ORIGINAL ARTICLE

Artesunate opens mitochondrial membrane permeability transition pore

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SUMMARY

The incidence of malaria is dramatically increasing, especially because parasites responsible for the majority of fatal malaria infections have now become resistant to commonly used antimalarial drugs such as chloroquine, mefloquine, and quinine. To combat this menace, the World Health Organization (WHO) introduced a new antimalarial drug called artesunate; a hemi-succinate derivative of artemisinin. The in vivo effects of artesunate on rat liver mitochondrial membrane permeability transition (MMPT) pore were investigated in Wister strain albino rats exposed to various doses of artesunate (1.5, 2.0, 3.0 and 5.0 mg per kg body weight per day) for five days. Membrane permeability transition was estimated under energized and de energized spectrophotometric method of Lapidus and Sokolove. The results revealed that artesunate tested at the various doses induced mitochondrial pore opening, induction being minimal (68%) at 5 mg/kg and maximal (240%) at 1.5 mg/kg. *In vitro*, artesunate at 30, 50 and 70 mg/ml also had an inductive effect in a concentration-dependent manner with minimum induction (18.1%) at 30 mg/ml and maximum induction (32.7%) at 70 mg/ml. Further, preincubation of mitochondria with artesunate for five minutes caused an induction of pore opening in a concentration-dependent manner, with minimum induction (7.9%) at 10 mg/ml and maximum induction (48.6%) at 70 mg/ml. In conclusion, these findings indicate that artesunate could be cytotoxic, opening mitochondrial membrane permeability transition pore, causing the release of cytochrome c and eventually apoptosis.

Key words: Artesunate, mitochondria permeability transition pore, mitochondria, oxidative stress, liver

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Introduction

The magnitude of malaria disease burden in Africa and South-East Asia is now of global concern as an estimated 1.5-2.7 million deaths occur every year with the highest mortality (more than 90%) in children less than five years of age.[1] Consequently, with the alarming spread of multidrug resistant *Plasmodium falciparum* WHO predicts that the number of people suffering from malaria will double by the year 2010 if new antimalarial strategies were not adopted.[2] Thus to circumvent this phenomenon of drug resistance, a team of Chinese researchers in 1971 discovered by extraction at low temperature from *Artemisia annua* "qinghao," a crystalline compound that they named quinghaosu (the name artemisinin is preferred by chemical abstract, RN 63968-64-9).[3] Artemisinin is a sesquiterpene lactone containing an endoperoxide bridge representing the active moiety of the molecule. Cleavage of this bridge generates organic free radicals. The radical molecules cause macromolecular damage by covalently alkylating and poisoning one or several malarial proteins.[4] Artemisinin is highly crystalline and does not dissolve in polar and nonpolar solvents. Hence it is chemically modified to yield these derivatives: artesunate, artemether, arteether, artelinic acid and dihydroartemisinin. The artemisinin class of antimalarial compounds has proven to be effective against multidrug–resistant *P. falciparum* and *P. vivax* strains.[5]

Interestingly, neurotoxicity of artesunate has been shown to occur in animal models after prolonged treatment with supratherapeutic doses.[6] The cytotoxic effects of this antimalarial *in vivo* and *in vitro* have been previously reported by Efferth *et al*.[7] These workers demonstrated that artesunate induced reactive oxygen species ROS-mediated apoptosis through the apoptotic intrinsic pathway (mitochondria-mediated). Therefore, the present study was set to investigate if artesunate would exhibit the same effect in mitochondria of normal

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cells through mitochondrial membrane permeability transition pore (MMPT) pore given the fact that the antimalarial has now become a drug of choice in the treatment of malaria fever.

Experimental Procedures

Animals

Male albino rats (Wister Strain) each weighing between 120-250 g were obtained from the preclinical Animal House, Faculty of Basic Medical Sciences, University of Ibadan, Ibadan, Nigeria. The animals were allowed to acclimatize for 15 days in the Animal House, Biochemistry Department, College of Medicine University of Ibadan. They were given water and rat chow *ad libitum,* and kept under standard condition of temperature and 12-hour dark\ light cycle.

Drug and reagents

Artesunate tablets were obtained from the Pharmacy Department of the University College Hospital, Ibadan. All chemicals and reagents were of the highest purity grade and purchased from Sigma-Aldrich Chemical Co., USA.

Experimental design

In the *in vivo* study, the experimental animals were divided into five groups of four per group. Groups I to IV were the treatment groups while Group V was control group. Various doses of artesunate (1.5, 2.0, 3.0 and 5.0 mg/kg/day) was orally administered (by intubation) to animals in groups I-IV while distilled water was given to the control group for 5 consecutive days. The animals had free access to water and purina chow throughout the period of the experiment and were subsequently sacrificed 24 hours after the last dose on the 5th day. For the *in vitro* study, mitochondria were isolated from the livers of normal rats and exposed to various concentrations of artesunate (30, 50 and 70 mg/ml) for swelling assay. In the case of preincubation, the mitochondria were incubated with various concentrations of artesunate (10, 30, 50 and 70 mg/ml) for 5 mins prior to swelling assay.

