International Journal of Biological Macromolecules 99 (2017) 358–364

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Characterization of the selective alkylation site in hemoglobin A by dihydroartemisinin with tandem mass spectrometry

Khomsan Tiensomjitr, Samran Prabpai, Palangpon Kongsaeree*

Department of Chemistry and Center for Excellence in Protein and Enzyme Technology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

ARTICLE INFO

Article history:
Received 30 December 2016
Received in revised form 24 February 2017
Accepted 26 February 2017
Available online 1 March 2017

Keywords:
Artemisinin
Hemoglobin
Selective modification

ABSTRACT

The reaction between the antimalarial drug dihydroartemisinin (DHA) and hemoglobin A (HbA) was investigated in vitro. A fluorescein-tagged artemisinin analog reacted with HbA and fluorescent HbA-drug adducts could be visualized on SDS-PAGE to confirm stable covalent reaction adducts and necessity of the endoperoxide moiety and Fe(II). Mass spectrometric analyses revealed that DHA favourably alkylated protein part rather than heme and the modification site was identified to be at Tyr35 of the beta globin chain.

1. Introduction

Artemisinin or qinghaosu (**1**) and its derivatives are potent antimalarial agents with rapid parasite clearance in malaria patients (Fig. 1) [1,2]. Artemisinin-based combination therapy (ACT) is recommended by the World Health Organization (WHO) as the first-line treatment for uncomplicated *Plasmodium falciparum* malaria. Remarkably, artemisinin-resistant malaria was reported along the western boarder of Cambodia [3], and mutations in the K13-propeller gene were discovered as a molecular marker of this resistance [4]. The endoperoxide moiety of artemisinin is crucial for its antimalarial activity as the initial reaction center while the absence of such endoperoxide moiety is deprived its potent bioactivity [1]. After activated by cellular ferrous iron (Fe^{2+}), more efficiently from heme than free Fe^{2+} [5], the endoperoxide undergoes a homolytic cleavage and the resulting reactive radical species finally alkylate biomolecules to exert its antimalarial function [1,6,7]. Covalent adducts between intra-parasite heme and **1** suggested that heme was a primary target of the radical intermediates, and these adducts indicated the alkylating capacity of artemisinin in vivo [8–10]. Several groups have characterized heme adducts from reactions of artemisinin with heme [11,12], with hemoglobin [7,13], and also in *P. vinckei*-infected mice [14].

Recently, proteins involved in glycolysis, hemoglobin degradation, antioxidant defense, and protein synthesis pathway of *P. falciparum* 3D7 were reportedly multiple targets of artemisinin and 1,2,4-trioxolane, a synthetic peroxide-based antimalarial agent [15,16]. Moreover, artemisinin targets multiple proteins in cancer cells by hemin activation [17]. Heme-containing proteins such as hemoglobins and catalase as well as a non-heme containing protein, translationally controlled tumor protein (TCTP) of *P. falciparum* were also found to interact with artemisinin [18–21]. Three possible mechanisms of artemisinin were recently proposed [17]: (1) artemisinin specifically and non-covalently binds proteins or (2) non-specifically attaches on surface of proteins before being activated by heme and covalent bond formation and (3) artemisinin alkylates heme-containing proteins on heme or nearby amino acids after being activated by heme. A specific protein modification site with artemisinin, however, has not been characterized until now. Herein, we report the characterization of HbA adducts with artemisinin derivatives and its modification site by utilization of fluorescent labelling and mass spectrometry.

