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Research Article

Additive *In Vitro* Antiplasmodial Effect of *N*-Alkyl and *N*-Benzyl-1,10-Phenanthroline Derivatives and Cysteine Protease Inhibitor E64

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Potential new targets for antimalarial chemotherapy include parasite proteases, which are required for several cellular functions during the *Plasmodium falciparum* life cycle. Four new derivatives of *N*-alkyl and *N*-benzyl-1,10-phenanthroline have been synthesized. Those are (1)-*N*-methyl-1,10-phenanthrolinium sulfate, (1)-*N*-ethyl-1,10-phenanthrolinium sulfate, (1)-*N*-benzyl-1,10-phenanthrolinium chloride, and (1)-*N*-benzyl-1,10-phenanthrolinium iodide. Those compounds had potential antiplasmodial activity with IC_{50} values from 260.42 to 465.38 nM. Cysteine proteinase inhibitor E64 was used to investigate the mechanism of action of *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives. A modified fixed-ratio isobologram method was used to study the *in vitro* interactions between the new compounds with either E64 or chloroquine. The interaction between *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives and E64 was additive as well as their interactions with chloroquine were also additive. Antimalarial mechanism of chloroquine is mainly on the inhibition of hemozoin formation. As the interaction of chloroquine and E64 was additive, the results indicated that these new compounds had a mechanism of action by inhibiting *Plasmodium* proteases.

1. Introduction

The erythrocytic life cycle of *Plasmodium*, which is responsible for all clinical manifestations of malaria, begins when free merozoites invade erythrocytes. The intraerythrocytic parasites develop from small ring-stage organisms to larger, more metabolically active trophozoites and then to multinucleated schizonts. The erythrocytic cycle is completed when mature schizonts rupture erythrocytes, releasing numerous invasive merozoites. Proteases appear to be required for cleavage of red blood cell ankyrin to facilitate host cell rupture and subsequent reinvasion of erythrocytes by merozoites, and for the degradation of hemoglobin ingested by intraerythrocytic trophozoites [1, 2].

Extensive evidence suggest that the degradation of hemoglobin is necessary for the growth of erythrocytic malaria parasite, apparently to provide free amino acids for parasite protein synthesis [3, 4]. In *P. falciparum*, hemoglobin degradation occurs predominantly in trophozoites and early schizonts, the stages at which the parasites are most metabolically active. Trophozoite ingest erythrocyte cytoplasm and transport it to a large central food vacuole. In the food vacuole, intact heme is released from hemoglobin to form the major component of malarial hemozoin pigment [5]. The food vacuole appears to be the site of action of a number of existing antimalarials and also offers opportunities for therapies directed against new targets. Antimalarial drugs such as chloroquine and primaquine appear to act by

preventing hemozoin formation. Other drugs, such as vinyl sulfones, act by preventing globin hydrolysis [6].

Hemoglobin degradation within the parasite is an ordered process involving at least three proteinases. Aspartic proteinase plasmepsin-I, is responsible for the initial cleavage of the hemoglobin tetramer at the hinge position, the Phe33-Leu34 bond in the α-globin chain. A second aspartic proteinase, plasmepsin-II, has also been identified and may have a role in the cleavage of denatured hemoglobin. Falcipain, a cysteine proteinase, is implicated in the cleavage of peptides from the denatured hemoglobin. The amino acids resulting from this process are presumably used by the parasite. Inhibitors of both cysteine and aspartic proteases have antimalarial effects [7, 8].

