THE INHIBITORY INFLUENCE OF ARTESUNATE ON MYELOPEROXIDASE ACTIVITY OF THE POLYMORPHONUCLEAR NEUTROPHIL

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ABSTRACT
Myeloperoxidase is the most abundant enzyme found in the polymorphonuclear neutrophil and is known to play a central role in the host defense system of the leukocyte. The enzyme has been reported to interact with some drugs to generate free radical which inhibits its activity. This study investigated the effects of artesunate on the activity of the enzyme. In investigating the effects of the drugs on myeloperoxidase, the influence of concentration, pH, partition ratio estimation and kinetics of inhibition were studied.
This study showed that artesunate is concentration-dependent inhibitor of myeloperoxidase with an IC_{50} of 0.078 mM. Partition ratio estimation showed that 60 enzymatic turnover cycles are required for complete inhibition of myeloperoxidase in the presence of artesunate. The influence of pH on the effect of artesunate on the enzyme showed least activity of myeloperoxidase at physiological pH. The kinetic inhibition studies showed that artesunate competitively inhibited myeloperoxidase with an increase in the Km value from 0.12 mM to 0.26 mM and no effect on the Vmax value. The Ki value was estimated to be 2.5 mM. The results obtained from this study show that artesunate is a potent inhibitor of myeloperoxidase and it is capable of inactivating the enzyme.
It is suggested that the inhibition of myeloperoxidase in the presence of artesunate as revealed in this study may partly explain the impairment of polymorphonuclear neutrophil and consequent reduction of the strength of the host defense system against secondary infections.

Keywords: myeloperoxidase, artesunate, inhibition, Polymorphonuclear neutrophils

INTRODUCTION
Polymorphonuclear neutrophils (PMNs) play a crucial role in a variety of infections caused by bacteria, fungi, and parasites (Shimada *et al*., 2009). Indeed, the involvement of PMNs in host defense mechanism against *Plasmodium falciparum* is well documented both *in vitro* and *in vivo* (Mikkel and Niel, 2003). For instance, alterations of the host defence system and in particular the depression of PMN function in acute human malaria have been reported (Nahrevanian, 2006). As a consequence, a decreased ability to deal with a secondary invading pathogen has been observed in malaria-suppressed mice and infected humans (Nahrevanian, 2006*).* Also, available evidences indicate that many of the antimalarial drugs such as artesunate currently used in the prevention or treatment of human malaria significantly reduce the immune response of the host *in vitro* and *in vivo* (White, 2004).

Artesunate which is known for the treatment of malaria is thought to interfere with hemoglobin digestion in the blood stages of the malaria parasites, as well as the immune system. It has also been shown to inhibit PfATP6, the parasite's SERCA- type enzyme (calcium transporter) expressed in Xenopus oocytes (Uhlemann *et al*., 2005). The isolates of malaria parasites identified a mutation in PfATP6 that was associated with resistance to artemether lending some support to idea that PfATP6 (Uhlemann *et al*., 2005) represents the main target for artemisinin. A study investigating the mode of action of artemisinin using a yeast model demonstrated that the drug acts on electron transport chain which generates local reactive oxygen species and causes the depolarization of the mitochondrial membrane (Li *et al*., 2005). Recently, the parasite's digestive vacoule has again been implicated in artemisinin action (Del-Pilar and Crespo, 2008) 2008). However, the specific effect of artesunate on myeloperoxidase which plays an important role on the immune system is yet to be verified.
Myeloperoxidase is an enzyme found in human leukocytes and has been known to function in the host defense system against microbial actions. The present study therefore investigates the effect of

concentration, pH, partition ratio estimation and kinetics of inhibition of artesunate on myeloperoxidase activity.

MATERIALS AND METHODS

Artesunate is a product of Laboratoire products Roche, Neuilly France and all other reagents which were of analytical grade were obtained from Sigma Chemical Co.Ltd. Polymorphonuclear neutrophils (PMNs) were isolated from human blood obtained from drug-free individual in accordance with modified methods of Boyum (1968) and Okuyama *et al*., (1995).

