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Heme Polymerization Inhibitory Activity (HPIA) of N-alkyl and N-benzyl-1,10 Phenanthroline Derivatives as Antimalaria

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ABSTRACT

Malaria parasite metabolizes hemoglobin and detoxifies the resulting toxic ferriprotoporphyrin IX by polymerization into a crystal insoluble pigment, known as hemozoin. A polymer identical to hemozoin, β-hematin, can be obtained in vitro from hematin at acidic pH. Quinoline-containing antimalarials (e.g. chloroquine) inhibit the formation of polymer. Thus, heme polymerization is an essensial and unique pharmacological target of antimalarial drugs. Previous study on in vitro and in vivo antiplasmodial activity showed that N-alkyl and N-benzyl-1,10-phenanthroline derivatives viz. (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 were potential as antiplasmodial. This study was conducted to investigate the posibility mechanism of action of those compounds on a simple in vitro micro assay of heme polymerization. The IC_{50} HPIA of N-alkyl and N-benzyl-1,10-phenanthroline derivatives were ranging from 35.54 mM to 62.08 mM while the IC_{50} HPIA of chloroquine was 3.49 mM. Our result showed that N-alkyl and N-benzyl-1,10-phenanthroline derivatives have effect on heme polymerization inhibitory activity.

Keywords: 1,10-phenanthroline derivatives, antiplasmodial, hemozoin, β-hematin, HPIA

INTRODUCTION

Malaria has long been a major killer of human mankind, especially in the tropical and subtropical regions of the world. More than half of the world population is at risk of being infected by malaria. Earlier hospes of malaria eradication by removal of its mosquito vector had only limited success due to the development of insecticide resistance by mosquitoes. However, the major jolt to human fight against malaria has come from the emergence of drug resistant strains of Plasmodium. Chloroquine, the most widely used drug for clinical treatment of malaria has lost its efficacy mainly due to its indiscriminate use as an over the counter drug in many countries (1-2).

The malaria parasite *Plasmodium falciparum* digests a large proportion of its host cell hemoglobin during its erythrocytic cycle, presumably as a source of essential nutrients. The digestion is a complex process involving three proteases, one cystein protease (falcipain) and two aspartic proteases (plasmepsin I and II). The digestion is also though to be initiated by the action of plasmepsin I on native hemoglobin, leading to the release of the iron II ferroprotoporphyrin IX (FPIX). The free FPIX is a toxic substance, and parasite which are lacking in heme oxygenase are unable to detoxify free FPIX by metabolism. Instead, malaria parasites have evolved an autocatalytic detoxification process in which FPIX is oxidized to iron III FPIX (hematin), which is then polymerized, forming inert crystals of hemozoin or malaria pigment (3).

Chemical formation of β-hematin is a non-physiological process. High resolution X-ray crystallographic studies and other analysis have constitutively proved that hemozoin (the pigment isolated from the malaria parasite lysate) and β-hematin (the synthetic pigment) are identical (4). The formation of β-hematin in malaria parasite is a spontaneous chemical reaction. It is proposed that formation of β-hematin can occur spontaneously between $6°C$ and $65°C$ in 0.1 to 4.5M acetate and pH 4.2-5.0.

Following early observations showed that free FPIX was able to form complexes with nitrogenous bases such as pyridines and quinolines, it was hypothesized that the quinoline-containing antimalarial agents exerted their effects by forming toxic complexes with free FPIX released *in situ*. Quinoline antimalarial agents are able to inhibit the spontaneous polymerization of hematin, suggesting a mechanism by which free FPIX or FPIX-chloroquine complexes may concentrate in the food vacuole and kill the parasite (3,5).