Isolation of rat liver mitochondria

Low ionic strength mitochondria were isolated according to the method of Schneider[8] and as modified by Johnson and Lardy.[9] Excised livers from Wister strain rats were minced in ice-cold homogenization buffer pH 7.4 (210 mM Mannitol, 70 mM sucrose, 5 mM Hepes-KOH and 1 mM EGTA) and centrifuged twice at 1,000 g for 5 mins at 4^{o}C to remove cell debris and the nuclear fraction. Mitochondria were isolated by subjecting the supernatant to centrifugation at 10,000 g for 10 mins at 4^{o}C. The pellet obtained was re-suspended in the isolation buffer (210 mM Mannitol, 70 mM sucrose, 5 mM Hepes-KOH, 0.5% BSA, pH 7.4) and then centrifuged at 10,000 g for 10 mins. This was done twice to eliminate extraneous debris. The mitochondria were immediately suspended in a solution of ice-cold MSH buffer (210 mM Mannitol, 70 mM Sucrose, 5 mM Hepes-KOH pH 7.4) then dispensed in Eppendorf tubes as aliquots and placed on ice. Fresh mitochondria were used for each experiment. Protein concentration was determined according to Lowry's method.[10] using bovine serum albumin (BSA) as standard.

Mitochondrial swelling assay

The sensitivity of the mitochondrial membrane transition pore (swelling) was determined by studying the rate of change in absorbance at 540 nm under energized and de energized conditions using SpectrumLab 752S uv-visible spectrophotometer.[11] Mitochondria (0.4 mg/ml) in MSH buffer were preincubated in the presence of 0.8 μM Rotenone and were energized with 5 mM sodium succinate to support swelling using Ca^{2+} and spermine as positive and negative control, respectively.

Statistical analysis

This was carried out with the aid of SPSS for windows; SPSS Inc., Chicago, Standard version 16.0 to determine difference between mean using One Way Analysis of Variance (ANOVA).

Results

The mitochondria of the control animals which were not administered artesunate were found to be intact as there was no swelling (pore opening) in the absence of triggering agent whereas a considerable swelling was observed when Ca^{2+} was used as the triggering agent. In this case, calcium induced opening of the mitochondrial permeability transition pore was inhibited by spermine as shown in [Figure 1]. However, the mitochondria of the artesunate-treated animals exhibited significant ($P < 0.05$) opening of the MMPT pore [Table 1] in a decreasing order of dose (5.0, 3.0, 2.0 and 1.5 mg per kg body weight) when compared with the mitochondria of control animals [Figure 2]. MMPT induction was minimal (68%) at 5 mg/kg and maximal (240%) at 1.5 mg/kg [Table 1].

In vitro, artesunate at 30, 50 and 70 mg/ml also had inductive effect on the MMPT pore in a concentration-dependent manner with minimum induction (18.1%) at 30 mg/ml and maximum induction (32.7%) at 70 mg/ml [Figure 3] and was found to be statistically significant at $P < 0.05$ [Table 1].

Table 1: The percentage and extent of MMPT induction by artesunate

Mitochondria (Absence of Ca2+)	% MMPT Induction (Swelling)	Extent of MMPT induction (Swelling) ART: Control (untreated)
Control (Untreated)	50	
1.5 mg/kg ART per day (*in vivo*)	240	5 × Control
2 mg/kg „	182	(Approximate)*
3 mg/kg „	123	4 × Control „
5 mg/kg „	68	3× Control „
		1 × Control „
Control (Untreated)	5.2	
30 mg/ml ART (*in vitro*)	18.1	3 × Control
50 mg/ml „	24.3	(Approximate)*
70 mg/ml „	32.7	5 × Control „
10 mg/ml ART (pre-incubation)	7.9	6× Control „
30 mg/ml „	20.6	2 × Control
50 mg/ml „	27.5	(Approximate)*
70 mg/ml „	48.6	4 × Control „
		5 × Control „
		9 × Control „

ART-indicates artesunate; MMPT-indicates mitochondrial membrane permeability transition. *Indicates a statistically significant difference at $P < 0.05$

