American Journal of

Biochemistry and Molecular Biology

ISSN 2150-4210

Academic
Journals Inc.

www.academicjournals.com

American Journal of Biochemistry and Molecular Biology 3 (1): 110-118, 2013
ISSN 2150-4210 / DOI: 10.3923/ajbmb.2013.110.118

Induction and Uncoupling of Rat Liver Mitochondria by Oral Administered Coartemether

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ABSTRACT

This study investigated the cytotoxic effect of varying doses of antimalarial coartemether (2.0, 4.0, 8.0 and 10.0 mg kg^{-1}) in the presence of ferrous sulphate (2800 mg kg^{-1}) for 3 days on normal rat liver mitochondrial membrane permeability transition pore opening and F_1F_0 ATPase activity. Swelling was estimated spectrophotometrically under succinate energized condition. Calcium ion treated mitochondria preloaded with coartemether induced swelling in a concentration dependent manner *in vitro*. Swelling was amplified in the presence of ferrous. Coartemether alone and combined coartemether-ferrous also induced mitochondrial swelling in the presence of spermine. *In vivo* study further showed that 10.0 mg kg^{-1} coartemether induced swelling in the presence of ferrous. Coartemether stimulated an increased activity of mitochondrial F_1F_0 ATPase in a concentration dependent manner. Thus, these findings indicate that coartemether at high dose in the presence of ferrous sulphate could be an inducer of mitochondrial mega pore opening and an uncoupler of oxidative phosphorylation initiating apoptosis.

Key words: Coartemether, mitochondria, F_1F_0 ATPase, rat, liver

INTRODUCTION

Coartemether or Coartem® (artemether-lumefantrine) is currently the most viable artemisinin combination therapy specifically indicated for the treatment of acute, uncomplicated malaria infections due to *Plasmodium falciparum* in patients of 5 kg b.wt. and above (WHO, 2006; Premji, 2009).

Coartemether is a fixed-dose combination tablet of 20 mg artemether and 120 mg lumefantrine in a ratio of 1:6 (Novartis Pharma AG., 2009). One of its components, artemether is a semisynthetic chiral acetal derivative of artemisinin that interferes with parasite transport proteins, disruption of mitochondrial function, inhibits angiogenesis and modulates host immune function. While, lumefantrine is a racemic mixture of a synthetic fluorine derivative formerly known as benflumetol and is structurally related to quinine, mefloquine and halofantrine. It interferes with the conversion of heme, the toxic intermediate produced during hemoglobin break-down to non-toxic hemozoin. The accumulation of heme and free radicals results in parasite death (Byakika-Kibwika *et al*., 2010).

Artemether, like other artemisinin-derived compounds, acts quickly to rapidly reduce the parasite burden, while lumefantrine serves as a longer-acting agent to eliminate remaining parasites. The combination is effective in parasite strains known to be resistant to traditional antimalarials such as chloroquine (Mwesigwa *et al*., 2010). Artemether is largely metabolized by cytochrome P450 (CYP) 3A4/5 but also by CYP2B6, CYP2C9 and CYP2C19. Metabolism through CYP3A4 produces an active metabolite, dihydroartemisinin (DHA) that contributes substantially to its antimalarial activity (Cousin *et al*., 2008). Lumefantrine is metabolized primarily by CYP3A4 and then undergoes glucuronidation (Hietala *et al*., 2010; Mwesigwa *et al*., 2010).

The highly reactive endoperoxide moiety in artemisinins is thought to be crucial for their mode of action but the exact mechanism remains controversial (Del Pilar Crespo *et al*., 2008). Several models have been proposed including a Fenton-type reaction where artemisinins generate reactive oxygen species and carbon-centered radical molecules that modify proteins of *Plasmodium* parasites. Other studies suggest that artemisinin inhibit the Ca^{2+}-dependent SERCA-like ATPase PfATP6 upon activation by Fe^{2+} from hemoglobin. Another mechanism is the disruption of the mitochondrial membrane potential as suggested from data of yeast model (Li *et al*., 2005).

Mitochondrial respiration depends on the flow of electrons through four oligomeric respiratory complexes that comprise the electron transport chain. The energy released by electron flow through the respiratory complexes is conserved in an electrochemical potential consisting of a proton gradient and membrane potential produced by the coupled translocation of protons through the inner mitochondrial membrane at Complexes I, II and IV. Energy stored in the electrochemical potential is coupled to ATP synthesis by translocation of protons into the mitochondrial matrix through complex V (ATP synthase) (Brand and Nicholis, 2011).

