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TITLE

Hypoxia modulates the effect of dihydroartemisinin on endothelial cells

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Abstract

Artemisinin derivatives, the current cornerstone of malaria treatment, possess also anti-angiogenic and anti-tumor activity. Hypoxia plays a crucial role both in severe malaria (as a consequence of the cytoadherence of infected erythrocytes to the microvasculature) and in cancer (due to the restricted blood supply in the growing tumour mass). However, the consequences of hypoxia onto the effects of artemisinins is under-researched.

This study aimed at assessing how the inhibition of microvascular endothelial cell (HMEC-1) growth induced by dihydroartemisinin (DHA, an antimalarial drug and the active metabolite of currently in-use artemisinins) is affected by oxygen tension.

Low doses of DHA (achieved in the patients' plasma when treating malaria) were more inhibitory in hypoxia, whereas high doses (required for anti-angiogenic or anti-tumor activity) were more effective in normoxia. The peroxide bridge is essential for cellular toxicity (deoxyDHA was inactive). High doses of DHA caused HMEC-1 apoptosis and G2 cell cycle arrest. Effects were mediated by the generation of oxidative stress as demonstrated by DCF-DA fluorescence and membrane lipid peroxidation analysis.

Overall, these results suggest that DHA inhibition of endothelial cell growth is related to the level of tissue oxygenation and drug concentration. This should be considered when studying both the effects of artemisinin derivatives as antimalarials and the potential therapeutic applications of these drugs as anti-tumor agents.

Keywords

hypoxia, dihydroartemisinin (DHA), endothelial cells, cell proliferation, malaria.

Abbreviations

BSO, buthionine sulfoximine; CPT, Camptothecin; DCF-DA, dihydrodichlorofluorescein diacetate; DeoxyDHA , dihydrodeoxyartemisinin; DHA, dihydroartemisinin; EC, endothelial cell; GSH, glutathione; HIF-1α, hypoxia inducible factor; HMEC-1, human microvascular endothelial cells; RBC, red blood cell; Tetraoxane, dimethyltetraoxodispiro-hexadecan; VEGF, vascular endothelial growth factor.

1. Introduction

Hypoxia is "a condition in which failure of either delivery or use of O_2 limits normal tissue function" [1]. Endothelial cell (EC) functions are influenced by hypoxia both in physiological and pathological conditions, indicating that EC are able to detect and respond to changes in oxygen tension. Responses to hypoxia are tissue-specific, depend on the EC type [2] and are mediated by enzymes such as oxygen-sensitive NADPH oxidases, endothelial nitric oxide synthase and heme oxygenase through the activation of transcription factors such as the hypoxia inducible factor (HIF-1α) [3]. The main effects produced by hypoxia on EC are the induction of morphological changes, cell growth and differentiation, which result in angiogenesis and sprouting of new vessels from pre-existing ones [4]. The production of autocrine factors such as vascular endothelial growth factor (VEGF) and cytokines is increased during angiogenesis [5, 6].

Angiogenesis is a physiological process associated with embryonic and adult development and wound healing, but also can be pathological in conditions such as cancer, especially in metastatic tumors. The uncontrolled growth of the tumor mass leads to inefficient tissue oxygenation, which in turn induces the formation of new vessels to restore adequate perfusion and nutrient supply. Therefore, an approach to anticancer therapy is to target tumor angiogenesis, usually in combination with chemo- or radiotherapy [7]. Most of the anti-angiogenic therapies approved for use in patients or in clinical trials are monoclonal antibodies against VEGF, the main tumor pro-angiogenic factor, or VEGF receptor inhibitors [8]. New classes of compounds are needed, targeting different stages of the angiogenic process such as tissue proteases or signal transduction molecules. Tissue hypoxia has recently been recognized also as an important factor which can influence the responsiveness to radiotherapy [9].

Artemisinin, the active principle from the plant *Artemisia annua*, and its semi-synthetic derivatives are the most important class of antimalarial drugs today. Among these, dihydroartemisinin (DHA) is both the intermediate in the semi-synthesis and the active metabolite of the other in-use artemisinin derivatives, as well as an antimalarial drug in its own right [10]. Compounds of this class possess also anti-tumor activity: they are toxic to tumor cell lines, induce tumor regression in animal models and can prolong the survival of terminally-ill patients with tumors resistant to conventional chemotherapeutic agents [11, 12].