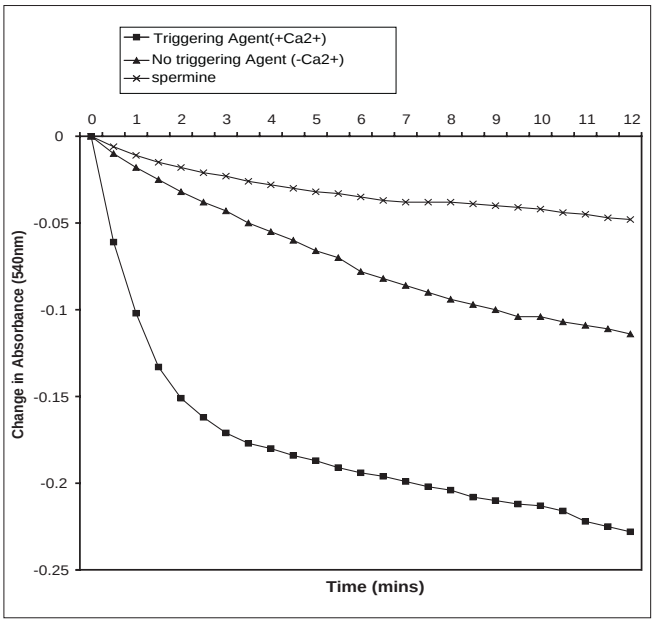

Figure 1: Opening of the MMPT pore of succinate-energenized mitochondria monitored as swelling at Δ540 nm for 12 mins in the presence and absence of triggering agent (Ca2+) and 0.1 mM spermine

Further, preincubation of mitochondria with artesunate for 5 mins caused an induction of pore opening in a concentration-dependent manner, also significant at $P < 0.05$ [Figure 4] with minimum induction (7.9%) at 10 mg/ml and maximum induction (48.6%) at 70 mg/ml [Table 1].

Discussion

In most tropical countries such as Sub-Saharan Africa, Southeast Asia and South America, multidrug resistance has become an increasing problem and poses a threat to the current management of malaria with inexpensive drugs such as chloroquine and pyrimethamine/sulfadoxine.[12] This has led to the use of sodium artesunate, an endoperoxide drugs derived from artemisinins with proven evidences to be extremely effective against multidrug-resistant *P. falciparum* malaria and severe malaria.[13]

In the present study, orally administered artesunate was observed to have induced mitochondrial membrane permeability transition (MMPT) pore opening in the livers of rat exposed to various doses of artesunate at 1.5, 2.0, 3.0 and 5.0 mg/kg body weight for five days. This was equivalent to the range of artesunate dose regimen (2 to 5 mg/kg/day) used as therapy for uncomplicated severe *P. falciparium* induced malaria as reported by Utzinger *et al*.[14] 1.5 mg/kg artesunate, equivalent of a single therapeutic dose normally taken by humans (100 mg / 70 kg/day body weight) was found to have the highest MMPT induction. This was further buttressed by

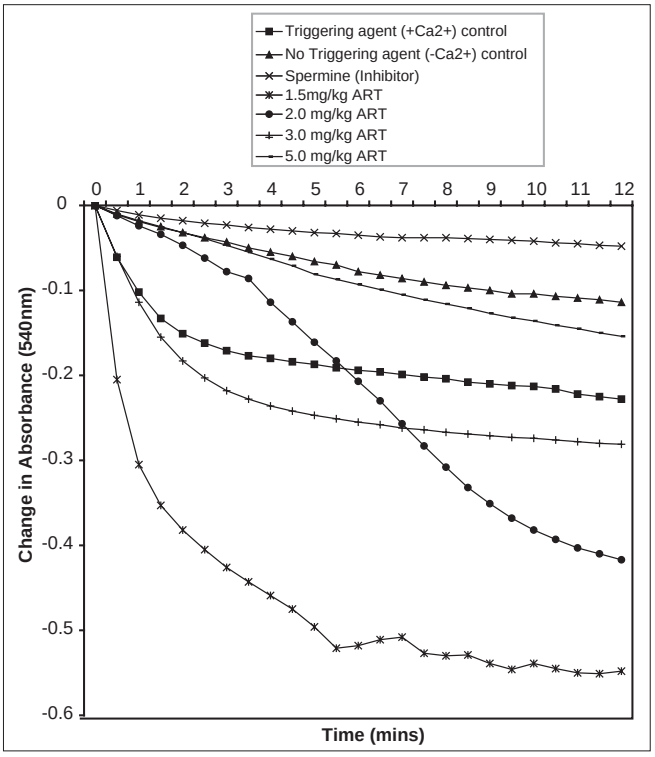

Figure 2: *In vivo* opening of the MMPT pore of succinate-energenized mitochondria monitored as swelling at Δ540 nm for 12 mins by various doses of artesunate (1.5, 2.0, 3.0 and 5.0 mg/kg body weight) in the presence and absence of triggering agent (Ca2+) and 0.1mM spermine.

in vitro and pre-incubation studies. These findings were found to be in agreement with previous works reported by Meshnick *et al*.[15] and Posner *et al*.[16] stating that the antimalarial activity of artemisinin and its derivatives