The list of described inducers of the Ca^{2+}-dependent mitochondrial mega channel (Membrane Permeability Transition (MPT) pore, is long and includes many different chemical and physical factors all synergistic to Ca^{2+}(Gunter and Pfeifer, 1990; Zoratti and Szabo, 1995). As a result of MPT pore opening intramitochondrial solutes of molecular mass lower than 1.5 kDa equilibrate with those in cytosol (Zoratti and Szabo, 1995). This is accompanied by the activation of mitochondrial respiration, the loss of ions accumulated in the matrix and high amplitude swelling of mitochondria. Recent findings suggest MPT involvement in either programmed mitochondrial destruction (Zorov *et al*., 1992; Brand and Nicholis, 2011) and hence, in mitochondrial selection (Skulachev, 1996) or in programmed cell death (Zamzami *et al*., 1995). The mitochondrial contribution to apoptosis opens a vast field for investigating new mitochondrial related diseases.

Several studies have tested coartemether and its components in a complete range of acute and subchronic animal toxicology studies, including reproductive toxicology, genotoxicity and juvenile animal studies (Raji *et al*., 2005; Efferth and Kaina, 2010; Onyesom and Agho, 2011). Mechanistic neurotoxicity studies were performed in both rats and dogs to evaluate functional and histopathologic changes (Oyemitan *et al*., 2007; Cousin *et al*., 2008; Ajibade *et al*., 2011). Despite these findings there is a paucity of large-scale clinical trials suitable to detect rare but significant toxicity especially when artemisinin combine therapy is prescribed alongside with ferrous in severe anemia conditions. Although, Mpiana *et al*. (2007) have reported that endoperoxide lactone based drugs could form complexes with heme and hemin. Therefore, attempts had been made to further investigate the effect of cytotoxic effect of coartemether on mitochondrial integrity.

MATERIALS AND METHODS

Chemicals: Coartemether tablets (Norvatis, Pharma AG, Switzerland), ferrous sulphate tablets (Pharmacy unit, University College of Health, Ibadan, Nigeria), mannitol, sucrose (BDH Chemicals

Ltd; Pools, England), HEPES (May and Baker Lab; USA), EGTA, bovine serum albumin (Sigma Chemical Co; USA), spermine (Research Biochemical, USA) and all other reagents used were of analytical grade.

Animal: Male albino Wistar rats (120-150 g) were obtained from Preclinical Animal House, Physiology Department, University of Ibadan, Ibadan, Nigeria. The animals were maintained in cages acclimatized for two weeks in accordance to good laboratory animal care practice at the departmental animal house. Tap water and commercial pelleted feed were provided under standard conditions of temperature 28±2°C and a 12 h light/dark cycle.

Experimental design: Oral coartemether tablets were dissolved in sunflower oil as vehicle and administered orally to the test groups using an oral dosing needle for 3 days in accordance to WHO recommendation for treatment of uncomplicated malaria. Animals were assigned randomly into six groups of three rats each. Group I: untreated normal rats; group II: (control) normal rats were given oral sunflower oil (1 cm^3), group III-VI were coadministered coartemether (2.0, 4.0, 8.0 and 10.0 mg kg^{-1} body weight) and ferrous sulphate (4.3 mg kg^{-1} b.wt.) respectively. The animals were euthanized 24 h after fasting overnight by cervical dislocation at the end of treatment. Subsequently, the rat liver was excised, trimmed of excess tissue and subjected through the standard protocol for mitochondria isolation. Mitochondria isolated from Group I were preloaded with varying concentrations of coartemether (600, 1200, 2400 and 3000 μg mL^{-1}) and ferrous sulphate (1280 μg mL^{-1}) to assess the *in vitro* effects on mitochondria MPT.

Mitochondrial isolation: Rat liver mitochondria isolated by conventional differential centrifugation in a buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH 7.4) and 1 mM EGTA (Schneider and Hogeboom, 1950); EGTA was omitted in the final wash solution. Protein content was estimated by Folin-Ciocalteu method using Bovine Serum Albumin as standard (Lowry *et al*., 1951).

Assessment of mitochondrial swelling: Mitochondrial swelling was assessed according to the method of Lapidus and Sokolove (1993). Changes in absorbance of mitochondria were monitored at 540 nm in a 6405 Jenway UV-visible spectrophotometer. Mitochondria (0.4 mg mL^{-1}) were suspended in a medium containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH (pH 7.4), 0.8 μM rotenone and 5 mM succinate. Swelling was triggered by Ca^{2+} while spermine serves as an inhibitor.

