IN VITRO ANTIPLASMODIAL ACTIVITY AND CYTOTOXICITY OF NEWLY SYNTHESIZED *N*-ALKYL AND *N*-BENZYL-1,10-PHENANTHROLINE DERIVATIVES

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Abstract. A previous study showed that the 1,10-phenanthroline skeleton was active *in vitro* against chloroquine-resistant and sensitive strains of *Plasmodium falciparum*. Based on this skeleton, 8 derivatives of *N*-alkyl and *N*-benzyl-1,10-phenanthrolines have been synthesized. This study was conducted to evaluate the *in vitro* antiplasmodial activity and cytotoxicity of these compounds. The *in vitro* antiplasmodial activity was tested on two strains of *P. falciparum,* FCR-3 chloroquine-resistant and D10 chloroquine-sensitive strains, while their cytotoxicity was tested on the Vero cell line. The parasite and cell growth were estimated by hypoxantine-[2,8-^{3}H] uptake after 24- and 72-hour incubation with each compound tested. The control parasite or cell free from any compounds was referred to as having 100% growth. For this radioactive method, the IC_{50} value showing concentration inhibiting 50% of the parasite growth was determined by probit analysis. The results showed that the highest antiplasmodial activity was observed with (1)-*N*-benzyl-1,10-phenanthrolinium iodide with the IC_{50} 0.18-0.45 µM, and the IC_{50} of the compound on Vero cells ranged from 2,582.30 to 7,057.71 µM. The cytotoxic/antiplasmodial ratio indicates that this compound has high selectivity (10,993 ± 330.79-38,965 ± 6,888.27).

INTRODUCTION

Malaria is by far the world's most important tropical disease. It has been estimated that 1.5-2.7 million people die due to malaria infection annually and nearly 500 million are infected, especially children under five years old and pregnant women (WHO, 1998). Malaria also imposes a huge economic burden on countries where the disease is rife. The management of malaria has become a major concern to health care providers because of the increasing wave of resistance to old and new antimalarial drugs (Winstanley, 2000).

Chloroquine-resistant *P. falciparum* has spread widely and quickly to almost all malaria endemic countries (Winstanley, 2000). Chloroquine is the most common first-line drug for malaria in some countries of the world and in Indonesia. Based on data from the Ministry of Health, Republic of Indonesia in 2003, the annual incidence of *P. falciparum* infection in Java-Bali was 0.22 among 1,000 population while the annual malaria incidence in outer Java-Bali was 21.8 among 1,000 population. Chloroquine resistance is widespread in some endemic areas of Indonesia and the percentage of resistance varies from 10-97% (TDC, 2004).

Halofantrine is an effective drug against

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chloroquine-resistant *P. falciparum*. However, the drug is incompletely and variably absorbed, being more bioavailable if taken with fatty food. Prolongation of the QT interval on electrocardiograph, a risk factor for ventricular arrythmias, has been documented in patients taking halofantrine (Winstanley, 2000).

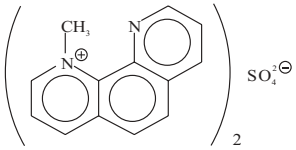
Based on disadvantages of halofantrine, Yapi *et al* (2000) have synthesized diaza-analogs of phenanthrene derived from 3-amino, 5-amino, 6-amino, 8-aminoquinoline and 5-aminoisoquinoline and have tested their *in vitro* antiplasmodial activity. The results showed that of the molecules tested, the 1,10-phenanthroline skeleton was the most active compound against both chloroquine-resistant (FcB1) and sensitive (Nigerian) strains *in vitro* with an IC_{50} of about 0.13 µM. Based on this skeleton, Mustofa *et al* (2003) synthesized thirteen derivatives of 1,10-phenanthroline and evaluated their *in vitro* antiplasmodial activities and structure-activity relationship. Based on the structure activity relationship model, 8 new compounds of *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives were synthesized (Hadanu, 2004; Supargiyono *et al*, 2004; Mustofa *et al*, 2005): 1) (1)-*N*-methyl-1,10-phenanthrolinium sulfate, 2) (1)-*N*-ethyl-1,10-phenanthrolinium sulfate, 3) (1)- *N*-t-butyl phenanthroline chloride, 4) (1)-*N*-benzyl-1,10-phenanthrolinium chloride, 5) (1)-*N*-benzyl-1,10-phenanthrolinium bromide, 6) (1)-*N*-benzyl-1,10-phenanthrolinium iodide, 7) (1)-*N*-(4-methoxy-benzyl)-1,10-phenanthrolinium chloride, and 8) (1)-*N*-(4-benzyloxy-3-methoxy-benzyl)-1,10-phenanthrolinium chloride. This study was conducted to evaluate the *in vitro* antiplasmodial activity and cytotoxicity of the *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives.

