Induction of cell cycle arrest and apoptosis in JAR trophoblast by antimalarial drugs

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ABSTRACT

Chloroquine, quinine, artemisinin, and pyrimethamine are generally considered safe drugs for treatment of malaria during pregnancy; however, high doses of these drugs are detrimental with adverse outcome of pregnancy. Since antimalarial drugs interaction with placental cells has not been addressed, in this study, we employed a non-radioactive proliferation assay and lactate dehydrogenase (LDH) release assays to investigate the effect of these drugs on JAR trophoblastic cell survival. All drug treatment resulted in inhibition of cell proliferation in a dose-dependent fashion \((p < 0.05)\) with IC50 at 6.96, 6.49, 6.69, and 6.89 \(\mu\)g/mL for chloroquine, quinine, artemisinin and pyrimethamine, respectively. In addition, the inhibition of cell proliferation was accompanied by increased cytotoxicity. Analysis of the progression of the cell cycle showed that these drugs triggered G0/G1 and S phase arrest. Furthermore, these antimalarial drugs induced apoptotic cell death as visualized by DNA fragmentation analysis techniques. Findings in this study revealed that cytotoxicity of these drugs on human placental trophoblast is mediated by both cell cycle arrest and induction of cell death and this could have important implications for the use of antimalarial drugs for treating malaria during pregnancy.

Malaria during pregnancy often resulted in serious risks for mother and the fetus, such as placental malaria and congenital malaria (7, 12, 15). All pregnant women diagnosed with malaria must be treated as soon as possible with a safe and effective drug regimen. Quinine, chloroquine, artemisinin, and their derivatives are examples of antimalarial drugs which have been used to treat malaria patients, including those with pregnancy. Administration of these drugs may result in adverse outcomes of pregnancy if administered in high doses and without precautions. Quinine is a quinolone antimalarial drug that was first discovered in Cinchona bark (22). In many countries, including Thailand, quinine has been used to treat patient during pregnancy where chloroquine-resistant \(P. falciparum\) exists. Although it is considered to be a safe drug, administration at abortifacient (high) doses result in teratogenic effects and nerve damage in the fetus (4, 18). There is also a risk of hyperinsulinemia and subsequent hypoglycemia (11). Since World War II chloroquine has been the most widely used antimalarial drug. This drug is generally considered to be safe with an appropriate dose, although it has gained a reputation as an inducer of abortion at high dose in pregnancy (20).

Artemisinin is an antimalarial ingredient derived from the Chinese weed “ging hao” (Artemisia annua). High dose usage of artemisinin in experimental animals can cause several abnormalities including...
neurotoxicity and embryotoxicity (5). Some studies have demonstrated that artemisinin used during pregnancy is safe and results in no adverse outcomes (14, 16). However, the use of this drug for malaria in pregnancies is still questionable since there is only limited information on this drug. Pyrimethamine is a dihydrofolate reductase inhibitor and slow-acting blood schizontocide for all malaria strains and is usually given in combination with sulphadoxine. Unlike other folic acid antagonist, pyrimethamine has no teratogenic effect and can be used for treatment and prophylaxis (2, 3, 25). Although these drugs are generally considered safe for treating malaria in pregnancies, drug interactions with human placenta still needs to be addressed. In this present study, we determined the effect of antimalarial drugs on cell proliferation, cytotoxicity, cell cycle progression and induction of cell death in JAR cells which display many characteristics of human placental trophoblasts.

MATERIALS AND METHODS

Antimalarial drugs. The antimalarial drugs used in this study were chloroquine (99% purity), quinine (91% purity), artemisinin (98% purity) (Sigma, St. Louise, MO), and pyrimethamine (> 90% purity, MP biomedicals, Aurora, OH). Chloroquine was prepared in phosphate buffer saline (PBS). Quinine, artemisinin, and pyrimethamine were prepared in dimethyl sulfoxide (DMSO). Drug concentrations were chosen, ranging from 0–50 μg/mL, according to a result of a preliminary cell proliferation test.