Assessment of mitochondrial F_1F_0 ATPase activity: Mitochondria isolated from untreated normal rats in another set of experiment were preloaded with varying concentrations of 10.0, 20.0, 30.0, 40.0, 50.0 and 60.0 mg mL^{-1} coartemether to assess the effects on mitochondrial F_1F_0 ATPase activity. Mitochondrial adenosine triphosphatase or F_1F_0 ATPase activity was determined by a modified method of Lardy and Wellman (1953). Each reaction vessel contained 65 mM tris-HCl (pH 7.4), 1 mM ATP and 25 mM sucrose. The reaction was started by the addition of mitochondrial fraction (0.4 mg mL^{-1}) vortex for 30 min at 25°C. The reaction was stopped by the addition of 8 mL of 10% trichloroacetic acid to each test tube then centrifuged at speed of 3000 g. The deproteinized supernatant was kept for phosphate determination.

Determination of inorganic phosphate: This was performed according to the method described by Fiske and Subbarow (1925) modified by Bababunmi and Bassir (1972). 0.4 mL of perchloric acid was added to 5.0 mL of the deproteinized supernatant in a test tube. This was followed by addition of 0.4 mL of 5% ammonium molybdate and 0.2 mL of a 0.2% freshly prepared solution of ascorbic acid. The tube was thoroughly mixed, gently shaken and allowed to stand for 20 min. A standard solution of potassium dihydrogen phosphate (0.2 mg Pi per 5 mL) was similarly treated. The intensity of the blue colour which developed was read at 680 nm using a spectrophotometer. Water blank was used to set the instrument at zero.

Calculation:

$$P_i\ (mg\ mL^{-1}) = \frac{\text{Reading of test} \times 0.02 \times 1}{\text{Reading of standard} \times 0.001}$$

$$P_i \text{ released mole} = \frac{\text{Pi mg mL}^{-1} \times 1000}{\text{Molecular weight of Pi} \times 1}$$

Mole P_i released mL^{-1} mitochondrial protein is given by the expression:

$$\frac{\text{Mole } P_i \text{ released}}{\text{mg mitochondrial protein} \times 30}$$

RESULTS

Triggering agent (Ca^{2+}) induced MPT or swelling in a succinate energized rat liver mitochondria under a normal respiration sucrose-phosphate buffer. However, mitochondrial membrane was intact without any observable swelling in the absence of a triggering agent (Fig. 1). *In vitro* study showed that mitochondria preloaded with 600, 1200, 2400, 3000 μg mL^{-1} coartemether induced opening of the MPT pore in a concentration dependent manner with minimal and maximal swelling inductions at 600 and 3000 μg mL^{-1} coartemether, respectively. Conversely, ferrous sulphate at 1280 μg mL^{-1} did not induced mitochondria swelling (Fig. 1). However, coartemether preloaded mitochondria in the presence of ferrous showed a significantly large amplitude ($p<0.05$) of swelling in a concentration dependent manner (Fig. 2).

Spermine inhibited swelling induced at low concentration of coartemether (600 μg mL^{-1}) and ferrous while high concentration of coartemether (3000 μg mL^{-1}) and ferrous induced mitochondria swelling in the presence of spermine (Fig. 3).

In the animal study, co-administered ferrous sulphate (4.3 mg kg^{-1}) and coartemether at 2.0, 4.0, 8.0 mg kg^{-1} did not induce mitochondria MPT both in the absence Ca^{2+}. However, there was significantly high ($p<0.05$) amplitude of liver mitochondria swelling in the animals co-administered with 10 mg kg^{-1} coartemether and ferrous sulphate (4.3 mg kg^{-1}) (Fig. 4).

The result also revealed that varying concentrations of coartemether (10, 30, 50 and 60 mg mL^{-1}) elevated the mitochondrial F_1F_0 ATPase activity in a concentration dependent manner with minimal (104.4 mol P_i/mg protein/ min) and maximal (146.4 mol P_i /mg protein/ min) activities at 10 and 60 mg mL^{-1}, respectively (Table 1).