Significant anti-angiogenic effects of different artemisinin derivatives have been reported using classical *in vitro* and *in vivo* models [13-15]. In particular, artemisinins reduce EC growth and migration, VEGF and VEGF-receptor expression and new vessel formation in mice bearing Kaposi's sarcoma xenograft tumors [14, 16-19]. A correlation between genes involved in angiogenesis and the response of tumor cells to artemisinins was demonstrated by transcriptional analysis [20].

Although widely used as antimalarials and despite their potential importance as anti-tumor agents, the mechanism of action of artemisinins is not completely elucidated. One common view is that artemisinins require ferrous iron, either in the 'free' state or from hemoglobin or heme, for activation. The peroxide is cleaved reductively to generate alkoxy, and thence carbon-centered radicals, that are supposed to provide the toxic effects [21]. The source of activation and the parasite target are still unknown. Multiple targets have been postulated, such as the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) as well as heme and-non heme containing proteins [22, 23]. Other authors reported that artemisinins can elicit oxidative stress and synergize the action of other redox active drugs without the involvement of carbon-centered radicals. In line with this theory, it has been found that artemisinins interfere with flavin cofactors involved in the function of disulfide reductase enzymes critical for maintaining redox homeostasis, thus enhancing oxidative stress [24, 25]. The same will

likely apply to cancer cells, in which several of the disulfide reductase enzymes such as thioredoxin reductase are over-expressed [26].

Artemisinin derivatives are more active on malaria parasites in normoxia than hypoxia [27], a finding that was explained by the hypothesis that oxidative stress is involved in their mechanism of action. On the contrary, the role of hypoxia in the anti-angiogenic or anti-tumor effect of artemisinins has not been investigated. Tissue hypoxia occurs also in malaria (particularly severe malaria), whereby infected red blood cells (RBC) sequestered in the peripheral circulation cause blood flow disturbance, especially in the post-capillary venules of the brain, through the combined contributions of reduced deformability, adhesion of parasitized RBC to the microvascular endothelium and the rosetting of uninfected RBC [28].

The standard *in vitro* experiments are usually performed in atmospheric oxygen tension (about 20%), defined as "normoxia". However, in human peripheral tissues the oxygen tension may range between 4-10% and is even lower in tumors [29]. The aim of the present work was to study the inhibition of EC growth by DHA (and the mechanisms thereof) in normoxia and hypoxia (20% vs. 1% oxygen tension, respectively).

2. Methods

2.1. Cell cultures and drugs

A long-term cell line of human dermal microvascular endothelial cells (HMEC-1) immortalized by SV 40 large T antigen was kindly provided by the Centres for Disease Control and Prevention, Atlanta, GA,USA [30]. Cells were maintained in MCDB 131 medium (GIBCO-BRL, Paisley, Scotland) supplemented with 10% foetal calf serum (HyClone, Logan, UT, USA), 10 ng/ml epidermal growth factor (PreproTech, Rocky Hill, NY, USA), 1μg/ml hydrocortisone (Sigma Italia, Milan, Italy), 2mM glutamine (EuroClone,

Pero, Italy), 100 U/ml penicillin, 100 μg/ml streptomycin (EuroClone) and 20mM Hepes buffer, pH 7.3 (EuroClone).

During the experiments HMEC-1 were cultured in normoxia (20% O_2, 5% CO_2, 75% N_2) or hypoxia (1% O_2, 5% CO_2, 94% N_2). The latter condition was achieved either by a humidified incubator Mini Galaxy A (RS Biotech, Scotland, UK) or by a humidified, sealed chamber (Billups-Rothenberg, Del Mar, CA), flushed for two min with the proper gas mixture.

Dihydroartemisinin (DHA) and dihydrodeoxyartemisinin (DeoxyDHA) were provided by Richard Haynes (The Hong Kong University of Science and Technology) and Diego Monti (ISTM-CNR, Milan, Italy), 1,10-dimethyl-7,8,15,16-tetraoxadispiro[5.2.5.2]hexadecane (tetraoxane) was synthesised by Diego Monti as described [31] (Figure 1).

