Inhibitory Activity and Docking Analysis of Antimalarial Agents from Stemona sp. toward Ferredoxin-NADP+ Reductase from Malaria Parasites

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1. Introduction

Malaria is still considered as a threat in the world, especially in Asia and Africa. Almost 97 countries in the world are malaria endemic. More than 3.3 billion people (almost 50% of the world’s population) are at risk of malaria with two Plasmodium species responsible for the majority of infections: P. falciparum and P. vivax [1]. Many antimalarial agents, such as chloroquine, pyrimethamine, quinine, proguanil, sulfadoxine, and artemisinin have been used for malarial therapy. Artemisinin, the first-line antimalarial drug has been used against P. falciparum in Cambodia, Thailand, Vietnam, and Myanmar, where artemisinin-resistant parasites have already been identified [1]. In Papua, Indonesia, resistance to artemisinin derivatives, artesunate and dihydroartemisinin, and semisynthetic 10-alkylamino-artemisinin derivatives, artesimone and artemiside, has also been observed in P. falciparum [2]. Ratcliffe, et al. (2007) studied 143 patients (103 and 40 patients) who were treated with chloroquine-sulfadoxine and chloroquine, respectively. The failure rate at 28 and 42 days was 65% and 48% for P. falciparum and P. vivax, respectively [3]. Pyrimethamine and sulfadoxine can inhibit dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) enzymes. These enzymes undergo gene mutations causing the resistance of antimalarial antifolate [4]. Double and quadruple mutations in the P. vivax dihydrofolate reductase (PvDHFR) found in Papua P. vivax isolates were 46 and 18%, respectively [5].

Plasmodium sp. has a unique organelle, called apicoplast, and it possesses metabolic pathways, which is not found
in human host [6, 7]. The apicoplast was firstly found in *P. falciparum* and *Toxoplasma* [8]. The function of plastid (apicoplast) in Apicomplexa is unclear, but many studies suggested its roles in the biosynthesis of fatty acids, haem, isoprenoids, and Fe-S cluster, based on the analysis of genome sequence. More importantly, the metabolism of apicoplast is necessary for the survival of the *Plasmodium* [6, 9, 10].

Ferredoxin (Fd) and Fd-NADP+ reductase (FNR) in apicoplast constitute a redox system [11, 12] that is homologous to those of plant chloroplast and algae, especially green and red algae [8, 13]. The involvement of apicoplast Fd has been indicated in the reactions of an Fe-S cluster assembly [14] and isoprenoid biosynthesis pathways [10, 15], fatty acid desaturation [5], and heme oxygenation [16].

In a previous study, *in silico* analysis showed that croomine and *epi*-croomine could form hydrogen bonds with the amino acid residue of Ala9 of the DHFR enzyme [17]. DHFR can be found in human and *Plasmodium*, but FNR is found only in *Plasmodium*. Fd and FNR were cloned from *P. falciparum* and their interactions were characterized [9, 18]. There has been no report on plant-alkaloid inhibiting FNR to date. Here, we describe the inhibitory effect of alkaloids from the roots of *Stemona* sp. on the PfFd/FNR redox system, and the results of their docking analysis.

### 2. Materials and Methods

#### 2.1. Isolation of Alkaloid from *Stemona* Species

The dry roots of *S. tuberosa* were cut in small size and ground into powder and extracted using ethanol. The filtrate was evaporated under vacuum and extracted by acid based method using 5% of HCl and conc of NH₄OH. Water fraction was partitioned using dichloromethane (DCM) and evaporated. The crude alkaloid was separated using column chromatography under silica gel as stationary phase and DCM-methanol as mobile phase with polarity gradient elution. The alkaloids were purified using preparative chromatography to give tuberostemonine, croomine, and *epi*-croomine (*epi*-10-hydroxycroomine or 3-*epi*-tuberospiroinine A) [19].

![Figure 1: Structure of alkaloids from the roots of *Stemona* sp.](image)

Isolation of isoprotostemonine and javastemonine A from *S. javanica* utilized the same method as described for the isolation of alkaloids from *S. tuberosa*. Previous study exhibited that tuberostemonine, croomine, and *epi*-croomine were treated to antifolate target of DHFR enzymes. Isoprotostemonine and javastemonine A from *S. javanica* were carried out against *P. falciparum* multidrug resistant and wild type parasites at Medical Molecular Research Unit, Thailand Science Park, Pathumthani, Thailand [20]. The structures of alkaloids were shown in Figure 1. Five alkaloids were made as 1000 ppm stock solution (in 0.2% of DMSO in suspension of phosphate buffer at pH 7.0). The final alkaloids solution concentration was 100 ppm.

#### 2.2. Preparation of Recombinant PfFNR and PfFd. Production and purification of recombinant PfFd and PfFNR were performed as reported by Kimata-Ariga et al. [9].