Halofantrine was an effective drug against chloroquine-resistant *P. falciparum*. The disadvantages of this drug were its variation in bioavailability and the side effect of ventricular arhythmia. To overcome those disadvantages, Yapi et al. [9] synthesized diaza-analogs of phenanthrene by substituting the two nitrogen atoms in the phenanthrene skeleton, and the 1,10-phenanthroline skeleton is the most active compound *in vitro* on both chloroquine-resistant (FcB1) and chloroquine-sensitive (Nigerian) strain with an IC_{50} of about 0.13 μM. Mustofa et al. [10] synthesized four new derivatives of *N*-alkyl and *N*-benzyl-1,10-phenanthroline: (1)-*N*-methyl-1,10-phenanthrolinium sulfate, (1)-*N*-ethyl-1,10-phenanthrolinium sulfate, (1)-*N*-benzyl-1,10-phenanthrolinium chloride, and (1)-*N*-benzyl-1,10-phenanthrolinium iodide. The *in vitro* antiplasmodial activity of those new compounds showed that four derivatives are active against *P. falciparum* FCR3 and D10 strains with an IC_{50} of 0.13–0.79 μM while the *in vivo* antiplasmodial activity against *P. berghei* in Swiss mice has an ED_{50} of 2.08–50.93 mg/kg [11, 12]. The activity of *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives on hemozoin formation inhibitory activity is lower than that of chloroquine [13]. The mechanism of action of halofantrine has been identified as at hemoglobin degradation, and the interaction between halofantrine and proteinase inhibitor Ro40-4388 and E64 are antagonistic [7]. Based on these results, further study was done to investigate the mechanism of action of those new compounds on the protease enzymes of *P. falciparum* FCR3 *in vitro*.

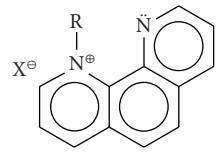
2. Materials and Methods

2.1. Molecules Tested. Four derivatives of *N*-alkyl and *N*-benzyl-1-10-phenanthroline were evaluated for their mechanism in inhibiting *P. falciparum* proteases *in vitro*. The four derivatives of *N*-alkyl and *N*-benzyl-1-10-phenanthroline have been synthesized by Mustofa et al. [14] and Hadanu et al. [15]. Identification of the compounds was carried out by means of infrared (IR) spectroscopy, proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, carbon nuclear magnetic resonance (^{13}C-NMR) spectroscopy and mass spectroscopy (MS). Quantitative structure-activity relationship (QSAR) of these 1,0-phenanthroline derivatives was also investigated. On the basis of the QSAR studies, there was a correlation between antiplasmodial activity and electronic parameters as represented by a linear function of activity versus atomic net charge of certain atoms on the 1,0-phenanthroline skeleton especially N-1 atom. Each molecule was different at the substituent on nitrogen atom in position 1 of the 1,10-phenanthroline skeleton (Figure 1). Cysteine proteinase, transepoxysuccinyl-L-leucylamido-(4-guanido)-butane (E-64) was purchased from Sigma and chloroquine diphosphate was obtained from Konimex-Indonesia. In this study, the mechanism of action of those compounds on the inhibition of *P. falciparum* protease *in vitro* was evaluated.

2.2. Parasite Cultivation. *P. falciparum* FCR3 was continuously cultured and maintained by standard methods [16] with type O erythrocytes suspended in complete culture medium (pH 7.3), which consisted of filtered sterilized RPMI 1640 solution supplemented with 500 mg of gentamycin, 2 g of sodium bicarbonate, 6.2 g of HEPES per liter, and 10% type-O human serum. Incubation was in a candle jar at 37°C under an atmosphere of 5% CO_2. The level of parasitemia in the culture was kept between 2–5% with 5% hematocrit. Parasite synchrony was maintained by serial treatments with 5% sorbitol.

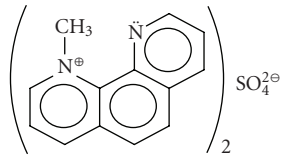
2.3. In Vitro Antiplasmodial Activity. Drug sensitivity assay was carried out in 96-well microtitration plates. *In vitro* antiplasmodial activity was determined as described by Contreras et al. [17] and Lebbad [18]. Drugs were dissolved in dimethyl sulfoxide and prediluted with serum-free medium. Dose response assay was carried out to obtain the 50% inhibitory concentration (IC_{50}) of the individual drugs. Microplate was preincubated with 100 uL of serially diluted test drugs. Ring stage-infected erythrocytes (100 μL per well with 3% hematocrit and 2% parasitemia) were incubated in triplicate with twofold serial dilution of each drug for 72 hours. Each experiment was performed in duplicate separate experiment. Parasitemia was measured microscopically on thin smear stained with 5% Giemsa. The number of parasitized red cells was counted in approximately 1,000 red cells and divided by 10 to calculate the percentage (%) of parasitized cells. Red cells infected with any stages of *Plasmodium* were counted as infected red cells. Growth inhibition was expressed as percent parasitemia compared with untreated control. Drug IC_{50} value was calculated from the log of the drug concentration-response relationship.