MATERIALS AND METHODS

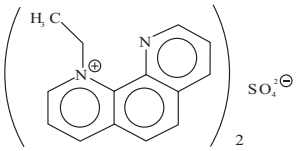
Eight derivatives of *N*-alkyl and *N*-benzyl-1,10-phenanthrolines were synthesized by Hadanu (2004), Supargiyono *et al* (2004), and Mustofa *et al* (2005), and the chemical structure is shown in Fig 1.

Two strains of chloroquine-resistant *P. falciparum*, FCR-3 (IC_{50}>100 nM) and chloroquine-sensitive, D10 strain (IC_{50}<100 nM) were obtained from Eijkman Institute for Molecular Biology, Jakarta. Parasites were cultured according to the modified method described by Trager and Jensen (1976). The parasites were maintained *in vitro* in human red blood cells ($O^{\pm}$), diluted to 1-2% hematocrit in RPMI-1640 medium (Sigma-Aldrich, USA), supplemented with 25mM HEPES (Sigma Chemical, USA) and 30 mM $NaHCO_3$ (Sigma-Aldrich, USA) and supplemented with 5% human $O^{\pm}$ serum. Parasite cultures were synchronized by 5% of D-sorbitol (Sigma-Aldrich, USA) in distilled water as reported by Lambros and Vanderberg (1979). The method used for *in vitro* antiplasmodial activity testing was adapted from a radioactive method (Desjardins *et al*, 1979). The compounds were tested in triplicate in 96-well plate (Nunclon™, Germany) cultures at a ring stage of 2% parasitemia (with 3% hematocrit). For each test, the parasite cultures were incubated with the compounds at decreasing concentrations for 24 and 72 hours. The first concentration of the compound (10 mg/ml) was dissolved in dimethyl sulfoxide (Merck, Germany) and then diluted with RPMI-1640 medium. Parasite growth was estimated using hypoxantine-(2,8-^{3}H) (Sigma-Aldrich, USA) uptake. The control parasite free from any compounds was referred to as having 100% growth. Concentrations inhibiting 50% of the parasite were determined by probit analysis using SPSS software.

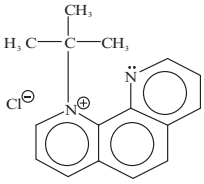
Cytoxicity of the compounds was assessed against the Vero cell line (kidney cells from the African green monkey) obtained from Integrated Research and Testing Laboratory, Gadjah Mada University, Indonesia. The Vero cell line was cultured in M199 medium (Gibco, Auckland) containing 10% fetal bovine serum



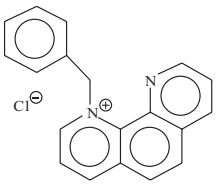
1) (1)-*N*-methyl-1,10-phenanthrolinium sulfate



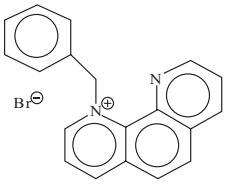
2) (1)-*N*-ethyl-1,10-phenanthrolinium sulfate



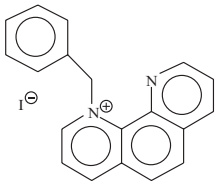
3) (1)- *N*-t-butyl phenanthroline chloride



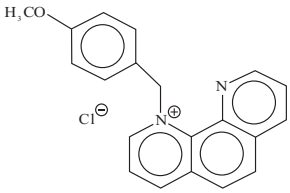
4) (1)-*N*-benzyl-1,10-phenanthrolinium chloride



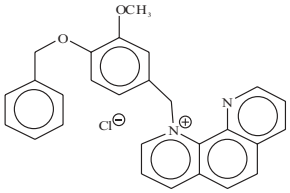
5) (1)-*N*-benzyl-1,10-phenanthrolinium bromide



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



7) (1)-*N*-(4-methoxy-benzyl)-1,10-phenanthrolinium chloride



8) (1)-*N*-(4-benzyloxy-3-methoxy-benzyl)-1,10-phenanthrolinium chloride

Fig 1–Eight derivatives of *N*-alkyl and *N*-benzyl-1, 10-phenanthroline.

(Sigma-Aldrich, USA). Subcultures were obtained after treatment with 0.125% trypsin (Gibco, Auckland) in phosphate buffer saline. To determine cytotoxicity, Vero cells were cultured in 96-well plates at 1.5 x 10^4 cells/well in 100 µl medium. One hundred µl of solution was added at various concentrations. Cell growth was estimated by the uptake of hypoxantine-(2,8-^{3}H) at 24 and 72 hours incubation and was compared with the control cultures without compounds.