The myeloperoxidase rich fraction was thereafter obtained after treating the neutrophil with Triton-X100 and stepwise fractionation by $(NH_4)_2SO_4$ (Andrews and Krinsky,1981). The concentration of myeloperoxidase was determined using $E_{480}91000M^{-1}cm^{-1}$(Odajimi and Yamazaki, 1970).

The assay procedure for determining activity of myeloperoxidase was carried out using the modified methods of Suzuki *et al* (1983) and Bozeman *et al* (1990). To investigate the dose influence of artesunate on myeloperoxidase, various concentrations (0-0.12 mM) of the drug were added to the reaction mixture which contains 10.0 µM myeloperoxidase, 0.3 mM hydrogen peroxide and 100 mM sodium phosphate buffer at 25ºC at pH 7.4 and the absorbance taken at 355 nm. The activity was expressed as percentage inhibition of the enzyme. The inhibition of myeloperoxidase by artesunate at various pH values was determined by incubating 10.0 µM myeloperoxidase with 0.1 mM artesunate, 0.3 mM hydrogen peroxide and 100 mM sodium phosphate buffer at 25ºC (pH 4.2-7.4). After 2 minutes aliquots were withdrawn from the mixture to determine the residual activity at 355 nm. The partition ratio was determined by incubating myeloperoxidase (10.0 µM) with 0.1 mM of artesunate at varying concentrations of hydrogen peroxide (0-120 µM) in phosphate buffer saline (pH 7.4) at 25ºC for 2 minutes after which aliquots were withdrawn from the mixture to determine the residual activity at 355 nm. The kinetic study was carried out with variation of initial reaction velocity with substrate concentrations (potassium iodide 0.02 mM- 0.12 mM). The experiments were carried out in the absence and presence of the artesunate. Results were analyzed using the double reciprocal plot of the Michaelis- Menten kinetic model to obtain the maximum velocity (V_{max}), the Michaelis constant (K_m) and inhibitor constants (K_i). At least four replicate experiments were carried out for the antimalarial drug.

RESULTS

Figure 1 shows the influence of various concentrations of artesunate on myeloperoxidase which is expressed as percentage inhibition compared with the control. A significant increase ($p<0.05$) in the percentage of inhibition is observed with increase in concentration of the antimalarial drug. This antimalarial drug inhibited the activity of myeloperoxidase with an IC_{50} value of 0.078 mM as determined from the dose-response curve. The maximum inhibition achieved was 54% at 0.08 mM after which there was no further increase in inhibition up to 0.12 mM artesunate concentration.

In Figure 2, the partition ratio of artesunate on myeloperoxidase activity was estimated. The results reveal that in the presence of artesunate, a significant reduction ($p<0.05$) in the enzyme activity was observed with increase in hydrogen peroxide concentrations. The enzyme still had activity up to 50 µM of hydrogen peroxide. The partition ratio extrapolation shows that 20 enzymatic cycles are required for total inactivation of the enzyme in the presence of the antimalarial drugs (Figure 2)

In the presence of artesunate, the activity of myeloperoxidase displayed a broad pH optimum between 5.2 and 6.0 (Figure 3). A significant increase ($p<0.05$) in the activity of the enzyme was observed between pH 4.2 and 5.2. There was no significant change ($p>0.05$) in the activity of the enzyme between pH 5.2 and 7.

Figure 4 reveals the result of double reciprocal plot of myeloperoxidase activity in the presence and absence of artesunate. It was observed that artesunate competitively inhibited myeloperoxidase with an increase in the K_m value from 0.12 to 0.26 mM while it has no effect on the V_{max} value (1.49 mM/min). The Ki value was also estimated to be 2.5 mM.

DISCUSSION

The results obtained from this study suggest that artesunate is a concentration-dependent inhibitor of myeloperoxidase. Myeloperoxidase utilizes several compounds as substrates and some of these subtrates have the potential to inhibit it through generation of radicals (Ator *et al*., 1987). Previous studies have revealed that artesunate generates free radicals which are probably responsible for their antimalarial actions (Nwanjo and Oze, 2007). The available results obtained from this investigation (Figure 1) suggest that artesunate might also be a possible substrate capable of inhibiting the enzyme through free radicals generation. The IC_{50} value of artesunate is closely related to the therapeutic achievable maximum concentration of the drug that gets to the plasma (Isavadharm *et al*., 2001) thus showing the relevance of the value in clinical situation.