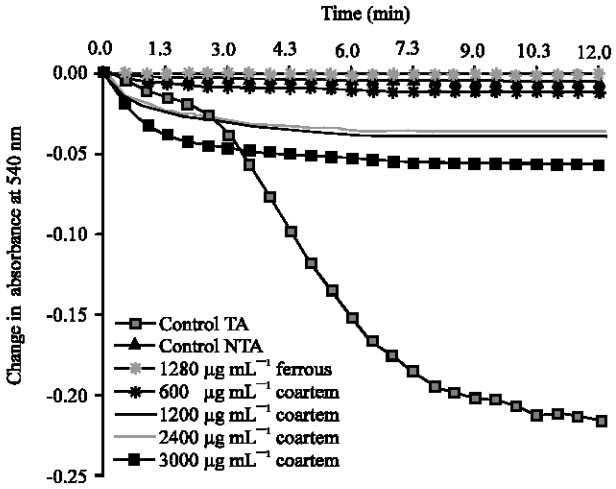

Fig. 1: Change in absorbance (540 nm) for 12 min by ferrous sulphate (Fe^{2+}), varying concentrations of coartemether on mitochondrial membrane permeability transition pore in the energized by sodium succinate, TA: Triggering agent, NTA: Non triggering agent

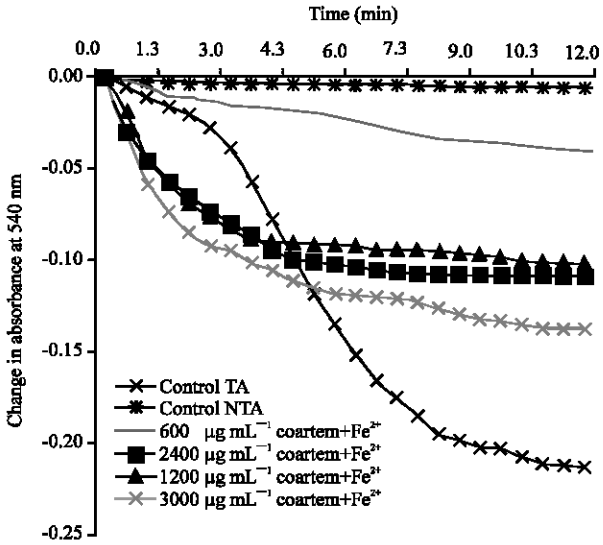

Fig. 2: Change in absorbance (540 nm) for 12 min by varying concentrations of coartemether in the presence of ferrous sulphate on mitochondrial membrane permeability transition pore energized by sodium succinate, TA: Triggering agent, NTA: Non triggering agent

Table 1: The Hydrolysis of ATP by mitochondrial F_1F_0 ATPase by varying concentrations of coartemether

Concentration (mg mL^{-1})	mg P_i released (mL^{-1})	Mole P_i released	Mole P_i released (mL^{-1})
10	17.0	125.2	104.3
20	19.0	139.4	116.2
30	19.6	144.1	120.0
40	20.7	152.2	126.8
50	23.5	172.9	144.1
60	23.9	175.7	146.4

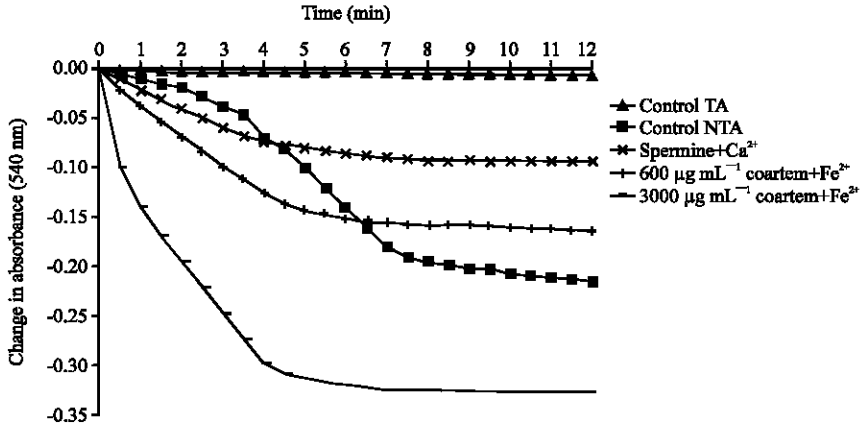

Fig. 3: Change in absorbance (540 nm) for 12 min by two concentrations of coartemether (lowest and highest) combined with ferrous sulphate on mitochondria permeability transition pore in the presence of spermine energized by sodium succinate, TA: Triggering agent, NTA: Non triggering agent