2.4. In Vitro Drug Combination Assay. Analysis of the combination effects of *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives with protease inhibitor E64, and interaction with chloroquine were determined by a modified fixed-ratio isobologram method [7, 8, 19]. *In vitro* antiplasmodial activity of each compound was determined before drug combination assay. The fractional inhibitory concentration (FIC; FIC = IC_{50} of drug in the combination/IC_{50} of drug when tested alone) of each drug was calculated and plotted as an isobologram. The isobologram analysis evaluates the

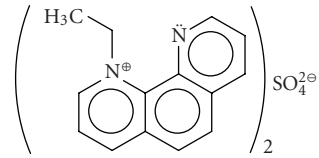


1,10- phenanthroline skeleton

(a)

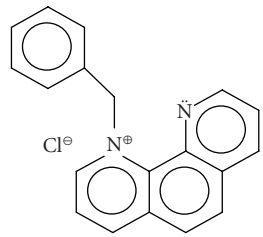


(1)-*N*-methyl-1,10-phenanthrolinium sulfate

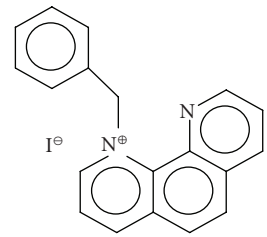


(1)-*N*-ethyl-1,10-phenanthrolinium sulfate

(b)



(1)-*N*-benzyl-1,10-phenantrolinium chloride



(1)-*N*-benzyl-1,10-phenanthrolinium iodide

(c)

Figure 1: *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives.

nature of interaction of two drugs, that is, drug A and drug B required to produce a defined single-agent effect (e.g., IC_{50}), when used as single agents, are placed on the x and y axes in a two-coordinate plot, corresponding to (C_A, 0) and (0, C_B), respectively. The line connecting these two points is the line of additivity. The concentrations of the two drugs used in combination to provide the same effect, denoted as (C_A, C_B), are placed in the same plot. Synergy, additivity, and antagonism are indicated when (C_A, C_B) is located below (concave line), a straight line and above (convex line), respectively. Combination Index (CI), similar to isobologram analysis, provides qualitative information on the nature of drug interaction, and CI, a numerical value calculated as described below, also provides quantitative measure of the extent of drug interaction. Drug combinations of either *N*-alkyl and *N*-benzyl-1,10- phenanthroline derivatives and E64 or chloroquine were expressed as the sum of the fractional inhibitory concentration (Σ FIC) or combination index (CI), according to method of Zhao et al. [20]

$$\mathrm{CI} = \frac{C_{A.x}}{IC_{x.A}} + \frac{C_{B.x}}{IC_{x.B}}. \quad (1)$$

$C_{A.x}$ and $C_{B.x}$ are the concentrations of drug A and drug B used in combination to achieve x% drug effect. $IC_{x.A}$ and $IC_{x.B}$ are the concentrations for single agents to achieve the same effect. $\sum$ FIC values were defined as synergism (<0.5), antagonism (>4), and additive (unity) [21].

Drug concentrations for these assay were 0–1,300 nM for *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives; 0–3,000 nM and 0–38.75 nM for E64 and chloroquine, respectively. Each combination was serially diluted and processed as for the sensitivity assay, therefore allowing IC_{50} to be calculated. To validate the effect, the activity of chloroquine in combination with E64 was assayed as a positive control. Layout of drug combination assay in 96-well plate is shown in Figure 2.

3. Results and Discussion

3.1. In Vitro Sensitivity of the Parasites to Antimalarial Drugs and Proteinase Inhibitor. The IC_{50} data for all *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives tested, chloroquine diphosphate and E64 against *P. falciparum* FCR3 are shown in Table 1. The ability of chloroquine to inhibit parasite growth was shown to be more potent than that of *N*-alkyl and *N*-benzyl-1-10 phenanthroline derivatives. Chloroquine diphosphate was used to identify the sensitivity of *P. falciparum* FCR3. As the IC_{50} was 24.28 ± 3.15 nM, therefore *P. falciparum* FCR3 used in this study was sensitive against chloroquine.

