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
chloroquine-resistant \textit{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 arrhythmias, has been documented in patients taking halofantrine (Winstanley, 2000).

Based on disadvantages of halofantrine, Yapi et al (2000) have synthesized diaza-analogues of phenanthrene derived from 3-amino, 5-amino, 6-amino, 8-aminquinoline and 5-aminisoquinoline and have tested their in vitro antimalarial 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 $\mu$M. Based on this skeleton, Mustofa et al (2003) synthesized thirteen derivatives of 1,10-phenanthroline and evaluated their in vitro antimalarial 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-phenanthroline sulfate, 2) (1)-N-ethyl-1,10-phenanthroline sulfate, 3) (1)-N-t-butyl phenanthroline chloride, 4) (1)-N-benzyl-1,10-phenanthroline chloride, 5) (1)-N-benzyl-1,10-phenanthroline bromide, 6) (1)-N-benzyl-1,10-phenanthroline iodide, 7) (1)-N-(4-methoxy-benzyl)-1,10-phenanthroline chloride, and 8) (1)-N-(4-benzylxy-3-methoxy-benzyl)-1,10-phenanthroline chloride. This study was conducted to evaluate the in vitro antimalarial 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 \textit{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$^+$), 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$^+$ 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 antimalarial activity testing was adapted from a radioactive method (Desjardins et al, 1979). The compounds were tested in triplicate in 96-well plate (Nunc\textsuperscript{TM}, 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.

Cytotoxicity 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.
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-^3H) 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 antimalarial 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/antimalarial ratio (CAR) at 24 and 72 hours incubation time are summarized in Table 2. All the compounds had low cytotoxicity 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 phenanthrene. Halofantrine is active against strains of P. falciparum that are resistant to chloroquine, pyrimethamine and quinine (Rang et al, 2003). However, halofantrine is known to have some unwanted side effects, such as abdominal pain, nausea, vomiting, diarrhea, orthostatic hypotension, prolongation of QTc intervals, pru-
Evaluation of N-alkyl and N-benzyl-1,10-phenanthroline Derivatives

Table 1
IC₅₀ (µM) of N-alkyl and N-benzyl 1,10-phenanthroline derivatives against FCR-3 and D10 of P. falciparum in vitro.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Incubation time</th>
<th>FCR-3</th>
<th>D10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hours</td>
<td>72 hours</td>
<td>24 hours</td>
</tr>
<tr>
<td>(1) (1)-N-methyl-1,10-phenanthroline sulfate</td>
<td>0.79 ± 0.16</td>
<td>0.32 ± 0.002</td>
<td>0.59 ± 0.43</td>
</tr>
<tr>
<td>(2) (1)-N-ethyl-1,10-phenanthroline sulfate</td>
<td>0.42 ± 0.06</td>
<td>0.29 ± 0.14</td>
<td>0.41 ± 0.001</td>
</tr>
<tr>
<td>(3) (1)-N-t-butyl phenanthroline chloride</td>
<td>1.84 ± 0.21</td>
<td>2.09 ± 0.08</td>
<td>7.15 ± 1.36</td>
</tr>
<tr>
<td>(4) (1)-N-benzyl-1,10-phenanthroline chloride</td>
<td>0.57 ± 0.004</td>
<td>0.36 ± 0.03</td>
<td>0.54 ± 0.06</td>
</tr>
<tr>
<td>(5) (1)-N-benzyl-1,10-phenanthroline bromide</td>
<td>0.13 ± 0.06</td>
<td>0.10 ± 0.04</td>
<td>0.86 ± 0.57</td>
</tr>
<tr>
<td>(6) (1)-N-benzyl-1,10-phenanthroline iodide</td>
<td>0.23 ± 0.007</td>
<td>0.18 ± 0.003</td>
<td>0.33 ± 0.07</td>
</tr>
<tr>
<td>(7) (1)-N-(4-methoxy-benzyl)-1,10-phenanthroline chloride</td>
<td>0.23 ± 0.004</td>
<td>0.33 ± 0.03</td>
<td>3.17 ± 2.79</td>
</tr>
<tr>
<td>(8) (1)-N-(4-benzyloxy-3-methoxy-benzyl)-1,10-phenanthroline chloride</td>
<td>1.19 ± 0.07</td>
<td>0.30 ± 0.007</td>
<td>2.19 ± 0.18</td>
</tr>
</tbody>
</table>

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 agent.

The 1,10-phenanthroline ring system is well known for its metalloprotease inhibition activities by chelating divalent metal ions. As a chelating metal compound, 1,10-phenanthroline 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 salicylaldehyde isonicotinoyl hydrazide and 2-hydroxy-1-naphthylaldehyde m-fluorobenzoylhydrazone had been promoted as antimalarial compounds (Tsafack et al., 1996).

A 1,10-phenanthroline was reported by Yapi et al (2000) to be an antimalarial compound after evaluating 13 of 1,10-phenanthroline 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-phenanthroline.

In our research, the chelating capacity of 1,10-phenanthroline was blocked by N-10 alkylation and N-10 benzylation. Of the 8 compounds tested, compounds (5) and (6) had the...
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highest activity against FCR-3

Compound (6) had the highest activity against D10 of P. falciparum. However, all the compounds were less active than chloroquine itself (Table 1). Based on structure, compounds (1), (2), and (3) 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 possess 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) are 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 cytotoxicity against the Vero cell line, however compound (3) had the lowest cytotoxic/antiplasmodial ratio (24.19-141.26) (Table 2). The other compounds showed higher cytotoxic/antiplasmodial ratios which indicates their high selectivity.

In conclusion, of the eight compounds tested, (1) N-alkyl-1,10-phenanthrolinium iodide (compound no. 6) had the highest antimalarial 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.

ACKNOWLEDGEMENTS

The study was funded by the Indonesian 10th Competitive Integrated Research fund.

Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC(_{50}) (µM) on Vero cell line</th>
<th>Cytotoxic/antiplasmodial ratio against FCR-3 and D10 calculated at 24 hours and 72 hours of incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hours</td>
<td>72 hours</td>
</tr>
<tr>
<td>(1)</td>
<td>859.50±26.16</td>
<td>3,200.90±45.227</td>
</tr>
<tr>
<td>(2)</td>
<td>376.56±185.12</td>
<td>570.00±113.13</td>
</tr>
<tr>
<td>(3)</td>
<td>168.81±112.79</td>
<td>295.40±108.07</td>
</tr>
<tr>
<td>(4)</td>
<td>4,216.58±288.30</td>
<td>474,475±421.02</td>
</tr>
<tr>
<td>(5)</td>
<td>&gt;10,000</td>
<td>94,740±19,154</td>
</tr>
<tr>
<td>(6)</td>
<td>2,582.30±747.78</td>
<td>7,057.71±3,143.10</td>
</tr>
<tr>
<td>(7)</td>
<td>&gt;10,000</td>
<td>13642±9,283.35</td>
</tr>
<tr>
<td>(8)</td>
<td>&gt;10,000</td>
<td>5,069.97±1,233.44</td>
</tr>
</tbody>
</table>

\(a\)Cytotoxic/antiplasmodial ratio against FCR-3 and D10 calculated at 24 hours and 72 hours of incubation time.
EVALUATION OF N-ALKYL AND N-BENZYL-1,10-PHENANTHROLINE DERIVATIVES

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