Cell line. The choriocarcinoma cell line (JAR) is derived from a human trophoblastic tumor of the placenta. This cell line exhibits some similarities to a normal human placental trophoblast. It produces estrogen, progesterone, gonadotrophin and lactogen in culture. Unlike isolated trophoblast from placenta, this cell line can be grown continuously under normal culture condition. It was, therefore, chosen for this study. The cells were maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) with 10% FBS and antibiotics (100 unit/mL of penicillin G and 100 μg/mL of streptomycin sulfate). The JAR cell line was purchased from ATCC (Manassas, VA) and cultured at 37°C in 5% CO₂.

Non-radioactive proliferation assay. JAR cells (10,000 cells/well) were cultured in 96-well culture plates, in a total volume of 200 μL of RPMI 1640 supplemented with 0.5% FBS. Cells were cultured in the presence of increasing concentrations (0–50 μg/mL) of chloroquine, quinine, artemisinin, or pyrimethamine. Both treatment and control groups were performed in 6–8 replicate wells. The relative number of viable cells was then determined after 72 h incubation, by adding 1 mg/mL of 3-[4,5-di-methylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and incubating for a further 4 h. The formazan crystals were then solubilized with acid isopropanol (90% isopropyl alcohol, 0.004 N HCl) for 1 h. The optical density of this solution was measured at 595 nm. These experiments were repeated at least three times to assure their reproducibility.

LDH assay. The release of LDH from JAR cells was used to detect cytotoxicity and was measured at the end of each proliferation experiment. Briefly, culture plates were centrifuged at 1500 rpm for 15 min at room temperature to ensure accumulation of cells at the bottom of the wells. The cell-free culture media (100 μL) was collected and then incubated with 100 μL of the reaction mixture Cytotoxicity detection kit (Boehringer Mannheim, Indianapolis, IN) for 30 min at room temperature in the dark. 1 N HCl (50 μL) was added into each well to stop the enzymatic reaction. The optical density of the solution was then measured by using an ELISA plate reader with a 490 nm filter. Percent cytotoxicity was determined relative to the control groups.

Study of cell cycle using flow cytometry. JAR cells were cultured in 3 mL of RPMI 1640 containing 10% FBS in 6-well culture plates (100 × 20 mm) until 70–80% confluence was achieved. The culture medium was then replaced with serum-free fresh medium and incubated with 0.1 mM hydroxyurea for 24 h. Synchronized JAR cells were then cultured in a fresh RPMI 1640 medium in the presence of 3% FBS with or without the antimalarial drugs (10 μg/mL) for 6, 12, 20 and 32 h. Cells were then trypsinized, centrifuged, and resuspended in PBS and stained with propidium iodide solution. Stained cells (30,000 counts) were analyzed by flow cytometry. The percent of cells in each phase of the cell cycle was then determined.

Cell death assays. In the DNA laddering assay, JAR cells were cultured in 3 mL of RPMI 1640 containing 10% FBS in 6-well culture plate until 70–80% confluence was achieved. Culture media were replaced with fresh media containing 0.5% FBS with or without the antimalarial drugs at various concen-
Antimalarial drugs and JAR
tations (0–50 μg/mL) and incubated for 24, 48, and 72 h. Cells were then washed with cold PBS and lysed with lysis solution. A DNA sample was then extracted from the solution using a phenol-chloroform-isoamyl alcohol extraction protocol. The DNA concentration was determined using a spectrophotometer (OD_{260}). DNA (2 μg) samples, along with positive controls, were then subjected to agarose gel (1.5%) electrophoresis. The DNA ladder was analyzed by ethidium bromide staining and visualized using a UV transilluminator.