Morphological effects following treatment with mefloquine, quinine, and halofantrine are similar to that observed following treatment with chloroquine, i.e., an initial swelling of the acid food vacuole. It is generally accepted that chloroquine exert its antimalarial effect
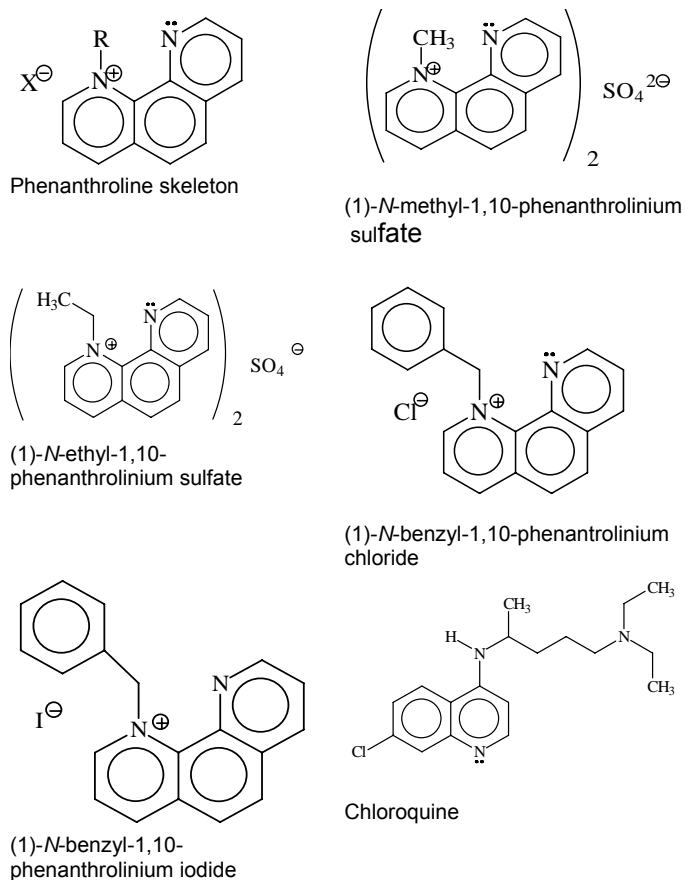
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Phenanthroline skeleton

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

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

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

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

Chloroquine

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

by interacting with the hemoglobin degradation process within the parasite, probably through an interaction with protease and heme detoxification (6-7).

Halofantrine has been identified as an effective drug against chloroquine resistant-*P. falciparum.* However this compound is remarkably expensive, and there is no parenteral formulation. In addition this compound is incompletely absorbed via the gastrointestinal tract and that the bioavailability varies. Halofantrine has also reported to prolong the electrocardiographic PR and corrected QT intervals. QT prolongation is a risk factor for ventricular arrythmias in patients consuming halofantrine (8).

Based on the disadvantages of halofantrine, Yapi *et al.*(9) have synthesized diaza-analogs of phenanthrene by substituting the two nitrogen atoms in the phenanthrene skeleton and proven that 1,10-phenanthroline skeleton was 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.*, (10-11) have synthesized four new derivatives of 1,10-phenanthroline : (1)-*N*-methyl-phenanthrolinium sulfate, (1)-*N*-ethyl-phenanthrolinium sulfate, (1)-*N*-benzyl-1,10-

phenanthrolinium chloride, and (1)-*N*-benzyl-1,10-phenanthrolinium iodide and evaluated their activity. The *in vitro* antiplasmodial activity among these compounds showed that four derivatives were active against *P. falciparum* FCR3 and D10 strains with IC_{50} ranged from 0.13 to 0.79 μM. Moreover, the *in vivo* study showed that these compound were also active against *P. berghei* on infected Swiss mice with an ED_{50} ranged from 2.08 to 50.93 mg/kg (12-13). This study was conducted to evaluate the heme polymerization inhibition activity of these compounds on a simple *in vitro* micro assay.

EXPERIMENTAL SECTION

Materials

Four derivatives of 1-10-phenanthroline were evaluated for their mechanism of action on the *in vitro* heme polymerization inhibition assay. Each molecule was different at the substituent on nitrogen atom in position 1 of the 1-10-phenanthroline skeleton (Figure 1). Hematin powder was purchased from Sigma and chloroquine diphosphate was obtained from Konimex-Indonesia. Drug stock solutions were prepared in aquadest or DMSO as required.

Procedure
***In Vitro* Heme Polimerization Inhibition Assay**

The ability of the tested compounds to inhibit heme polymerization was assessed by a modified of protocol described by Basilico *et al.* (14). The modification was that it used 1.5 mL Eppendorf tube instead of 96-well U-bottom microplates. Hematin was freshly dissolved to 4 mM in 0.2 M NaOH solution and 100 μL of this solution were mixed in an Eppendorf tube with 50 μL of glacial acetic acid and 50 μL of invarious concentration *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives or chloroquine (final concentration from 1.94 to 51.39 mM). Aquadest or dimethylsulfoxide (DMSO) was used as negative control. The final pH was 2.88 to 3.60. After 24 hours incubation at 37°C the tubes were centrifuged at 3000 rpm for 15 minutes. After discarding the supernatant, the pellet was washed three times with 200 μL DMSO. The pellet was then completely dissolved in 200 μL of 0.1 NaOH, and 100 μL of each sample was placed into 96-microwells plate and the optical density was read at 405 nm wave length with micro-ELISA reader (Bio-Rad Lab), and the β-hematin concentration was calculated using standard curve of hematin concentration against optical density value. Prior the assay, the standard curve was prepared by dissolving 4mM hematin in 0.1M NaOH and the optical density was read in micro-ELISA reader, the result was create as a standard curve by plotting the mean optical density for each standard concentration on the ordinate against the