The partition ratio estimation shows that artesunate could also not only inhibit the activity of myeloperoxidase but could completely inactivate the enzyme thus, artesunate could cause an irreversible damage to the Polymorphonuclear neutrophils function

The inhibition of myeloperoxidase in the presence of artesunate is pH-dependent and the drug can inhibit the enzyme effectively at physiological pH. This therefore suggests that artesunate is a highly likely to contributes to the impairment of the functions of the polymorphonuclear neutrophil in the leucocytes, since the inhibition of myeloperoxidase (an important enzyme in the neutrophil) is highly favoured at physiological pH.

The trend of results observed in the presence of artesunate (figure 4) indicated that the drug is a competitive inhibitor of myeloperoxidase. This is likely to suggests that the drug may have attached to the substrate-binding portion of the active site and subsequently blocked the access to the substrates. This shows that they both likely compete with substrate at the active site of the enzyme. The low Ki values when compared with Km of the substrate also reveal that artesunate is an effective inhibitor of myeloperoxidase. Some drugs such as dapsone and sulphapyridine which have structural resemblance with artesunate have been reported to show similar pattern of inhibition of myeloperoxidase (Kettle *et al*., 1993). It was revealed that dapson and sulphapyridine showed competitive inhibition of the enzyme by converting it to oxyferryprotein (compound II). This might be true for artesunate since they possess similar structural features.

This study therefore shows that the inhibition of myeloperoxidase in the presence of artesunate may partly explain the impairment of polymorphonuclear neutrophil and consequent reduction of the strength of the host defense system against secondary infections.

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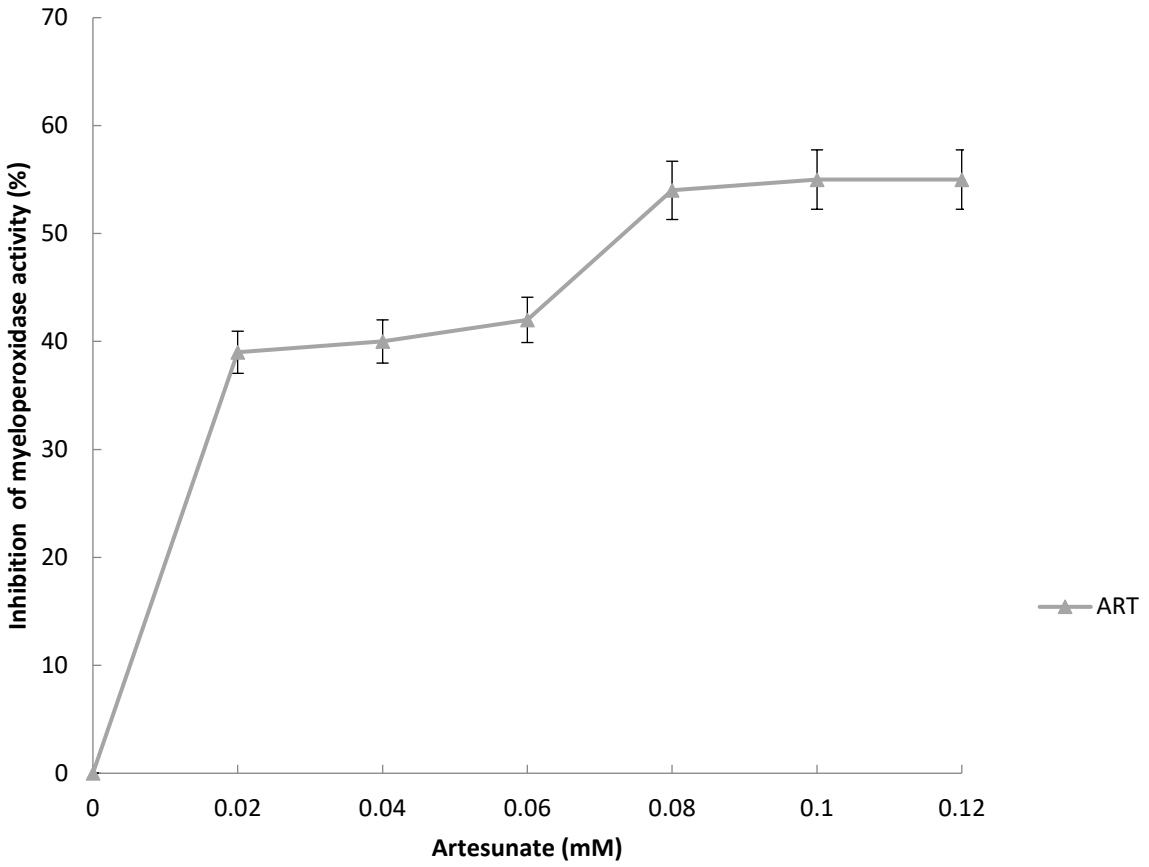

