes: Host-parasite interactions," *Int. J. Parasitol.*, vol. 34, no. 2, pp. 163–189, 2004, doi: 10.1016/j.ijpara.2003.09.011.

[8] Z. Zhou, V. R. Vorperian, Q. Gong, S. Zhang, and C. T. January, "Block of HERG potassium channels by the antihistamine astemizole and its metabolites desmethylastemizole and norastemizole," *J. Cardiovasc. Electrophysiol.*, vol. 10, no. 6, pp. 836–843, Jan. 1999, doi: 10.1111/j.1540-8167.1999.tb00264.x.

[9] Y. E. Karapetyan *et al.*, "Unique drug screening approach for prion diseases identifies tacrolimus and astemizole as antiprion agents," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 110, no. 17, pp. 7044–7049, Apr. 2013, doi: 10.1073/pnas.1303510110.

[10] C. C. Musonda, G. A. Whitlock, M. J. Witty, R. Brun, and M. Kaiser, "Chloroquine-astemizole hybrids with potent in vitro and in vivo antiplasmodial activity," *Bioorganic Med. Chem. Lett.*, vol. 19, no. 2, pp. 481–484, Jan. 2009, doi: 10.1016/j.bmcl.2008.11.047.

[11] C. R. Chong, X. Chen, L. Shi, J. O. Liu, and D. J. Sullivan, "A clinical drug library screen identifies astemizole as an antimalarial agent," *Nat. Chem. Biol.*, vol. 2, no. 8, pp. 415–416, 2006, doi: 10.1038/nchembio806.

[12] S. M. Corsello *et al.*, "The Drug Repurposing Hub: A next-generation drug library and information resource," *Nature Medicine*, vol. 23, no. 4. Nature Publishing Group, pp. 405–408, Apr. 07, 2017, doi: 10.1038/nm.4306.

[13] P. E. Meissner *et al.*, "Methylene blue for malaria in Africa: Results from a

dose-finding study in combination with chloroquine," *Malar. J.*, vol. 5, Oct. 2006, doi: 10.1186/1475-2875-5-84.

[14] V. I. Mwangi, R. M. Mumo, D. M. Kiboi, S. A. Omar, Z. W. Ng'ang'a, and H. S. Ozwara, "Methylene blue inhibits lumefantrine-resistant Plasmodium berghei," *J. Infect. Dev. Ctries.*, vol. 10, no. 6, pp. 635–642, Jun. 2016, doi: 10.3855/jidc.7556.

[15] D. Amantea, M. Certo, and G. Bagetta, "Drug repurposing and beyond: The fundamental role of pharmacology," *Functional Neurology*, vol. 30, no. 1. CIC Edizioni Internazionali s.r.l., pp. 79–81, Jul. 30, 2015, doi: 10.11138/FNeur/2015.30.1.079.

[16] A. Nzila, Z. Ma, and K. Chibale, "Drug repositioning in the treatment of malaria and TB," *Future Medicinal Chemistry*, vol. 3, no. 11. Future Med Chem, pp. 1413–1426, Sep. 2011, doi: 10.4155/fmc.11.95.

[17] Q. L. Fivelman, I. S. Adagu, and D. C. Warhurst, "Modified fixed-ratio isobologram method for studying in vitro interactions between atovaquone and proguanil or dihydroartemisinin against drug-resistant strains of Plasmodium falciparum," *Antimicrob. Agents Chemother.*, vol. 48, no. 11, pp. 4097–4102, Nov. 2004, doi: 10.1128/AAC.48.11.4097-4102.2004.

[18] M. Kumar *et al.*, "Multistage Antiplasmodium Activity of Astemizole Analogues and Inhibition of Hemozoin Formation as a Contributor to Their Mode of Action," *ACS Infect. Dis.*, vol. 5, no. 2, pp. 303–315, Feb. 2019, doi: 10.1021/acsinfecdis.8b00272.

[19] M. Akoachere *et al.*, "In vitro assessment of methylene blue on chloroquine-sensitive and -resistant Plasmodium falciparum strains reveals synergistic action with artemisinins," *Antimicrob. Agents Chemother.*, vol. 49, no. 11, pp. 4592–4597, Nov. 2005, doi: 10.1128/AAC.49.11.4592-4597.2005.

[20] A. U. Krettli, V. F. Andrade-Neto, M. D. G. L. Brandão, and W. M. S. Ferrari, "The Search for New Antimalarial Drugs from Plants Used to Treat Fever and Malaria or Plants Ramdomly Selected: A Review," *Mem. Inst. Oswaldo Cruz*, vol. 96, no. 8, pp. 1033–1042, 2001, doi: 10.1590/S0074-02762001000800002.

[21] J. Dormoi and B. Pradines, "Dose responses of proveblue methylene blue in an experimentalmurine cerebral malaria model," *Antimicrobial Agents and Chemotherapy*, vol. 57, no. 8. American Society for Microbiology (ASM), pp. 4080–4081, Aug. 2013, doi: 10.1128/AAC.00634-13.

[22] J. H. Lee *et al.*, "Assessment of general and cardiac toxicities of astemizole in male cynomolgus monkeys: Serum biochemistry and action potential duration," *Toxicol. Res.*, vol. 24, no. 4, pp. 289–295, Dec. 2008, doi: 10.5487/TR.2008.24.4.289.

[23] S. Aykul and E. Martinez-Hackert, "Determination of half-maximal inhibitory concentration using biosensor-based protein interaction analysis," *Anal. Biochem.*, vol. 508, pp. 97–103, Sep. 2016, doi: 10.1016/j.ab.2016.06.025.

[24] D. T. A. Te Dorsthorst, P. E. Verweij, J. F. G. M. Meis, N. C. Punt, and J. W. Mouton, "Comparison of fractional inhibitory concentration index with

response surface modeling for characterization of in vitro interaction of antifungals against itraconazole-susceptible and -resistant Aspergillus fumigatus isolates," *Antimicrob. Agents Chemother.*, vol. 46, no. 3, pp. 702–707, Mar. 2002, doi: 10.1128/AAC.46.3.702-707.2002.

[25] R. Y. Huang *et al.*, "Isobologram analysis: A comprehensive review of methodology and current research," *Frontiers in Pharmacology*, vol. 10, no. OCT. Frontiers Media S.A., p. 1222, Oct. 29, 2019, doi: 10.3389/fphar.2019.01222.

[26] M. A. Wambaugh, S. T. Denham, M. Ayala, B. Brammer, M. A. Stonhill, and J. C. S. Brown, "Synergistic and antagonistic drug interactions in the treatment of systemic fungal infections," *Elife*, vol. 9, pp. 1–26, May 2020, doi: 10.7554/eLife.54160.