Table 1. Inhibitory effect of N-alkyl and N-benzyl-1,10-phenanthroline derivatives and chloroquine on β-hematin formation

Compound	Substituent	Maximum inhibition (%)	IC$_{50}$ β-hematin formation (mM)
(1)-N-methyl-1,10-phenanthrolinium sulfate	-CH$_3$	94.21	46.29 ± 0.35
(1)-N-ethyl-1,10-phenanthrolinium sulfate	-CH$_2$CH$_3$	99.15	35.54 ± 0.42
(1)-N-benzyl-1,10-phenanthrolinium chloride	PhCH$_2$-	62.25	62.08 ± 10.24
(1)-N-benzyl-1,10-phenanthrolinium iodide	PhCH$_2$-	62.26	47.55 ± 14.94
Chloroquine	-	99.09	3.49 ± 0.26

hematin concentration on the abscissa. Linear regression of the standard curve value was Y=0.061 + 0.008X, R^2= 0.998. The heme polymerization inhibition activity was expressed as the percentage of inhibition calculated by the following equation :

% inhibition = 100 x (1-(A/B)

A = Concentration of β-hematin in tested compound.
B = Concentration of β-hematin in negative control.

The data are expressed as the molar equivalents of test compounds required to inhibit heme polymerization by 50% (IC$_{50}$).

RESULT AND DISCUSSION

An *in vitro* assay was used to asses the abilities of the N-alkyl and N-benzyl-1,10-phenanthroline derivatives compounds to inhibit β-hematin formation. In the assay hematin was allowed to form β-hematin under acidic condition. All the heme polymerization inhibition activity of the four tested compounds were calculated using the standard curve as the reference. Of the four tested compounds, all showed measurable inhibition activity of β-hematin formation with the IC$_{50}$ ranged from 35.54 to 62.08 mM compared to chloroquine with IC$_{50}$ of 3.49 mM (Table 1). This result in agreement with those reported by Hawley *et al.* (3) on heme polymerization and antimalarial drug potency assays of chloroquine and halofantrine, with IC$_{50}$ of 24.4 μM and 184.5 μM for chloroquine and halofantrine respectively. These result indicated that halofantrine and 1,10-phenanthroline derivatives have potency on inhibition of heme polymerization. In the preliminary experiment the effect of possible interfering non-physiological aqueous DMSO substance was examined. DMSO was added to the

standard assay mixture at concentration of 10-100% (v/v) and did not inhibit the hematin polymerization reaction at the concentrations tested but enhanced the hemozoin-forming activities. This might be caused by an increased solubility of hematin, which tends to precipitate in aqueous solutions at the pH of the digestive vacuole (pH 4.8 to 5.2). This result support previous study reported by Korosawa *et al.* (15) on the effect of DMSO on hematin polymerization activity, the result showed that 0.5 to 10% (v/v) DMSO did not inhibit hematin polymerization reaction and the percentage of inhibition was -37%.

The different substituent of 1,10-phenanthroline skeleton seems has effect on the inhibition activity of β-hematin formation. The presence of phenanthroline skeleton itself appeared to be favorable for this activity, since all compounds possessing this group. Modification of drug structure is one possible method to get higher activity. This study showed that alkylation on 1,10-phenantroline ring influenced on its effect. (1)-N-ethyl-1,10-phenantrolinium sulfate which had greater proportion of non-polar molecule, had higher activity on inhibition of hematin polymerization than (1)-N-methyl-1,10-phenantrolinium sulfate. Halogen counter ion substitution on (1)-N-benzyl-1,10-phenantroline also influenced the compounds on its effect, the activity of Iod (I⁻) counter ion was higher than Chloride (Cl⁻) counter ion. Iod also known as a living group which is better than chloride therefore Iod is easier to kick out and replaced by other nucleophile.