#### 2.3. Enzymatic Activity Analysis

Enzyme activity of PfFNR was measured using a grating microplate reader (model SH-1000Lab, CORONA, Japan). Diaphorase activity of FNR with DCPIP as an electron acceptor (Scheme 1) was measured as reported by Onda [21] with modification in the reaction mixture of 50 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 0.2 mM DCPIP, and 5 nM FNR. The reaction was initiated by adding 5-100 µM of NADPH and the decrease of the absorbance was monitored for 5 min with 10-second intervals at 600 nm. The activity of NADPH-dependent electron transfer from FNR to Fd (Scheme 2) was measured using cytochrome c (cyt c) as an arbitrary electron acceptor as reported by Onda [21] with modification in the reaction mixture of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 200 µM cyt c, and 10 nM PfFNR. Electron transfer reaction was initiated by adding 0.5-40 µM PfFd and 100 µM NADPH, and the absorbance was monitored for 5 min with 10-second intervals at 550 nm. The kinetic parameters for the Michaelis constant (*Kₘ*) for these reactions were determined.
Scheme 1 (NADPH \rightarrow FNR \rightarrow DCPIP). Scheme 1 is used for measuring the NADPH-dependent catalytic activity of FNR.

Scheme 2 (NADPH \rightarrow FNR \rightarrow Fd \rightarrow cyt c). Scheme 2 is used for measuring the NADPH-dependent electron transfer activity from FNR to Fd.

2.4. Inhibition Assay. Inhibition of diaphorase activity of PfFNR was measured in the reaction of Scheme 1 as described above, by the addition of each alkaloid at 100 ppm, NADPH at 100 uM, and 5 nM FNR. Inhibition of the electron transfer from PfFNR to PfFd was measured in the reaction of Scheme 2 as described above, by the additions of each alkaloid at 100 ppm and PfFd at 1 \mu M. A 5 mM solution of DMSO was used as a control.

2.5. Docking Procedures. Molecular docking was performed using Dock 6.8 software (http://dock.compbio.ucsf.edu/DOCK6/index.htm) [22]. Docking studies were carried out on an Intel Core i5 2.4 GHz PC, with 16 GB memory and with Windows 10 operating system.

3. Ligand and Receptor Preparations

The receptor model was obtained from Protein Data Bank (PDB) server (http://www.rcsb.org/) with ID 1GAQ [23]. This complex consists of three chains, there are enzyme *Plasmodium falciparum* (PfFNR), flavin adenine dinucleotide (FAD), and some crystals of water. Crystals of water were not involved in the reaction mechanism directly and were stripped out from the complex. In contrary, FAD was used as a part of the receptor. Hydrogen and charge atoms were added to PfFNR and FAD complex using molecular mechanics method with AMBERff14SB force field [24]. Hydrogen atom was stripped out from PfFNR and hydrogen-free PfFNR was saved in pdb format to generate molecular surface of the receptor.

Ligand models were drawn using HyperChem6 software of HyperCube, Inc (http://www.hyper.com/). All the ligand models were preoptimized using MM+ molecular mechanics methods before being optimized utilizing semiempirical method AM1 to generate 3D conformation also in HyperChem 6 as final process. These optimized ligands were saved in mdl mol file format. Hydrogen and charge atoms were added and loaded using the AM1-BCC charge calculation [25] and UCSF Chimera (https://www.cgl.ucsf.edu/chimera/) [26] methods, respectively. All these protonated and charged ligands were saved in Sybil mol2 files format.

4. Ligands Docking

The molecular surface of the receptor was generated with the dms tools which was included in the DOCK 6.8 package program. The molecular surface was achieved by dms tool and spheres of the receptor were generated using program sphgen [27]. Binding site was represented by the largest cluster of the spheres generated of sphgen. Grid box for docking simulation was created around the binding site with extra margin 5 Å. Grid space was set in 0.2Å, while the dimension is 47.814Å x 63.571Å x 39.732Å. The location of the largest cluster of the spheres as the representation of the binding site and grid box on the receptor was shown in Figure 2. Each ligand was docked within the binding site of the receptor using Dock 6.8 resulting in binding (Grid_score) and van der Waals (Grid_vdw) energies, electrostatic interaction (Grid_es), and internal energy of the complex (Int_energy). Int_energy was determined based on force of field energy calculated.

5. Results and Discussion

The structures of alkaloids from the roots of *Stemona* species are shown in Figure 1.