For in situ detection of apoptotic cell death, JAR cells were cultured in 3 mL of RPMI 1640 containing 10% FBS in 6-well culture plate containing a glass slide until 70–80% confluence was achieved. The culture medium was replaced with fresh medium containing 0.5% FBS with or without the antimalarial drugs and incubated for 24 or 48 h. Apoptosis staining was carried out using an in situ detection of apoptotic cell death kit (R&D systems, Minneapolis, MN) on culture slides as followed. Glass slides were washed with ice cold PBS then fixed using freshly prepared paraformaldehyde solu-
tion 3.7% for 10 min at room temperature. Slides were then rinsed with PBS and incubated in permeabilisation solution for 30 min at room temperature. Slides were then rinsed with washing buffer and incubated with the TdT reaction mixture for 60 min at 37°C. The labeling reaction was stopped followed by the incubation with streptavidin-FITC for 10 min at room temperature. Slides were then washed with PBS and visualized using fluorescent microscopy.

Statistical methods. All experiments were repeated at least three times to ensure their reproducibility. An IC50 was calculated using regression analysis with four-parameter logistic equation of Sigmaplot software. Data are presented as means ± standard error (SE). Differences in data between treated and non-treated control groups in the cell cycle studies, cell proliferation and cytotoxicity experiments were analyzed using the Dunnett’s method of One-way analysis of variance (ANOVA).

Fig. 1 Inhibition of JAR cell proliferation and induction of cytotoxicity by antimalarial drugs. JAR cells were cultured in the presence of increasing concentrations (0–50 μg/mL) of chloroquine, quinine, artemisinin and pyrimethamine for 72 h. Relative cell numbers were measured by MTT assays. Cell decreased relative to the control (mean ± SE) was then determined (a-d). LDH release in conditioned media was measured at the end of each proliferation assays and the percent cytotoxicity (mean ± SE) relative to the control groups was determined (e–h). Asterisk (*, p < 0.05) represents significant difference between the treatment and control groups as analyzed by One Way ANOVA.
## RESULTS

### Anti-proliferative and cytotoxic effect of antimalarial drugs on JAR cells

When JAR cells were cultured in the presence of antimalarial drugs, all drug treatments resulted in inhibition of cell proliferation in 72 h, in a dose-dependent fashion (Fig. 1a–d). Concentration as low as 3.13 μg/mL of chloroquine, quinine, artemisinin and pyrimethamine decreased cell numbers significantly compared to the non-treated control groups (p < 0.05). Moreover, there was no decrease of cell number observed in cells treated with DMSO at a concentration that corresponded to those of the

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**Fig. 2** Modulation of cell cycle progression by antimalarial drugs. Synchronized JAR cells were restimulated with 3% FBS with or without the antimalarial drugs at (10 μg/mL) for 6, 12, 20 and 32 h. Percent cell (mean ± SE) in each cell cycle phase was analyzed using flow cytometric analysis. Asterisk (*, p < 0.05) represents significant difference between the antimalarial drug treated and control groups as analyzed by One Way ANOVA.

**Fig. 3** Induction of DNA fragmentation in JAR by chloroquine. JAR cells were cultured in RPMI 1640 in the presence or absence of increasing concentrations of chloroquine for 24, 48, and 72 h. DNA samples are then extracted and fractionated by agarose gel (2%) electrophoresis and DNA ladders were visualized after ethidium bromide staining. Lane 1, 22 = 100bp marker, Lane 2–7 = chloroquine 3.12, 6.25, 12.5, 25, 50 μg/mL, 24 h, Lane 9–14 = chloroquine 3.12, 6.25, 12.5, 25, 50 μg/mL, 48 h, Lane 15–21 = chloroquine 3.12, 6.25, 12.5, 25, 50 μg/mL, 72 h, Lane 8, 15 = positive control.

![DNA Fragmentation](image21)
Antimalarial drugs and JAR

drugs used (data not shown). The calculated IC50 value for chloroquine, quinine, artemisinin, and pyrimethamine were 6.96, 6.49, 6.69, and 6.89 μg/mL, respectively. At the highest dose tested (50 μg/mL), the cell number was decreased by more than 90%. In a related study, we analyzed the cytotoxicity of these drugs on JAR cells; by measuring LDH release in conditioned media paralleled to proliferation assay. As anticipated, LDH release was induced by these drugs in a dose dependent manner (p < 0.05). The results (Fig. 1e–h) are expressed as percent cytotoxicity relative to the non-treated control group. The highest dose of each drug (50 μg/mL) induced almost 100% cytotoxicity.