Chloroquine, the classic hemozoin-targeted agents, is a moderately hydrophobic base possessing titratable protons that confer net positive charge in the acidic environments of digestive vacuole. Thus, chloroquine is thought to diffuse in its non-protonated forms across the vacuolar membrane, and be trapped in the acidic compartment of the digestive vacuole. Once in the vacuole, chloroquine prevents sequestration of toxic heme into hemozoin by binding heme. The ability of chloroquine-like drugs to act as inhibitors of heme aggregation may be dependent upon two factors: formation of drug-heme complex (a) and interaction of drug-heme complex with the heme polymer (b). Derivatized 4-amino-quinolines interact with hematin-μ-oxo-dimers resulting in a cofacial π-π sandwich type complex. Cation-π type interactions have been recognized as an important non-covalent binding force, and have been postulated to be a dominant force in biological environment with a number of protein systems. Therefore, based upon differences in the cation-π type non-covalent interaction between aromatic rings (choloquine and mefloquine), it has been demonstrated that π electrons of chloroquine ring are better suitable for various interactions exemplified as π-cation, π-π, π-charge, and π-dipole interactions compared with mefloquine that posses highly
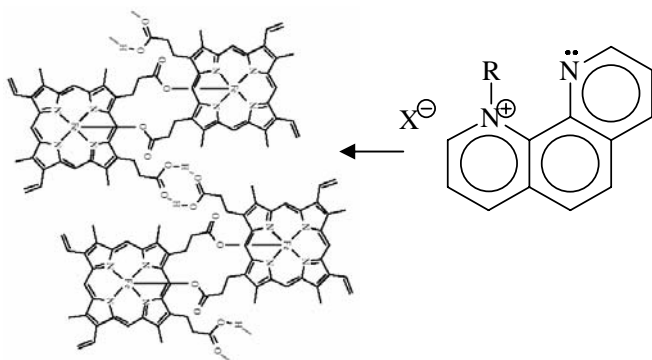
Figure 2. Structure of hemozoin or β-hematin based upon current X-ray model (2,4,5) and the possibility inhibition mechanism of *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives on heme polymerization. Heme units in β-hematin are linked to each other through a coordination bond, between ferric ion of one unit to propionate carboxyl group of another. These dimmers are linked via an extensive network of hydrogen bonds contributed by the second propionic acid group of FPIX.

electronegative subtituents such as trifluoromethyl. Resultantly, chloroquine does bind to hematin due to favorable electronic profiles and this type of chemical interaction is absent in the case of mefloquine (2). Chloroquine binds non covalently to the growing face of hemozoin crystal and prevents its further growth (4). Hemozoin was originally considered to be formed by the polymerization of heme, but it has now been demonstrated to be a crystalline cyclic dimmer of ferriprotoporphyrin IX. Antimalarials such as chloroquine can be considered crystallization inhibitors or agents that act to divert heme from participating in the crystallization process, leading to the accumulation of free heme, which is potentially toxic (16).

N-alkyl and *N*-benzyl-1,10-phenanthroline derivatives have two nitrogenous bases. One of them is a cation (positive charge) which has ability to bind with electronegative ions (oxygen on ferriprotoporphyrin IX) while the other nitrogen is electronegative which binds to electropossitive ion such as hydrogen. The ability of *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives bind to dimmer of ferriprotoporphyrin IX prevent its crystallization (Figure 2). This condition support the work of Ziegler *et al.*(1) on drug-heme binding and correlation with antimalarial activity. They explain the observation that chloroquine and halofantrine which had intraerythrocytic antimalarial activity, inhibit *in vitro* heme polymerization. During intraerythrocytic phase of the malaria life cycle hemoglobin is utilized as a predominant source of nutrients. The amino acid derived from digestion of hemoglobin are incorporated into parasite proteins and may also be utilized for energy

metabolism. Massive degradation of about 5mM hemoglobin releases large amount of toxic free heme. Continuous degradation of hemoglobin and concomitant detoxification of heme are absolutely necessary for uninterrupted growth and proliferation of the parasite. Therefore, the metabolic functions related to hemoglobin digestion and heme detoxification pathway may be potential targets for new antimalarial drug discovery. By binding to hematin, antimalarial drugs inhibit this process, resulting in higher concentration of free heme and antimalarial drug-heme complexes in the food vacuole. It is further postulated that this free heme and or antimalarial drug-heme complexes must somehow cause parasite death (4,17).

CONCLUSION

The activity of *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives to interfere with the hematin polymerization process could be one of the possible mechanism of action of these compounds as antimalarial drugs. Our result showed that heme polymerization inhibitory activity of *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives were lower than that of chloroquine. The IC_{50} HPIA of *N*-alkyl and *N*-benzyl-1,10-phenanthroline derivatives (35.54 mM to 62.08 mM) were higher compare to IC_{50} of chloroquine (3.49 mM).

ACKNOWLEDGEMENT

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