Figure 1:Effect of different concentrations of artesunate(ART) on purified human myeloperoxidase
Each plotted value is a mean of four determinations **± SD**

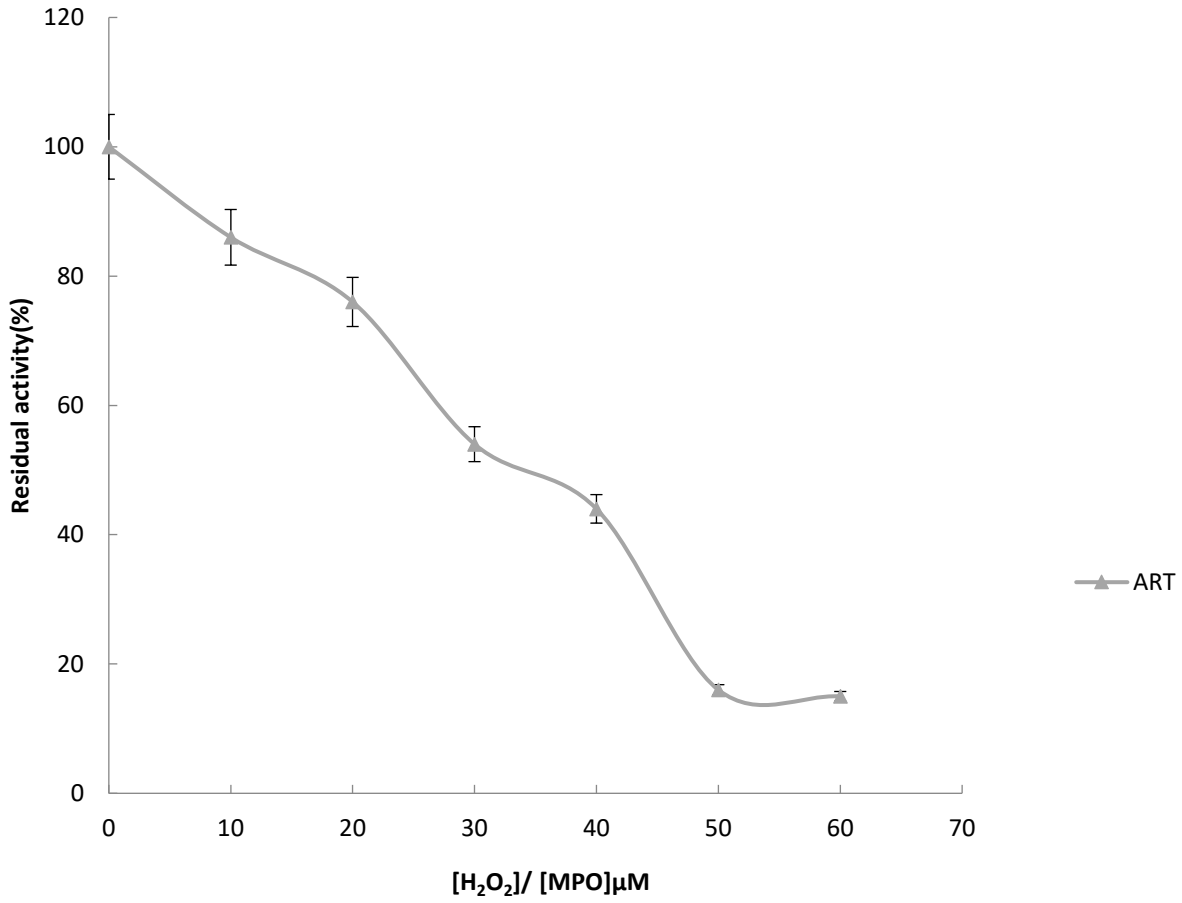

Figure2:Percentage partition ratio of artesunate (ART) on purified human myeloperoxidase