2.2. Cell viability assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) cytotoxicity assay was used to measure cell viability as described elsewhere [32]. Briefly, 10^4 cells/well were seeded in 96-well flat bottom tissue culture clusters (Costar, NY, USA). After 24h, cells were treated with the different drugs at the doses of 0.012, 0.049, 0.195, 0.781, 3.125, 12.5, 50 and 200μM in triplicate for 24-48-72h, in normoxic or hypoxic conditions. In some experiments cells were pre-treated (4h) with 0.25mM buthionine sulfoximine (BSO; Sigma) or 1mM ascorbic acid (Sigma), then supernatants were discarded and cells treated with DHA. At the end of the treatment, cell viability was measured as the reduction of MTT to blue formazan. Formazan crystals were dissolved in lysing buffer: 20% (w/v) of SDS (Sigma), 40% of N,N-dimethyl formamide (Sigma) in water. The absorbance was read at 550nm with 650nm reference (automatic microplate reader Molecular Devices, Sunnyvale,

CA, USA) and the data are expressed as the percentage of inhibition of cell viability compared to control cells cultured in the same oxygen tension.

2.3. Cell cycle analysis

HMEC-1 were seeded in 25 cm^2 flasks (Corning, NY, USA) ($6.5x10^5$, $4.0x10^5$ or 2.5 $x10^5$ cells/flask for 24-48-72h experiments, respectively) and let adhere overnight. Cells were treated with 1μM or 12.5μM DHA and maintained in hypoxia or normoxia for different times (24-48-72h). Cell cycle profile was evaluated by propidium iodide staining (CycleTEST PLUS kit, Becton Dickinson, Milan, Italy) and flow cytometry (FACS analysis). Cells were trypsinized, prepared according to manufacturer instructions and analyzed using a cytofluorimeter Beckman Coulter FC500 (Beckman Coulter, FL, USA). The percentage of cells in the different phases of cell cycle (G_0/G_1, S, G_2) was determined.

2.4. Apoptosis assays

2.4.1. Annexin V binding assay

The exposure of phosphatidylserine (PS) at cell surface, was measured by Annexin V staining and FACS analysis. HMEC-1 were seeded in 25 cm^2 flasks (Corning) ($5x10^5$ or $2x10^5$ cells/flask for 24h and 48h experiments, respectively), treated with DHA 2.5μM or 50μM and incubated in hypoxia or normoxia for 24 or 48h. Cells were trypsinized and stained with the AnnexinV-FITC/7AAD kit (Becton Dickinson) according to the manufacturer's instructions. FACS analysis was performed using a cytofluorimeter Beckman Coulter FC500 (Beckman Coulter). The percentage of AnnexinV positive cells was calculated and the data are expressed as the fold-increase compared to control cells cultured in the same oxygen tension.

2.4.2. Caspase activity assay

Caspase 3 and 7 activation was evaluated using the fluorimetric kit Apo-ONE homogenous Caspase-3/7 Assay (Promega Italia, Milan, Italy), which measures the fluorescence released following the caspase-induced cleavage of ZVAD-rhodamine, a pro-fluorescent substrate of caspases (Exc 499nm, Em. 521 nm). HMEC-1 were seeded at $2x10^4$ per well in 96-well plates (Costar) and let adhere overnight. After 24h, cells were treated with 1μM or 50μM DHA or 4μM camptothecin (CPT, used as reference compound) for 5h and then caspase activity was evaluated following the manufacturer instructions. Data are expressed as fold-increase compared to untreated control cells cultured in the same oxygen tension.

2.5. Reactive Oxygen species (ROS) production

HMEC-1 were seeded at 10^4 cells/well in 96-well flat bottom tissue culture clusters (Costar). After overnight adhesion, cells were washed with PBS and treated with 10μM dihydrodichlorofluorescein diacetate (H_2DCF-DA; Sigma) in PBS for 15 minutes at 37°C in the dark. Cells were washed with PBS and DHA (0.5-50μM) was added. After 24h incubation in normoxia or hypoxia, cell supernatants were transferred in dark plates (Costar) and read (λ exc 504nm; λ em 529nm) using a Synergy4 microplate reader (BioTek, Bad Friedrichshall, Germany).

