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Differential interactions of the broad spectrum drugs artemisinin, dihydroartemisinin and artesunate with serum albumin

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ABSTRACT

Artemisinin is a drug, widely used in malaria treatment. As the binding affinity of artemisinin and its derivatives dihydroartemisinin and artesunate to blood serum proteins might influence the effectiveness of the drug, binding of artemisinin and derivatives to serum albumin was studied under near physiological conditions. Binding kinetics indicate a simple, single-step association process for all artemisinin derivatives. The determined changes in enthalpy and entropy upon drug binding clearly indicate that hydrophobic forces are most important for artemisinin and dihydroartemisinin binding, whereas binding of artesunate is governed by both hydrophilic and hydrophobic forces. Key residues, which are most likely involved in binding of the respective compounds, were identified in subsequent protein/drug docking studies. The obtained results not only explain differences in between artemisinin and derivatives but generally illustrate how slight modifications in a drug can significantly affect principles underlying drug binding to target proteins.

Introduction

Artemisinin (qinghaosu), a sesquiterpene endoperoxide isolated from the Chinese traditional herb *Artemisia annua*, is a widely used drug targeting the malaria pathogen *Plasmodium falciparum* (Miller and Su 2011). In addition, artemisinins display activity against a wide range of trematodes (Keiser and Utzinger 2007), and recent studies indicate that artemisinin and derivatives have significant anticancer and antiviral effects (Dell'Eva et al. 2004; Efferth 2005; Efferth et al. 2001, 2002; Hou et al. 2008). Artemisinin-type drugs inhibit the activation of nuclear factor kappa-B (NF-κB), an important activator protein in cancer development and virus replication (Aldieri et al. 2003; Efferth et al. 2002), and the artemisinin derivative dihydroartemisinin targets human metastatic tumor cells (Cabello et al. 2011; Rasheed et al. 2010). Furthermore, the artemisinin derivative artesunate induces oxidative DNA damage and repair (Berdelle et al. 2011) and inhibits tumor angiogenesis *in vitro* and *in vivo* (Anfosso et al. 2006; Chen et al. 2004; Dell'Eva et al. 2004).

Serum albumin (SA) is the most abundant protein in the human blood plasma, and the binding affinity of artemisinin and derivatives to SA might influence the effectiveness of these (and other) drugs at their active site (Kragh-Hansen 1981; Otagiri 2005). Thus, especially when considering the broad spectrum of biological activity, it is crucial to gain detailed knowledge on the mechanism of interaction between artemisinin and its derivatives with SA.

Bovine SA (BSA) is widely used as a model SA protein to investigate protein–drug interactions in detail as it has 76% sequence identity to human SA and is experimentally well accessible (Gelamo et al. 2002). SA proteins have three linearly arranged domains (I–III) with two major drug binding sites in the subdomains IIA (site I) and IIIA (site II), which are widely known as warfarin and diazepam binding sites in BSA, respectively (Ni et al. 2006). Thus far, solely the interaction of an ethanolic artemisinin solution with BSA has been reported, although only under non-physiological conditions (Bian et al. 2006). Thus, while crucial for understanding potential differences in their respective pharmacokinetics, binding of artemisinin derivatives, such as dihydroartemisinin or artesunate, to SA proteins under physiological conditions has never been investigated yet. Nevertheless, the reported experiments clearly indicate that artemisinin binds to site I on BSA. BSA contains two tryptophan residues, and while Trp134 is localized in the outer hydrophilic environment, Trp213 resides in the hydrophobic binding pocket of site I (Kragh-Hansen 1981; Peters 1985). Therefore, Trp213 is widely used as an intrinsic fluorescent probe to study drug binding to site I under physiological conditions

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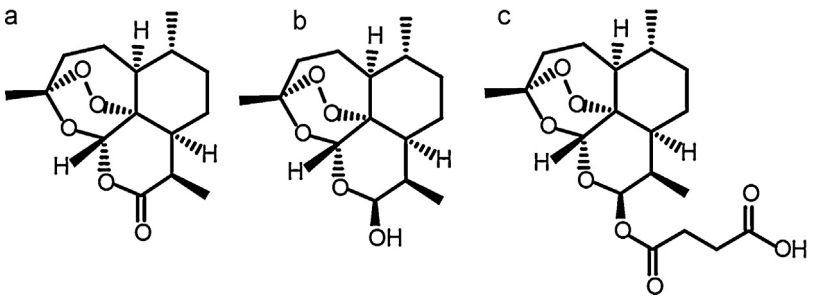

Fig. 1. Artemisinin and derivatives in clinical use. Structure of (A) artemisinin, (B) dihydroartemisinin and (C) artesunate.

by fluorescence spectroscopy. Based on the presented analyses, hydrophobic forces appear to be most important for artemisinin and dihydroartemisinin binding, whereas artesunate binding is different and governed by both hydrophilic and hydrophobic forces. Nevertheless, the binding kinetics indicates a simple, single-step association process for all artemisinin derivatives. However, artesunate appears to bind to BSA at a faster rate and dissociates sooner, and thus artesunate might be released easier at a target site. The obtained results nicely illustrate how slight modifications in a drug can significantly affect principles underlying drug binding to a target protein and the efficiency of drug release at a target site.