Each plotted value is a mean of four determinations **±SD**

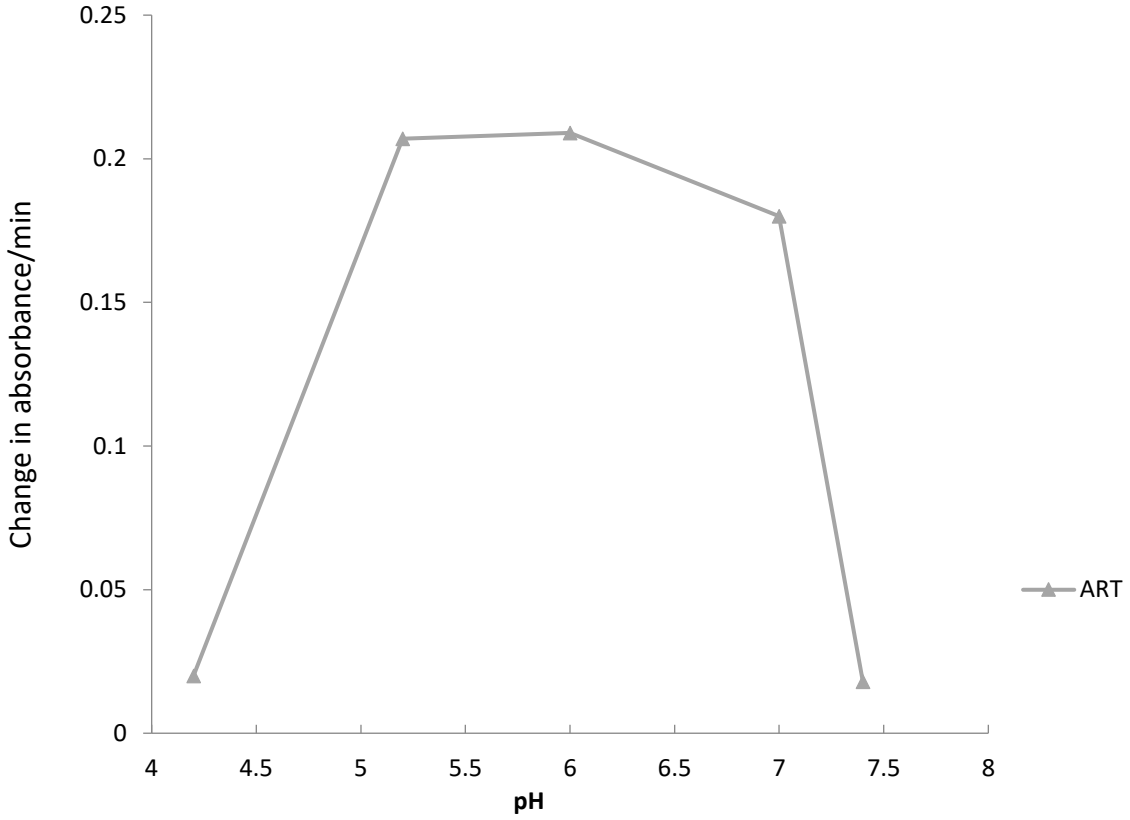

Figure3:pH-dependent effect of artesunate (ART) concentrations on purified human myeloperoxidase
Each plotted value is a mean of four determinations **± SD**