Antimalarial drugs modulation of JAR cell cycle progression
For studying the involvement of antimalarial drugs on modulating the progression of the JAR cell cycle, we performed cellular DNA staining to measure the percent cell in each phase of the cell cycle. After synchronization for 24 h, cells were restimulated using 3% FBS with or without the antimalarial drugs at (10 μg/mL) and stained with propidium iodide. We found that the majority of synchronized cells (approximately 61%) were arrested in the S phase, leaving a small number of cells in the G2/M phase (approximately 15%). Restimulation of synchronized cells allowed us to study a control of cell cycle progression by antimalarial drugs up to 32 h, or approximately 2 cell division cycles.

At 6 h after restimulation (Fig. 2), cells in the non-treated control group did exit S phase arrest, however, chloroquine, quinine, artemisinin and pyrimethamine delayed the exit for at least 6 h (p < 0.05) as more cells accumulated in the S phase. At 12 h, quinine and artemisinin resulted in a significant accumulation of cells in G0/G1 phase. At 20 and 32 h after treatment, chloroquine, quinine and pyrimethamine but not artemisinin induced accumulation of cells in the S phase compared to the non-treated control group (p < 0.05). Interestingly, pyrimethamine could delay the exit from S phase arrest for up to 32 h. However, this phenomenon was reversed after the withdrawal of pyrimethamine (data not shown).

Induction of apoptotic cell death in JAR cells by antimalarial drugs
Since the antimalarial drugs used in this study inhibited JAR cell proliferation and induced cytotoxicity, we, therefore, further analyzed if the cytotoxicity is mediated by the induction of apoptotic cell death. We employed 2 methods to illustrate DNA fragmentation of apoptotic cells. Starting from 24 h (Fig. 3), we observed fragmented DNA after all drugs treatments (data not shown for quinine, artemisinin and pyrimethamine), as well as JAR cells treated with actinomycin D (1 μg/mL) for 5 h as a positive control for apoptosis. At the highest dose of each drug tested (50 μg/mL) at 72 h, no fragmented DNA was observed. This phenomenon

Fig. 4  In situ detection of JAR apoptotic cell death. JAR cells were cultured in RPMI 1640 in the presence of antimalarial drugs (50 μg/mL) for 48 h. In situ cell death detection was then performed and apoptosis was visualized by fluorescent microscopy. Apoptotic cells showed a fluorescent nucleus. A = nuclease treated (positive staining), B = non-treatment control, C = chloroquine, D = quinine, E = artemisinin, F = pyrimethamine.
could be attributed to the non-fragmented DNA being isolated from the small number of non-apoptotic surviving attached cells. In addition, at 72 h, fragmented DNA could be seen in the non-treated control group, suggesting the occurrence of spontaneous cell death.

The induction of apoptosis was confirmed using enzymatic in situ labeling (Fig. 4). This method can reveal apoptosis in a single cell level. JAR cells were cultured on glass slides in the media with or without the antimalarial drugs (50 μg/mL) for 48 h and stained for DNA fragmentation. Chloroquine (C), quinine (D), artemisinin (E) or pyrimethamine (F) treatments of these cells did cause apoptosis cell death as visualized by fluorescent microscopy, whereas there was little spontaneous cell death observed in the non-treated control group (B).