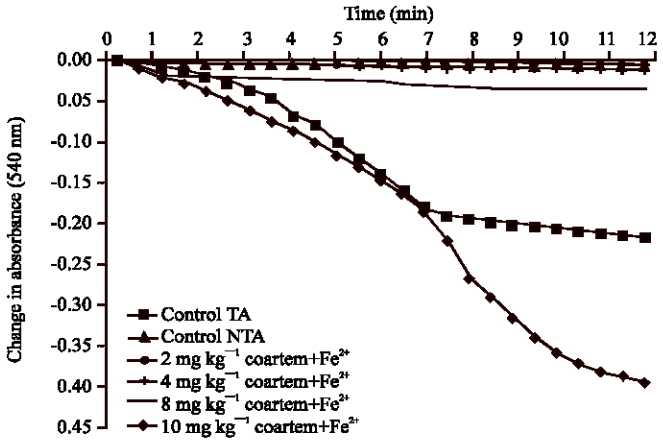

Fig. 4: Change in absorbance (540 nm) for 12 min *in vivo* effect of co-administered Fe^{2+} (4.3 mg kg^{-1}) and varying dose of coartemether on mitochondria permeability transition pore in the absence of a triggering agent energized by sodium succinate, TA: Triggering agent, NTA: Non triggering agent

DISCUSSION

In the present study, rat liver mitochondria preloaded with varying concentrations of coartemether *in vitro* induced mitochondrial Membrane Permeability Transition (MPT) pore in a concentration dependent manner. This supports the theory implicating mitochondrial MPT pore formation (swelling) as the possible mode of action for artemisinin-related compounds (Eckstein-Ludwig *et al*., 2003; Wang *et al*., 2010). Studies had shown that the parasiticidal activity of artemether resides on the peroxyl ring of dihydroxyartemisinin metabolite (Krishna *et al*., 2004; Efferth and Kaina, 2010). The cleavage of an endoperoxide bridge generates free radicals (Cumming *et al*., 1997). Free radicals have been associated with mitochondrial MPT pore opening (Del Pilar Crespo *et al*., 2008). Mitochondrial membrane permeabilization results in the release of

cytochrome c that trigger apoptosis by caspase-cascade pathways, consequently leading to cell death (Galluzi *et al*., 2009). Furthermore, the addition of varying concentrations of coartemether to the calcium ion preloaded mitochondria in the presence of ferrous sulphate further increased the swelling of mitochondria. Previous studies reported that iron bioactivates artemisinin into a free radical through an iron-mediated cleavage (Dhingra *et al*., 2000; Balint, 2001; Noori *et al*., 2004). This study also showed that coartemether in the presence of iron could serve as a cytotoxic agent against cancerous cells. Other studies had proved that the combined administration of dihydroxyartemisinin and iron retarded the growth rate of tumor (Moore *et al*., 1995).

The induction of mitochondrial MPT by coartemether at high concentrations in the presence of a natural inhibitor spermine *in vitro* suggests that the cytotoxic effect of coartemether could be at high concentrations.

Animal study showed that coartemether at the therapeutic dose range of 2-4 mg kg^{-1} b.wt. did not induce mitochondrial swelling in the absence of a calcium ion. This seems to be in agreement with Norvatis Pharma claim on the clinical safety of the therapeutic dose range (Novartis Pharma AG., 2009). This observation might have resulted from the action of CYP3A4 isoenzyme metabolizing and degrading the total amount of drug that reaches the receptor/active site to elicit a pharmacological response (Cousin *et al*., 2008; Mwesigwa *et al*., 2010). However, swelling was induced at high dose of 10 mg kg^{-1} b.wt. coartemether.

Further studies indicated that coartemether elevated the hydrolysis of ATP to ADP and inorganic phosphate by mitochondrial F_1F_0 ATPase in a concentration dependent manner. This was determined spectrophotometrically by the increase in concentration of released inorganic phosphate P_i. This observation could also be accounted through the induction of mitochondrial MPT by coartemether *in vitro* which have already compromised the intactness of the mitochondria. The mitochondrial F_1F_0ATPase or ATP synthase is known to harnesses the proton gradient generated during the transfer of electron along the respiratory chain and couples it to the oxidative phosphorylation of ADP and inorganic phosphate to produce ATP required for diverse biochemical and cellular functions (Vinogradov, 2000; Nelson and Cox, 2008). The collapse in mitochondrial electrochemical gradient could result in the hydrolysis of ATP by F_1F_0-ATPase for the proton gradient recovery. This turns F_1F_0-ATPase into a consumer rather than being a producer of ATP in failing cells (Zablockaite *et al*., 2007; Nelson and Cox, 2008).

CONCLUSION

This study indicates that coartemether at high dose in the presence of iron could have a profound cytotoxic effect on mitochondrial membrane permeability transition pore and it also could serve as an uncoupler of mitochondria respiration. However, additional studies are required to probe further into the effect of coartemether on cytochrome c release and mechanisms associated with apoptosis to gain more insight into the toxicological pathway.

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