2.6. Lipid peroxidation

HMEC-1 were seeded ($2x10^5$ cells/well) in 24-well plates (Costar) and let adhere overnight. Cells were treated with 0.5 or 50μM DHA for 24h in normoxia or hypoxia. Supernatants were removed and cells washed once with PBS. Cell lysis was induced by 0.1% Triton (Sigma) and freeze-thawing of the samples. Endogenous and DHA-induced lipid peroxidation was measured by determining the levels of thiobarbituric acid reactive

substances (TBARS) in cell homogenate according to the method of Wey slightly modified [33]. Briefly, 250µl of cell homogenate were added to 500µl of 2-thiobarbituric acid (TBA, Sigma) reagent (6g TBA previously dissolved in 4ml NaOH 5N - Merck, Milan, Italy- brought to 100 ml in 3%$HClO_4$ - Sigma) and heated at 100°C for 10 minutes. After cooling, TBARS were extracted with 1N butanol (Merck). Fluorescence of the upper alcoholic phase, separated by a brief centrifugation, was determined in a Varian Cary Eclipse spectrofluorimeter (Varian instruments, CA, USA) (λ exc 520nm; λ em 553nm). A standard curve was obtained by dissolving tetraethoxypropane (Sigma) in 0.01N HCl (Merck) to produce malondialdehyde, used as standard compound. Data are expressed as pmoles of TBARS per micrograms of proteins, evaluated by the Bradford protein assay [34].

2.7. Statistical analysis

Cell inhibition was evaluated at each time point (24h, 48h and 72h) for each product by using a linear model for repeated data (at each drug concentration). Culture conditions (hypoxia and normoxia) were included as explanatory variable, together with the interaction between the condition and compound concentration. A generalized linear model was used to compare compounds in either conditions at the different time points. Data were analyzed over the entire 0.012-200 µM range of concentrations tested, as well as for the 0.012-3.125 µM range for DHA, that can be achieved during malaria treatment at maximum concentration (C_{max}) and along the elimination curve. Significance was set at $P < 0.05$.

All the other data were analyzed using a 2-tailed Student *t* test with the level of significance at *P* less than 0.05 and expressed as mean ± S.D.

3. Results

3.1. HMEC-1 growth in normoxic and hypoxic conditions

The growth of HMEC-1 in normoxia (standard 20% oxygen tension) or hypoxia (1% oxygen) was assessed for 96h. Cell growth rate was linear in normoxia throughout the period under observation. No significant differences in cell growth rate were observed in the first 48h in either oxygen tension condition (Generalized Estimated Equation, GEE -0.0004, p = 0.5). After 48h, cell growth in hypoxia was significantly slower (GEE -0.0015, p <0.001) (Figure 2).

3.2. The inhibition of HMEC-1 growth by DHA is dependent on oxygen availability

DHA inhibited cell growth in a dose and time-dependent manner, both in normoxia and hypoxia. The dose-response curves are displayed in Figure 3 (DHA: left panel). The curves under the two culture conditions cross with an equipoise at about 3.1μM. The linear model applied to compare cell growth inhibition under normoxic and hypoxic conditions showed, when considering the entire dose range for DHA, no significant effect of the culture condition (oxygen tension), a significant effect of the dose, and a significant interaction between dose and oxygen concentration (meaning that the dose-response curves are different between normoxia and hypoxia) at all the time points tested (24, 48 and 72h). When restricting the analyses to the range of clinically-relevant concentrations (i.e. $\leq$ 3.1μM to cover those achievable at C_{max} after a single oral dose of DHA as well as at t ½), there was a significant effect of the dose, borderline significance for the culture condition at 24h and 72h, and a significant interaction at 48h and 72h but not 24h (Table 1).

3.3. The peroxide bridge is essential for activity, but does not explain the different behaviour of DHA in normoxia or hypoxia

The antimalarial and antitumour activities of the artemisinins are due to the presence of the peroxide pharmacophore [25, 35]. To investigate this aspect, HMEC-1were treated with

deoxyDHA, identical to DHA but lacking the peroxide bridge, and tetraoxane, which contains two peroxides embedded in a chemical structure different from DHA (Figure 1).