**DISCUSSION**

In this study, the JAR trophoblastic cell line was used as a model for the behavior of human placental trophoblasts treated with antimalarial drugs (chloroquine, quinine, artemisinin, and pyrimethamine). All these drugs had cytotoxic effect and inhibited cell proliferation. Despite of the differences in their chemical structure, all drugs exerted a similar degree of cytotoxicity on the JAR trophoblast. Further study of the effect of antimalarial drugs on other human cancer cell lines was conducted in our laboratory. A comparison of drug sensitivity among three cell lines revealed that JAR cells are the most susceptible to quinine and artemisinin treatment compared to a human breast cancer cell line (MCF-7, IC50 = 35 μg/mL for quinine, and 16.25 μg/mL for artemisinin) and a human oral carcinoma cell line (KB, IC50 = 13.75 μg/mL for quinine and 9.38 μg/mL for artemisinin). In contrast, JAR cells are the least susceptible to pyrimethamine treatment compared to MCF-7 (IC50 = 5.62 μg/mL) and KB (IC50 = 5.8 μg/mL) cell lines. In addition, the sensitivity of JAR cells to chloroquine falls in between that of MCF-7 (IC50 = 5.31 μg/mL) and KB (IC50 = 8.8 μg/mL) cell lines.

Though the IC50 value of these antimalarial drugs on JAR cells (from 6.49–6.96 μg/mL) are higher than the effective antimalarial concentration [plasma level ranging from 0.2–5.0 μg/mL (1, 8, 21, 24)] for treatment, a detrimental effect on the trophoblasts was observed at the lowest dose of the drugs used (3.13 μg/mL). Interestingly, it had been reported that the quinine treatment regimen for patients in Thailand, could lead to a plasma level of quinine as high as 20 μg/mL (10). However, clinical studies at the Thai-Burmese border demonstrated that a therapeutic dose of quinine (30 mg salt/kg/day for 7 days) in 376 pregnancies was safe to use during the first trimester of pregnancy (17), although during second or third trimester a treatment with artemesunate-ataquaquine-proguanil is better than with quinine to treat multiple drug resistant P. falciparum infections (13).

Our experiments show that the mode of action of antimalarial drugs on inhibition of JAR cell proliferation is due to the induction of cell cycle progression arrest. Drugs used in this study have shown the ability to delay the cell cycle by slowing the exit from the S phase arrest and/or the induction of G0/G1 phase arrest. It has been demonstrated in several studies that S phase arrest is often accompanied by the interference of the proteins that control cell cycle. For example, hypoxia-induced S phase arrest in human breast (T-47D) and cervical (NHK 3025) cancer cell lines paralleled the down-regulation of cyclin A (23). A study with rat fibroblasts showed that in a low serum condition, the cells entered S phase arrest with a decreased amount of cyclin A, cyclin B, and cdc2, whereas the level of cyclin D2, cdk4, and cdk2 persisted at a high level (9). A study using JAR cells showed that cell cycle progression was modulated by hyperglycaemia (26). Although the mechanisms of G0/G1 and S phase arrest in JAR cells has not been determined, we anticipate that this may be attributed to the interference of cell cycle control proteins, the inhibition of DNA synthesis, and the process of mitosis.

In addition to inducing cell cycle arrest, our study has demonstrated that antimalarial drugs induced apoptotic cell death in the JAR cell line. We utilized an agarose gel electrophoresis DNA laddering method to detect fragmented DNA, while it was confirmed using in situ detection of cell death. Both methods confirmed that antimalarial drugs triggered apoptosis in the JAR trophoblast. In contrast, a study using human trophoblastic JEG-3 cells, when treated with cigarette smoke hydrocarbon benzo[a]pyrene, cell proliferation was inhibited along with a cell cycle arrest in G2/M phase, however there was no accompanying apoptosis (6). Furthermore, in a study of the biological activity of Nodal, a member of TGF-β superfamily, on HTR8/ SVneo trophoblasts, this cytokine could act as a growth regulator by inhibiting proliferation, inducing cell cycle G1 phase arrest and apoptosis (19). Our findings in this present study have clearly shown that induction of a delay in cell cycle pro-
gression was accompanied by the induction of apoptosis.

Collectively, the data presented in this report have, for the first time, illustrated the interaction of antimalarial drugs with JAR trophoblastic cells and this could have important implications for the use of antimalarial drugs for treating malaria during pregnancy.

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