5.1. Inhibition of NADPH-Dependent Catalytic Activity of PfFNR by Alkaloids from the *Stemona* sp. The effect of these alkaloids from the roots of *Stemona* spp. (Figure 1) on the NADPH-dependent catalytic activity (diaphorase activity) of PfFNR (Table 1) was inquired. In this reaction, the effects on the FNR catalytic activity and/or interaction with NADPH were to be considered. In the absence of the inhibitors, the $K_m$ value for NADPH was 3.57 \mu M. The inhibition assay was performed under the same conditions used in the $K_m$ determination employing the following conditions: the concentration of NADPH was 100 \mu M, the concentration of PfFNR was 5 nM, and the concentration of tested compounds
was 100 ppm. The % inhibition was calculated according to the following equation, and the results are listed in Table 1.

\[
\text{% Inhibition} = \left( \frac{(dA/dt)_{\text{control}} - (dA/dt)_{\text{sample}}}{(dA/dt)_{\text{control}}} \right) \times 100\%
\]

The results showed that three out of the five tested compounds were able to inhibit the reaction by between 0.7 and 33.9%, while the other two compounds exhibited a negative inhibition, which means that these compounds accelerated the activity. Croomine alkaloid showed 33.9%, the largest inhibition, and epi-croomine showed lower but considerable inhibition. Isoprotostemonine and javastemonine A exhibited a negative inhibition. This might be because both alkaloids have double bonds conjugated to carbonyl group and, thus, might considerably trigger electron delocalization and stabilisation of resonance. Stabilisation of resonance in isoprotostemonine and javastemonine A, which means that these compounds accelerated the electron transfer from PfFNR to PfFd, will make \textit{P. falciparum} parasites live because electron transfer occurs in respiratory process \[28\].

### 5.2. Inhibition of the Electron Transfer from PfFNR to PfFd by Alkaloids from the \textit{Stemona} sp.

The effect of alkaloids from the roots of \textit{Stemona} sp. (Figure 1) on the electron transfer from PfFNR to PfFd (Table 2) was investigated. In the absence of the inhibitors, the \(K_m\) value of PfFNR for PfFd was 0.56 \(\mu\)M, which was consistent with previous studies \[28\]. The inhibition assay was performed under the same conditions used in the \(K_m\) determination employing the following conditions: the concentration of PfFd was 1 \(\mu\)M, the concentration of PfFNR was 10 nM, and the concentration of tested compounds was 100 ppm. The % inhibition was calculated according to (1) and the results are listed in Table 2.

In this case, the same three compounds inhibited the reaction, while the two compounds exhibited a negative inhibition, but to different extents as compared to the results in Table 1. Croomine showed the largest inhibition on the reaction the FNR activity and tuberostemonine showed slight inhibition. This could be due to the inhibition of either FNR catalysis or NADPH binding to FNR. Croomine was previously shown to inhibit the activity of dihydrofolate reductase (DHFR, an enzyme for antifolate drug mechanism). The \(IC_{50}\) of croomine to the DHFR was 5.29 \(\mu\)M, while tuberostemonine was not active to the enzyme \[17\].

### 5.3. Docking Analysis of Alkaloids toward Protein

The interaction between the alkaloids from the \textit{Stemona} sp. and PfFd-PfFNR was studied by molecular docking. The structure of PfFNR was obtained from the Protein Data Bank (PDBID 1GAlQ)[23]. Five alkaloids from \textit{Stemona} sp. were expected to bind to PfFNR. Figure 2 showed the location of the largest cluster of the spheres as the representation of the binding site and grid box on the receptor. This result indicated that the parameters used in the procedure could be extended to search for the binding conformation to the active site for the other ligands (alkaloids). Figure 3 showed that interaction between receptors of PfFd (blue), PfFNR (brown), and ligand (red). FAD (green) is not involved in bonding receptors-ligand.

Electron transfer is going on in \textit{Plasmodium} from PfFNR to PfFd through electrostatics interaction during respiration process \[9\]. Position of croomine displayed at the middle between PfFNR and PfFd, while epi-croomine, tuberostemonine, isoprotostemonine, and javastemonine A were on the edge of the receptors (Figure 3). Croomine showed inhibition 33%, which was higher than tuberostemonine and epi-croomine. Croomine could obstruct electron transfer from PfFNR to PfFd. This result was supported by research report foregoing that croomine inhibited competitively the human DHFR enzyme at 10 ppm \[17\].

Tuberostemonine is stenine alkaloid, while croomine and epi-croomine are spironine, and isoprotostemonine and javastemonine A are stemoamide alkaloids types \[29\]. The characteristic of stenine type is 4-fused rings in its structure, and spironine type has only 2-fused rings. N atom in 4-fused rings structure is rigid and stable. It is likely that the N atom in 4-fused ring is important for the diaphorase activity.

### 6. Conclusions

Croomine could inhibit the activity of FNR enzyme and electron transfer from PfFNR to PfFd. Thus, croomine from \textit{S. tuberosa} appeared to have potential as an antimalarial agent. Tuberostemonine inhibited diaphorase for redox reaction.
Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors’ Contributions

The authors contributed equally in isolation and inhibition of enzyme activities, molecular docking, and writing of this paper.

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