	1	2	3	4	5	6	7	8	9	10	11	12
A	O	O	O	O	O	O	O	O	O	O	O	
B	O										O	
C	O										O	
D	O										O	
E	O										O	
F	O	1	1	1	2	2	2	3	3	3		
G	O	•	•	•	•	•	•	•	•	•	O	
H	O	O	O	O	O	O	O	O	O	O	O	
F_2		4	4	4	5	5	5	6	6	6		

Combination solution	Ratio of the drug	
	Drug A	Drug B
1	5	0
2	4	1
3	3	2
4	2	3
5	1	4
6	0	5

Figure 2: Layout of a combination experiment in a 96-well plate with the concentration ratios of drug A and drug B prepared as six solutions. When plates were prepared as described in the text, clear wells serve as aquadest, dot black wells serve as a parasite control (no drug, 0% growth inhibition), and wells labeled 1 to 6 serve as drug wells for six drug combination solution, in triplicate, with the wells in row F holding the lowest drug concentration. Both 96-well plates were prepared similarly, with row F_2 representing solutions 4 to 6 in the second 96-well plate. Drug A was (1)-*N*-alkyl and (1)-*N*-benzyl-1,10-phenanthroline derivatives, drug B was either E64 or chloroquine.

Table 1: *In vitro* antiplasmodial activity of *N*-alkyl and *N*-benzyl-1-10 phenanthroline derivatives, chloroquine diphosphate and E64 against *P. falciparum* FCR3.

Compound	Mean IC_{50} (nM) ± SD
(1)-*N*-methyl-1,10-phenanthrolinium sulfate	260 ± 40
(1)-*N*-ethyl-1,10-phenanthrolinium sulfate	465 ± 57
(1)-*N*-benzyl-1,10-phenanthrolinium chloride	328 ± 44
(1)-*N*-benzyl-1,10-phenanthrolinium iodide	273 ± 27
Chloroquine diphosphate	24 ± 3
E64	836 ± 13

3.2. Interaction between N-Alkyl and N-Benzyl-1-10 Phenanthroline Derivatives and E64. Isobolograms for *N*-alkyl and *N*-benzyl-1-10 phenanthroline derivatives-proteinase inhibitor E64 are shown in Figure 3. The combination between *N*-alkyl and *N*-benzyl-1-10 phenanthroline derivatives and E64 against the FCR3 isolate demonstrated additive antimalarial effect.

The isobologram describing the cooperative inhibition of parasite growth by *N*-alkyl and *N*-benzyl-1-10 phenanthroline derivatives and proteinase inhibitor E64 showed a slightly concave line and on the line of additivity, indicating an additive effect. The combination index (CI) values ranged from 0.89 ± 0.18 to 1.07 ± 0.18 (Table 2). Thus, the interactions between (1)-*N*-methyl-1,10-phenanthrolinium sulfate; (1)-*N*-ethyl-1,10-phenanthrolinium sulfate; (1)-*N*-benzyl-1,10-phenanthrolinium chloride, and (1)-*N*-benzyl-1,10-phenanthrolinium iodide with E64 were additive.

3.3. Interaction between N-Alkyl and N-Benzyl-1-10 Phenanthroline Derivatives and Chloroquine. Isobolograms for *N*-alkyl and *N*-benzyl-1-10 phenanthroline derivatives and chloroquine are shown in Figure 4. The interaction of those compounds against FCR3 isolate were additive with CI values ranging from 0.89 ± 0.18 to 1.07 ± 0.18 (Table 2). Chloroquine and protease inhibitor E64 in this study interacted additivily against *P. falciparum* with CI value 1.17 ± 0.19. The isobologram describing the additive inhibition of parasite growth by *N*-alkyl and *N*-benzyl-1-10 phenanthroline derivatives and chloroquine showed slightly convex line.