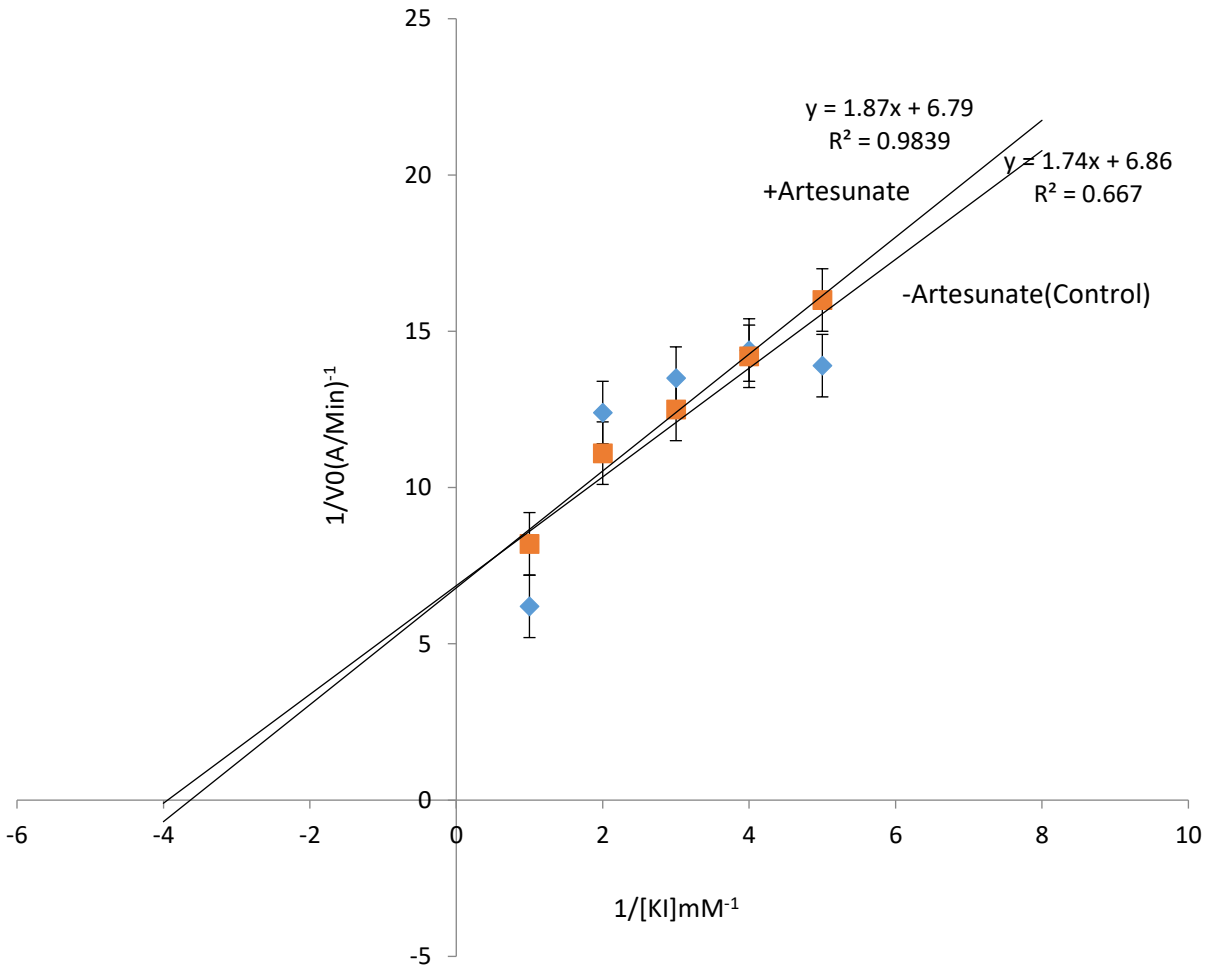

Figure4:Double reciprocal plot of purified human myeloperoxidase activity using potasium iodide as substrate
Each plotted value is a mean of four determinations **±SD**