DeoxyDHA was significantly less toxic than DHA, both in normoxia and hypoxia over the entire 0.012-200 μM range (Figure 3, central panel), indicating that the peroxide is crucial for toxicity. The tetraoxane (Figure 3, right panel) inhibited HMEC-1 growth in a dose- and time-dependent manner. However, contrary to DHA, no significant differences were observed in the dose-response curves when cells were cultured in normoxia or hypoxia.

3.4. DHA affects HMEC-1 cell cycle

The HMEC-1 cell cycle was analyzed both in normoxia and hypoxia. The percentages of untreated HMEC-1 in each phase of the cell cycle (G_0/G_1, S, G_2) were not significantly different in normoxia versus hypoxia (Figure 4, black bars). After treatment with 0.05μM of the reference compound camptothecin (CPT), most of the cells appeared dead (cell debris at microscopic observation) and the surviving ones were mostly in S or G_2 phase of cell cycle at all tested times (> 80% of the total, data not shown). Following DHA treatment in normoxia (Figure 4, upper panel) or hypoxia (Figure 4, lower panel), the percentage of cells in the G_2 phase increased, whereas that of cells in the G_0/G_1 decreased. This effect was dose- and time-dependent, significantly different from controls at 48 and 72h with 12.5μM DHA, only at 48h with 1μM DHA. The oxygen tension did not influence the percentages of the cells in the different phases of cell cycle.

3.5. High doses of DHA induce HMEC-1 apoptosis

Since artemisinin derivatives are known to induce apoptosis of cancer and EC [36], we investigated whether the extent of apoptosis was affected by different oxygen tensions.

Measuring Annexin V binding and caspases 3 and 7 activation, untreated control cells showed similar levels of apoptosis in normoxia and hypoxia at all times (data not shown).

Data in Figure 5A are expressed as fold-increase in the percentage of apoptotic cells compared to untreated control at the same oxygen tension. CPT (4μM) induced a significant increase in AnnexinV binding. At 24h, 50μM DHA induced a 3.4-fold increase of apoptotic cells in normoxia, whereas in hypoxia apoptosis was not significantly different from control. At 48h, 50μM DHA induced more apoptosis in normoxia than hypoxia, with 4.3 and 2.7 fold-increase, respectively. Low doses of DHA (1μM) did not induce significant apoptosis in either condition.

Caspases 3 and 7 were significantly activated in HMEC-1 treated with 50μM DHA, as shown by the fold -increase calculated versus control cells cultured at the same oxygen tension (Figure 5B). As expected, CPT (4μM) induced a significant increase in caspase activity. Differences between normoxia and hypoxia were not significant, although the increase in caspase activity was slightly higher in hypoxia.

Cells pre-treated with the caspase inhibitor Z-VAD were partially protected (30%) from DHA-induced death (data not shown), confirming that caspase activation partly contributed to DHA toxicity.

3.6. High doses of DHA induce oxidative stress on HMEC-1

3.6.1. DHA induces ROS production

Since it has been suggested that reactive oxygen species may be generated by artemisinins through oxidation of susceptible biomolecules [11, 25], we evaluated the production of reactive oxygen species (ROS) in our model using the fluorescent probe DCF-DA (Figure 6A). Under hypoxic conditions a decrease, although not significant, in basal DCF-DA fluorescence was observed in untreated control cells. Treatment with 50μM DHA

for 24h induced a significant increase in ROS production both in normoxia and hypoxia (1.6±0.4 fold-increase and 2.0 ± 0.5, respectively). No effects were observed at the low dose (0.5μM). Hematin (80μM), used as positive control [37], induced a 5-fold increase in ROS production (data not shown).

3.6.2. DHA induces lipid peroxidation

Oxidative stress was also evaluated by measuring membrane lipid peroxidation as production of thiobarbituric reactive substances (TBARS). No differences in control cells cultured in normoxia or hypoxia were observed. In agreement with the enhanced ROS production, 24h treatment with 50μM DHA induced also a significant increase in TBARS production by HMEC-1, both in normoxic and hypoxic conditions (Figure 6B). The low dose of DHA (0.5μM) did not increase lipid peroxidation. As a positive control, cells were treated with 1mM hydrogen peroxide, which induced a significant increase (2-fold) in lipid peroxidation (data not shown). Lipid peroxidation was not affected by the oxygen tension.