E64 is an irreversible cysteine protease inhibitor that inhibit *Plasmodium* growth at the trophozoite stage, causing accumulation of undegraded hemoglobin in the food vacuole. Additivity of *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives and cysteine protease inhibitor E64 has been proposed for those new compounds. This inhibition has an effect on the source of amino acids needed for *Plasmodium* growth.

The observations of an additive effect between chloroquine and cysteine protease inhibitor E64 or *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives would lead to both reduction of hemozoin and inhibition of cysteine proteases, such as those involved in host cell invasion by merozoites [2]. Mungthin et al. [7] observed an antagonism between cysteine protease inhibitor E64 and chloroquine, amodiaquine, quinine, mefloquine, and halofantrine. This different result could be caused by the different criteria used to interpret CI value. They used criteria of CI of less than, equal to, and more than 1 to indicate synergy, additive, and antagonism, respectively.

The IC_{50} of protease inhibitor E64 was 836 ± 13 nM. This result indicated that E64 inhibited the *Plasmodium* protease enzymes. Similar study conducted by Mungthin et al. [7], showed the IC_{50} of E64 against chloroquine-resistant isolate *P. falciparum* K1 and chloroquine-sensitive isolate *P. falciparum* HB3 of 8,932 ± 744 nM and 9,65 ± 1,677 nM, respectively. The effect of cysteine protease inhibitor E64 against chloroquine-resistant *P. falciparum* W2 and chloroquine-sensitive *P. falciparum* D6 was also performed by Semenov et al. [8] and the IC_{50} values are about 8,000 nM. The IC_{50} of E64 on our study was much lower than the previous studies and this could be due to the differences in *P. falciparum* isolates used for assay. Based on those studies it showed that the effect of protease inhibitor E64 is not influenced by the chloroquine sensitivity of *P. falciparum*.