2. Materials and methods

2.1. Materials

Artemisinin and dihydroartemisinin (DHA) were kind gifts from Prof. Y. Thebtaranonth, Department of Chemistry, Mahidol University. Sequencing grade modified trypsin was purchased from Promega Corporation (Madison, WI). Human methemoglobin A (metHbA), 5(6)-carboxyfluorescein, sodium dithionite, L-tyrosine, and all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

* Corresponding author.
E-mail address: palangpon.kon@mahidol.ac.th (P. Kongsaeree)

http://dx.doi.org/10.1016/j.ijbiomac.2017.02.094
0141-8130/

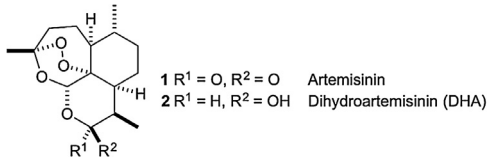
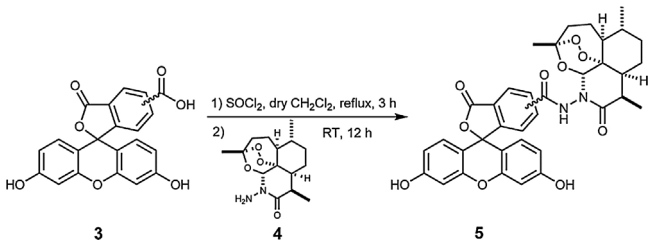


Fig. 1. Artemisinin and its derivative.



Scheme 1. Synthesis of a fluorescein-tagged artemisinin.

2.2. Methods

2.2.1. Synthesis of N-5(6)-carboxyfluorescein-11-azaartemisinin (5) and N-5(6)-carboxyfluorescein-10-azadeoxyartemisinin (S2)

According to the previous report [22], *N*-amino-11-azaartemisinin (**4**) and *N*-amino-10-azadeoxyartemisinin (**S1**) were prepared and further used to synthesize *N*-5(6)-carboxyfluorescein-11-azaartemisinin (**5**) (Scheme 1) and *N*-5(6)-carboxyfluorescein-10-azadeoxyartemisinin (**S2**) (Scheme S1), respectively. To a stirred solution of 5(6)-carboxyfluorescein (100 mg, 0.27 mmol) in 10 mL CH_2Cl_2, $SOCl_2$ (158 mg, 1.33 mmol) was added. The mixture was refluxed for 2 h and then allowed to cool down to room temperature. *N*-Amino-11-azaartemisinin (75.6 mg, 0.27 mmol) in 5 mL CH_2Cl_2 was then added to the reaction which was further allowed to stir at RT for 12 h. The reaction was diluted with H_2O (100 mL) and then extracted with CH_2Cl_2 (3×100 mL). The combined organic layers were evaporated under reduced pressure to give yellowish oil which was purified by column chromatography over Sephadex LH-20 using 100% MeOH as an eluent to obtain a yellow solid of *N*-5(6)-carboxyfluorescein-11-azaartemisinin. For the synthesis of *N*-5(6)-carboxyfluorescein-10-azadeoxyartemisinin, the above procedure was repeated by using *N*-amino-10-azadeoxyartemisinin.

2.2.2. Determination of quantum yield of fluorescein-tagged artemisinins

A stock solution (8 mM) of compound **5** and **S2** were prepared in EtOH. The solutions were diluted to various concentrations (0.4–20.0 μM) in 10% EtOH-NH_4OAc buffer (10 mM, pH 7.4). For all samples, absorption spectra were analyzed using an Agilent 8453 diode array UV–vis spectrophotometer and fluorescence spectra was measured using a Cary Eclipse fluorescence spectrophotometer. The absorbance and integrated fluorescence intensity for each concentration was plotted and the positive slope of linear fitting was calculated. The quantum yield was calculated against fluorescein as a standard reference ($\Phi = 0.93$) [23] according to the following equation [24];

$$\Phi_X = \Phi_{ST}\left(\frac{Grad_X}{Grad_{ST}}\right)\left(\frac{\eta_X^2}{\eta_{ST}^2}\right)$$

Where Φ is the quantum yield, Grad is the slope from the plot of integrated fluorescence intensity and absorbance, η is the refractive index of the solvent, and subscripts ST and X denote standard and sample, respectively.