RESULTS

Two strains of *P. falciparum* were used to evaluate the *in vitro* antiplasmodial activities of compounds (1)-(8): the chloroquine-resistant FCR3 and sensitive D10 strains. The results are summarized in Table 1. Of the 8 compounds tested, compounds (5) and (6) had the highest activity (IC_{50}= 0.10-0.13 and 0.18-0.23 µM, respectively) against FCR-3. Compound (6) had the highest activity (IC_{50} = 0.33-0.34-µM) on D10 *P. falciparum*.

The *in vitro* cytotoxicity assay on Vero cells and calculation of the cytotoxic/antiplasmodial ratio (CAR) at 24 and 72 hours incubation time are summarized in Table 2. All the compounds had low cytotoxity toward the Vero cells, and compound (3) had the lowest cytotoxic/antiplasmodial ratio (24.19-141.26). The other compounds had higher cytotoxic/antiplasmodial ratios, probably an indication of high selectivity.

DISCUSSION

The increasing spread of drug resistant malaria motivates to develop new, more sensitive antimalarial agents. One of these agents is phenanthrene, which when developed into halofantrine is more active than phenanthene. Halofanthrine is active against strains of *P. falciparum* that are resistant to chloroquine, pyrimethamine and quinine (Rang *et al*, 2003). However, halofanthine is known to have some unwanted side effects, such as abdominal pain, nausea, vomiting, diarrhea, orthostatic hypotension, prolongation of QTc intervals, pru-

Table 1
IC_{50} (μM) of *N*-alkyl and *N*-benzyl 1,10-phenanthrolines derivatives against FCR-3 and D10 of *P. falciparum in vitro.*

Compound	Incubation time			
	FCR-3		D10	
	24 hours	72 hours	24 hours	72 hours
(1) (1)-*N*-methyl-1,10-phenanthrolinium sulfate	0.79 ± 0.16	0.32 ± 0.002	0.59 ± 0.43	0.13 ± 0.002
(2) (1)-*N*-ethyl-1,10-phenanthrolinium sulfate	0.42 ± 0.06	0.29 ± 0.14	0.41 ± 0.001	0.15 ± 0.02
(3) (1)- *N*-t-butyl phenanthroline chloride	1.84 ± 0.21	2.09 ± 0.08	7.15 ± 1.36	2.24 ± 0.05
(4) (1)-*N*-benzyl-1,10-phenanthrolinium chloride	0.57 ± 0.004	0.36 ± 0.03	0.54 ± 0.06	0.14 ± 0.004
(5) (1)-*N*-benzyl-1,10-phenanthrolinium bromide	0.13 ± 0.06	0.10 ± 0.04	0.86 ± 0.57	0.74 ± 0.20
(6) (1)-*N*-benzyl-1,10-phenanthrolinium iodide	0.23 ± 0.007	0.18 ± 0.003	0.33 ± 0.07	0.34 ± 0.07
(7) (1)-*N*-(4-methoxy-benzyl)-1, 10-phenanthrolinium chloride	0.23 ± 0.004	0.33 ± 0.03	3.17 ± 2.79	3.60 ± 1.36
(8) (1)-*N*-(4-benzyloxy-3-methoxy-benzyl)-1, 10-phenanthrolinium chloride	1.19 ± 0.07	0.30 ± 0.007	2.19 ± 0.18	1.08 ± 0.02

ritus, rash (Karbwang and Na-Bangchang, 1994), and hepatotoxicity (Bassi *et al*, 2006). Halofantrine also has large intersubject variability in plasma drug concentrations due to its poor and inconsistent bioavailability. This may be the cause of causing some treatment failures rather than true resistance (Karbwang *et al*, 1991; Karbwang and Na-Bangchang, 1994). The disadvantages of halofantrine limited its use. Halofantrine is used in only a few countries, especially in Africa. In order to obtain a more active antimalarial compound from phenanthrene derivatives with a lower toxic effect, we modified the phenanthrene skeleton and obtained 1,10-phenanthroline, which has the potential to be developed into an antimalarial agents.

The 1,10-phenantroline ring system is well known for its metalloprotease inhibition activities by chelating divalent metal ions. As a chelating metal compound, 1,10-phenantroline has been used as an antimicrobial agent against bacterial species, such as *Prevotella ruminicola, Fibrobacter succinogenes, Lachnospira multipara* and *Megasphaera elsdenii* (Wallace *et al*, 1996). Other chelating metal compounds, such as salycylaldehyde isonicotinoyl hydrazone and 2-hydroxy-1-naphthylaldehyde m-fluorobenzoylhydrazone had been promoted as antimalarial compounds (Tsafack *et al*, 1996).