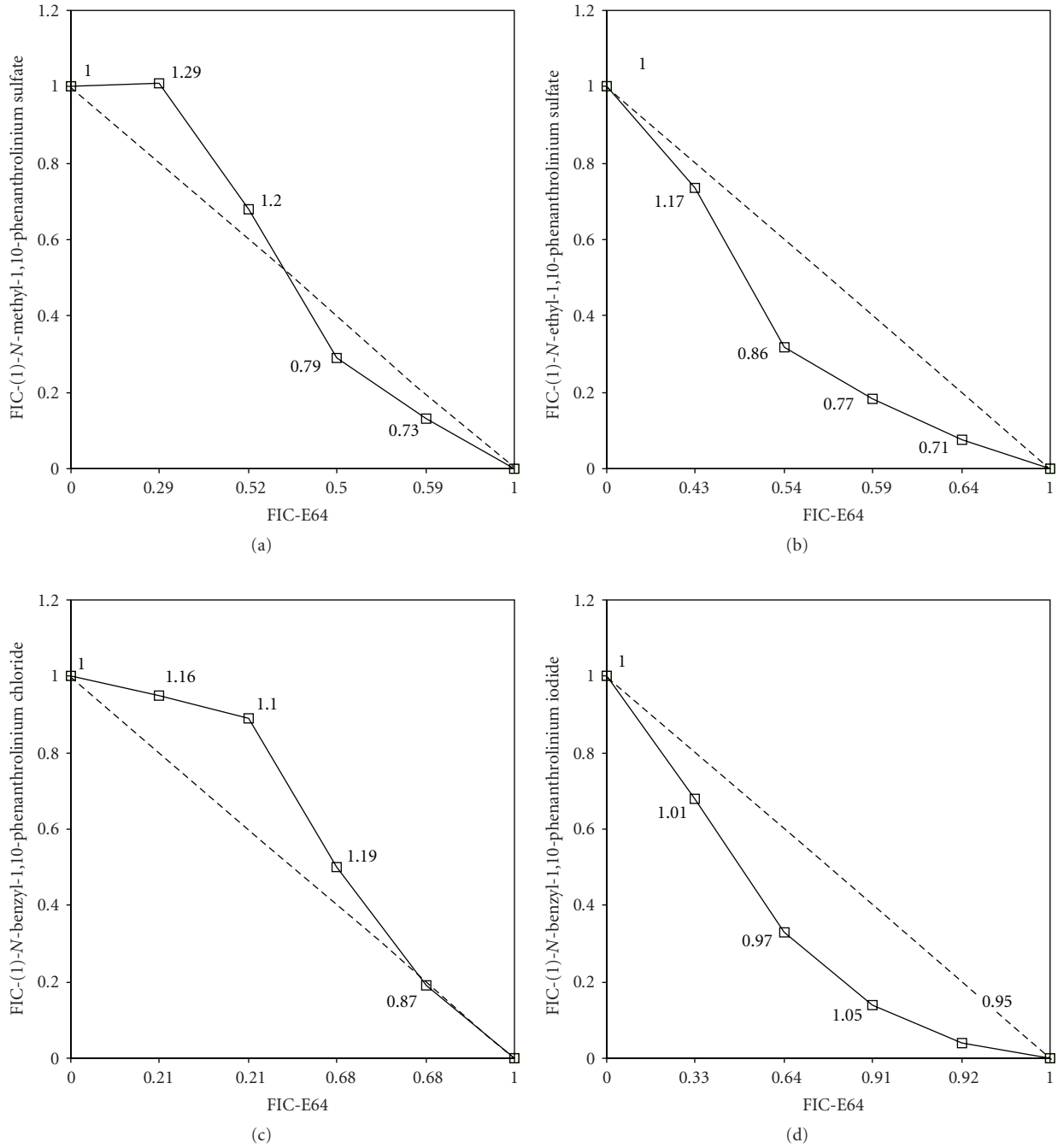


Figure 3: Isobologram showing the relationship between the FICs of E64 and *N*-alkyl and *N*-benzyl-1-10 phenanthroline derivatives against *Plasmodium falciparum* FCR3.

Based on this study we concluded that the interaction between *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives and E64 was additive as well as their interaction with chloroquine. This result is different from the previous study by Mungthin et al. [7] that showed an antagonistic interaction between halofantrine and proteinase inhibitor E64. 1,10-Phenanthroline derivatives have phenanthrene skeleton with substitution of two nitrogen atoms and alkylation's or benzylation on N-1 atom. Different structures between phenanthroline and 1,10-phenanthroline derivatives seem to contribute to the different mechanisms of action of those compounds.

Combination drug regimens for the treatment of malaria often achieve a therapeutic efficacy greater than that achieved with monotherapy. Other benefits may include decreased toxicity, delay, or prevention of drug resistance development, and favorable effects of synergistic drug interaction [22]. *N*-alkyl and *N*-benzyl-1,10-phenantroline derivatives and protease inhibitor E64 appear to act cooperatively to inhibit hemoglobin degradation and merozoites reinvasion. Based