Materials and methods

Materials

Artemisinin, dihydroartemisinin, artesunate, and fatty acid free BSA was obtained from Sigma. For binding analysis, stock solutions (2 mM) of artemisinin, dihydroartemisinin and artesunate were prepared in 10 mM phosphate buffer, 150 mM sodium chloride, pH 7.4. In the case of artemisinin and dihydroartemisinin, concentrated solutions were prepared in ethanol and subsequently diluted in phosphate buffer, in order to have an ethanol content below 10%. The concentration of BSA was determined spectroscopically using a molar extinction coefficient of 43,284 M^{-1} cm^{-1} at 280 nm.

Artemisinin binding to BSA

Fluorescence measurements were performed using a Fluormax-4 spectrofluorimeter at constant temperatures (±0.5 °C). The excitation wavelength was 290 nm and emission spectra were recorded from 305 nm to 400 nm. The decrease in the Trp fluorescence at 339 nm was recorded to monitor the BSA–artemisinin interaction.

Binding of artemisinin and derivatives to BSA was analyzed in detail by adding small aliquots of the drug from a concentrated stock solution into a 4 μm BSA solution. Fluorescence emission spectra were recorded after 2 min equilibration. All titrations were repeated at least three times and the mean values of K_a are reported. All binding experiments were performed in 10 mM phosphate buffer, pH 7.4, containing 150 mM sodium chloride.

Stopped-flow spectroscopy

Kinetics of the interaction of artemisinin derivatives with BSA was investigated by stopped-flow spectroscopy using an Applied Photophysics SX20 stopped-flow instrument. At a fixed BSA concentration of 3 μM, the drug concentration was varied from 0.4 μM to 1.6 μM (both concentrations after mixing). Changes in the fluorescence emission after excitation at 290 nm were monitored at 25 °C using a 310 nm cut-off filter after mixing equal volumes of protein and compound. Kinetic traces were analyzed using the Pro-K global analysis software provided by the manufacturer. All traces are mean values calculated from 5 to 10 independent kinetic profiles.

Molecular modeling

The x-ray crystallography-based structure of bovine albumin (PDB-ID: 3V03) was obtained from the Protein Data Bank (www.PDB.org) and was set as the rigid receptor molecule upon which flexible ligands were docked. The three dimensional artemisinin structures were downloaded from PubChem database (http://www.pubchem.ncbi.nlm.nih.gov/). All molecules were prepared using AutoDockTools for further docking with AutoDock 4.2. (Morris et al. 2009) A grid box covering the amino acids Trp134 and Trp213 in BSA was generated in order to allow ligands to find the best binding site in silico if both binding sites are covered by search area. For the calculation of artemisinin binding sites, dockings were performed with standard parameters except for the number of evaluations (2,500,000) and runs (100), which were reset to increase docking accuracy. Visualization of docking results and the measurement of distances was achieved by using VMD (Humphrey et al. 1996).

Results and discussion

Interaction of artemisinin, dehydroartemisinin and artesunate with BSA

Binding of drugs to serum proteins, such as SA, is a determinant of the actual drug efficacy, and the amount of unbound drug can correlate with drug activity. Weak binding to serum proteins may explain the activity of unbound artemisinin-type drugs, but also their rapid metabolization. The structures of the artemisinin derivatives analyzed in this study are shown in Fig. 1. Fluorescence spectra of BSA in the absence as well as in presence of different concentrations of artemisinin, dihydroartemisinin and artesunate are given in Fig. 2. Importantly, addition of the drugs neither changed the shape nor the maximum of the BSA fluorescence emission spectra, indicating that under the given experimental conditions no conformational changes are involved in drug binding to BSA. Furthermore, drug binding to BSA does not affect the far-UV circular dichroism spectra, indicating no substantial changes in the protein secondary structure upon drug binding (data not shown). As can be seen in Fig. 2, due to quenching of the BSA intrinsic fluorescence, the fluorescence intensity at 339 nm constantly decreases after successive addition of the drugs. Artemisinin binds to site I in BSA, (Bian et al. 2006) and thus the observed quenching can be attributed to changes of the Trp213 environment. Together, artemisinin and its derivatives bind to the BSA binding site I and binding might be quantified by monitoring quenching of the Trp213 fluorescence.

To determine the association constants K_a, we assumed that the detected fluorescence quenching is based on formation of a