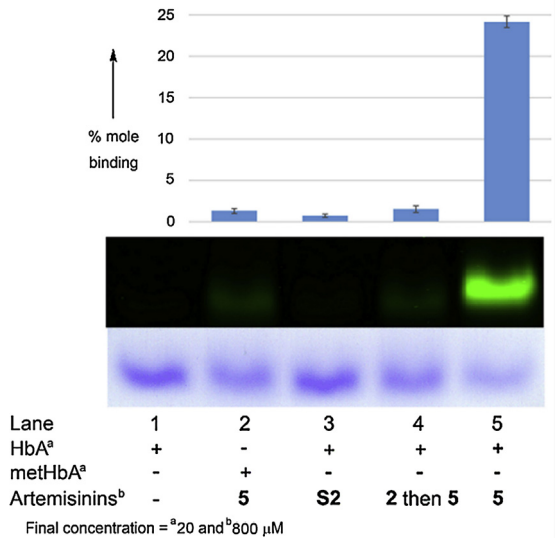


Fig. 2. SDS-PAGE analysis of fluorescein-tagged hemoglobin A. Fluorescent visualization (upper, $\lambda_{ex} = 488$ nm, $\lambda_{em} = 526$ nm) and Coomassie Blue staining (lower). Binding percentage of the reactions, measured from absorption at 496 nm, are shown on the top. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2.3. Antimalarial assay

Antimalarial activity was performed in vitro against *Plasmodium falciparum* K1, the multidrug resistant strain [25], using the microculture radioisotope technique [26] by the Bioassay Laboratory (http://www.biotec.or.th/bioassay) at the National Science and Technology Development Agency (NSTDA), Thailand. Effective concentration (EC_{50}) represents the concentration which causes 50% reduction in parasite growth as indicated by the in vitro uptake of [^{3}H]-hypoxanthine by *P. falciparum*. DHA and mefloquine were used as positive controls for the antimalarial activity with IC_{50} values of 2.57 nM and 32.8 nM, respectively.

2.2.4. Reduction of metHbA

The reduction of methemoglobin (metHbA) was modified from the previous protocol [27]. A desalting column of 5×1.5 cm (Sephadex G-25) equilibrated with 20 mM phosphate buffer, pH 7.0 was loaded with a mixture of metHbA (5.3 mg, 0.08 μmol = 0.32 μmol of heme) and $Na_2S_2O_4$ (5.57 mg, 3.2 μmol) in 20 mM phosphate buffer, pH 7.0 in a final volume of 1 mL. The loaded mixture was eluted with a running buffer the eluted HbA solution was then collected. The Bradford protein assay was used to measure HbA concentration before storing at −80 °C [28].

2.2.5. Reaction between HbA and artemisinin derivatives

The standard condition for the reaction between HbA and artemisinin derivatives was modified from the previous report [7]. HbA (1.3 mg, 0.021 μmol = 0.084 μmol of heme) was dissolved in 10 mM NH_4OAc buffer, pH 7.4 and 5(6)-carboxyfluorescein-11-azaartemisinin (**5**) (0.8 μmol in EtOH) was then added. The final HbA concentration was 20 μM, HbA and drug ratio = 1:40, and the co-solvent was 10% EtOH in 10 mM NH_4OAc buffer, pH 7.4. The reaction was incubated at 37 °C for 2 h (Fig. 2, lane 5). For negative control experiments, the above procedure was repeated by using metHbA (1.3 mg, 0.021 μmol) instead of HbA (Fig. 2, lane 2), the deoxy **S2** analog (0.8 μmol in EtOH) instead of **5** (Fig. 2, lane 3). DHA (0.8 μmol in EtOH) was allowed to react with HbA (1.3 mg, 0.021 μmol) for 2 h before the reaction was subsequently added with **5** and further stirred for 2 h (Fig. 2, lane 4). To establish the binding efficiency of the reaction between HbA and **5**, non-reacted drugs in the reaction mixture were separated from HbA part