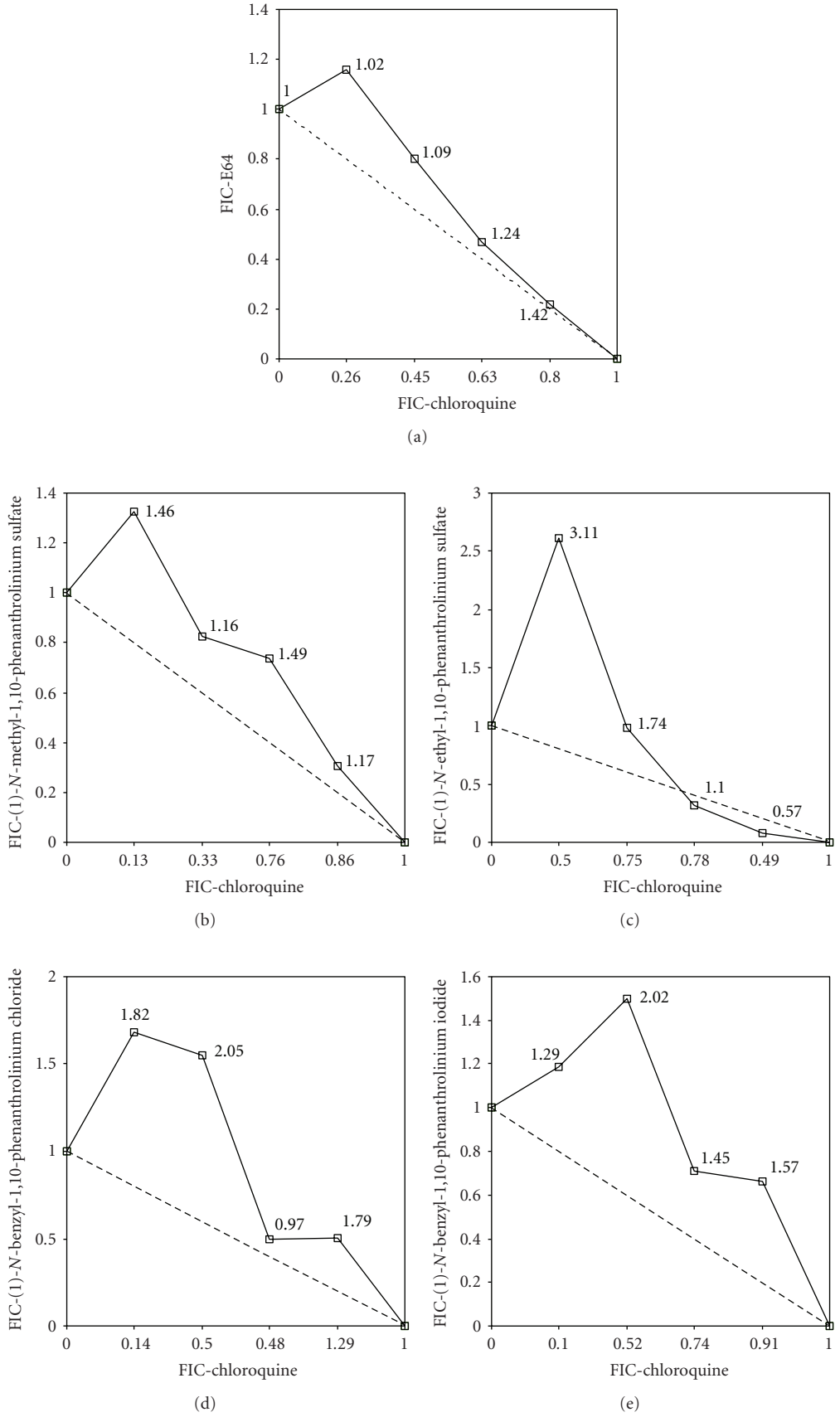


Figure 4: Isobologram showing the relationship between the FICs of chloroquine and *N*-alkyl and *N*-benzyl-1-10 phenanthroline derivatives against *Plasmodium falciparum* FCR3.

Table 2: Combination index of *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives with E64 or with chloroquine on *P. falciparum* FCR3 *in vitro*.

Compound combination			Combination Index (CI)	Interaction
(1)-*N*-methyl-1,10-phenanthrolinium sulfate	--	E64	1.00 ± 0.26	Additive
(1)-*N*-ethyl-1,10-phenanthrolinium sulfate	--	E64	0.89 ± 0.18	Additive
(1)-*N*-benzyl-1,10-phenanthrolinium chloride	--	E64	1.07 ± 0.18	Additive
(1)-*N*-benzyl-1,10-phenanthrolinium iodide	--	E64	0.99 ± 0.03	Additive
Chloroquine	--	E64	1.17 ± 0.19	Additive
(1)-*N*-methyl-1,10-phenanthrolinium sulfate	--	Chloroquine	1.28 ± 0.30	Additive
(1)-*N*-ethyl-1,10-phenanthrolinium sulfate	--	Chloroquine	1.54 ± 0.96	Additive
(1)-*N*-benzyl-1,10-phenanthrolinium chloride	--	Chloroquine	1.56 ± 0.58	Additive
(1)-*N*-benzyl-1,10-phenanthrolinium iodide	--	Chloroquine	1.53 ± 0.44	Additive

on this study it may be appropriate to use combinations of such inhibitors to treat malaria. This study has only identified the mechanism of action of *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives on *P. falciparum* FCR3, further studies should be performed with other chloroquine-resistant and -sensitive isolates in order to obtain more comprehensive information concerning the mechanism of action of these phenanthroline compounds.

Acknowledgments

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