A 1,10-phenantroline was reported by Yapi *et al* (2000) to be an antimalarial compound (2000) after evaluating 13 of 1,10-phenantroline derivatives. The antiplasmodial activity of the 1,10-phenanthrolines is increased when the two pyridinic rings are joined toward the phenyl ring (phenanthrolines), especially in the case of the 1,10-phenanthroline ring system. Yapi *et al* (2000) demonstrated that the antiplasmodial activity of these compounds does not correspond with its chelating capacity in the metalloprotease inhibition process, and it is different from their antimalarial activity as showed by Scheibel and Adler (1981, 1982). Its antiplasmodial activity was demonstrated by blockade of the potential chelating site by *N*-alkylation of 1,10-phenantroline.

In our research, the chelating capacity of 1,10-phenantroline was blocked by *N*-10 alkylation and *N*-10 benzylation. Of the 8 compounds tested, compounds (5) and (6) had the

Table 2
IC_{50} of *N*-alkyl and *N*-benzyl-1,10-phenanthrolines derivatives on the Vero cell line and cytotoxic/antiplasmodial ratio against FCR-3 and D10 of *P. falciparum*.

Compound	IC_{50} (μM) on Vero cell line		CAR[a]			
			FCR-3		D10	
	24 hours	72 hours	24 hours	72 hours	24 hours	72 hours
(1)	859.50 ± 26.16	3,200,900 ± 45,227	1,108.13 ± 208.54	10,028,966 ± 628,036	2,012.32 ± 1,495.27	25,219,219 ± 5,486,978
(2)	376.56 ± 185.12	570.00 ± 113.13	908.31 ± 121.76	1,429.30 ± 97.74	912.98 ± 2.65	3,828.68 ± 692.25
(3)	168.81 ± 112.79	295.40 ± 108.07	92.20 ± 10.5	141.26 ± 5.73	24.19 ± 4.84	131.73 ± 3.27
(4)	4,216.58 ± 288.30	474,475 ± 421.02	7,389.32 ± 634.00	131,475 ± 12,592	7,789.95 ± 909.63	328,739 ± 11,260
(5)	> 10,000	94,740 ± 19,154	> 50,000	907,623 ± 42,990	> 6,000	134,139 ± 33,434
(6)	2,582.30 ± 747.78	7,057.71 ± 3,143.10	10,993 ± 330.79	38,965 ± 6,888.27	8,091.77 ± 1,690.56	21,216 ± 4,412.51
(7)	> 10,000	13642 ± 9,283.35	> 30,000	40,777 ± 4,370.10	> 1,500	4,121.22 ± 1,349.04
(8)	> 10,000	5,069.97 ± 1,233.44	> 8,000	1.66×10^4 ± 388.03	> 4,000	4,683.38 ± 118.67

[a]Cytotoxic/antiplasmodial ratio against FCR-3 and D10 calculated at 24 hours and 72 hours of incubation time

highest activity against FCR-3 *P. falciparum*. Compound (6) had the highest activity against D10 *P. falciparum*. However, all the compounds were less active against the two strains than chloroquine itself (Table 1). Based on structure, compounds (5) and (6) are more nonpolar then compounds (1), (2), and (3) because of the existence of benzyl moieties in the structure. These nonpolar compounds may penetrate cell membranes more easily than the polar ones. When compared to compound (4) which exists as a chloride salt, compounds (5) and (6) should be more effective in interacting with cell membranes as these two compounds posses softer anion conjugates (Br^- and I^-). Likewise, the antimalarial activities of compounds (7) and (8) are lower than those compounds (5) and (6) as the molecular size of compounds (7) and (8) may be too bulky to interact with the cell membrane.

In order to evaluate their toxicity, all 8 compounds were tested on the Vero cell line. Toxicity testing showed that all the compounds had low cytotoxity against the Vero cell line, however compound (3) had the lowest cytotoxic/antiplasmodial ratio (24.19-141.26) (Tabel 2). The other compounds showed higher cytotoxic/antiplasmodial ratios which indicates their high selectivity.

In conclusion, of the eight compounds tested, (1)-*N*-benzyl-1,10- phenanthrolinium iodide (compound no. 6) had the highest antiplasmodial activity against *P. falciparum* with a high selectivity which indicates it may have the potential to be used as an antiplasmodial compound and should be studied further. Studies concerning the mechanism of action for *in vivo* antiplasmodial activity in mice and monkeys, and also its pharmacokinetic in rats are being conducted.

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