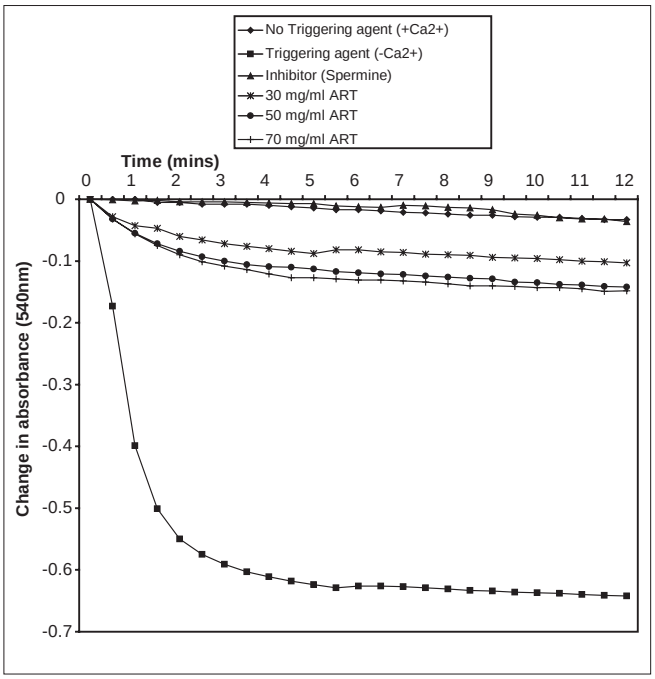

Figure 3: *In vitro* opening of the MMPT pore of succinate-energenized mitochondria monitored as swelling at Δ540 nm for 12 mins by various doses of artesunate (30, 50 and 70 mg/ml) in the presence and absence of triggering agent (Ca2+) and 0.1mM spermine

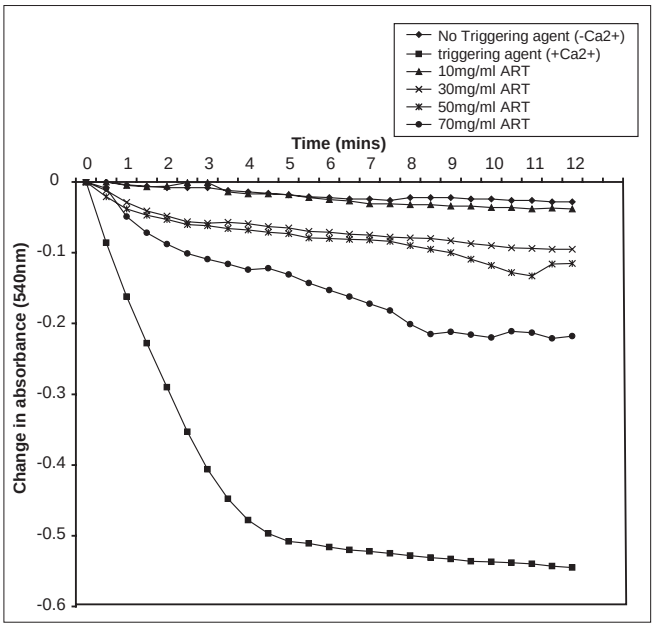

Figure 4: *In vitro* (pre-incubation for 5 mins) opening of the MMPT pore of succinate-energenized mitochondria monitored as swelling at Δ540nm for 12 mins by various doses of artesunate (10, 30, 50 and 70 mg/ml) in the presence and absence of triggering agent (Ca2+) and 0.1mM spermine.

depend on the cleavage of an endoperoxide bridge leading to the generation of carbon-centered free radicals and oxidative stress. Coincidentally, free radicals and oxidative stress had been implicated in mitochondrial membrane permeability transition pore opening[17] while the potent antimalarial activity of artesunate is due to its ability to generate free radicals.[18] This suggests that this property of artesunate could have a negative effect on mitochondrial membrane permeability transition by causing the ROS-mediated opening of the MMPT pore and consequently the release of cytochrome c which will trigger the apoptotic process. Furthermore, this study also supports the view that artesunate could serve as a cytotoxic agent against cancerous cells[7,19] as well as antiangiogenic effect reported by Chen *et al*.[20] because artesunate induces ROS-mediated apoptosis in doxorubicin-resistant T leukemia cells.

However, with increase in dose of artesunate, there was a decrease in MMPT pore opening. This suggests that the cytotoxic property of artesunate could be concentration dependent and the claim that artesunate was considered a safe drug with no obvious adverse reactions or noticeable side effects, even when given to children[21] is questionable. This study concludes that the artesunate could possibly also have an effect also on normal mitochondrial membrane permeability transition leading to pore opening and then causing apoptosis.

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