3.6.3. Glutathione (GSH) deprivation increases DHA toxicity on HMEC-1

To assess the role of oxidative stress in DHA-induced cell toxicity, HMEC-1 were pre-treated with buthionine sulfoximine (BSO), an inhibitor of glutamyl cysteine synthase, a key enzyme in the synthesis of the endogenous antioxidant GSH. As shown in Figure 6C, pre-treatment with 0.25mM BSO significantly enhanced the toxicity of the 24h treatment with 50μM DHA: the percentage of growth inhibition increased from 58% to 94% in normoxia and from 38.5% to 96% in hypoxia. At 72h, 50μM DHA was too toxic (>80% inhibition) to observe the effect of BSO. For this reason, the 72 h experiments were performed with 12.5μM DHA: the inhibition of cell growth was increased from 62% to 100% in normoxia and from 59% to 83% in hypoxia. These results were dose-dependent by using a non toxic range of BSO concentrations (0.01-0.5mM) (data not shown). The pre-treatment with BSO did not influence the effect of 0.5μM DHA at either 24h or 72h.

3.6.4. Ascorbic acid partially protects HMEC-1 from DHA toxicity

Ascorbic acid was used as an antioxidant to counteract the effect of ROS and oxidative stress. It has been reported that ascorbic acid has no effect itself on DHA [24]. As shown in Figure 6D, pre-treatment with ascorbic acid (1mM) decreased by 23% the toxicity of 50μM DHA on HMEC-1 cultured in normoxia but not in hypoxia. This protection was significant at 24h, but disappeared by prolonging the treatment for 72 h (data not shown). The effect of 0.5μM DHA was not influenced by pre-treatment with ascorbic acid.

4. Discussion

DHA inhibits the growth of HMEC-1 in a dose-dependent manner. The exposure-response relationship is different in hypoxic or normoxic conditions. These findings offer a potential explanation for some of the effects of DHA and other artemisinins when used for treating malaria and (prospectively) cancer, as well as embryotoxicity.

The experimental conditions are biologically and clinically pertinent. Hypoxia occurs physiologically in peripheral tissues and specifically in relevant zones in malaria, solid tumors and the embryo. Prolonged hypoxia affects the replication of EC. In the present experiments, growth rates were comparable in normoxic and hypoxic conditions for the first 48h, but thereafter, while growth continued unabated for normoxic cultures, it slowed down in hypoxia. The proportion of cells in the different phases of the cell cycle was similar in either conditions at all times.

The compound tested, DHA, is both an antimalarial drug on its own and the metabolite of the in-use artemisinin derivatives. The experiments were planned to cover a wide range of doses. At the low end ($\leq$3μM or 0.850μg/ml) are those corresponding to the plasma levels at peak concentration (C_{max}) and during drug clearance in malaria patients receiving either DHA or artesunate (an artemisinin derivative that is readily hydrolyzed to DHA), orally or

parenterally (see for example [38, 39]). The high concentrations (≥10μM or 2.84μg/ml) are similar to those of *in vitro* anti-tumor and anti-angiogenic studies [36]. In animal models, the doses used as antitumour or antiangiogenic are 10-20 times higher than those as antimalarial [14, 40].

The dose-response to DHA in terms of HMEC-1 growth inhibition was different in hypoxia and normoxia. At low doses DHA was more active in hypoxia, whereas at high doses it was more effective in normoxia. The curves under the two culture conditions have a different shape which suggests different mechanism of action and/or the availability of different targets for low and high doses with high or low oxygen tensions.

DHA, like other artemisinins, has a very short half life of about 1h or less when given orally (summarized in [41]). *In vitro*, the half life depends on culture conditions such as temperature, pH and medium and is about 4h at 37 °C at pH 7.6 (P.Olliaro; Basilico unpublished data). This means that measuring *in vitro* the effects of DHA at 24h or longer times after treatment reflects the consequences of the first hours of exposure.

The peroxide bridge is essential for the biological activity of all the artemisinin derivatives [36, 42, 43]; here deoxyDHA, which lacks the peroxide, was inactive except at very high concentrations (likely through a different mechanism of cell toxicity). However, the peroxide bridge alone cannot explain DHA sensitivity to oxygen tension as the activity of the tetraoxane, which contains two peroxide units embedded in a different molecular structure (see Figure 1), was similar in either conditions. This differential mechanism of action is supported by observations from Kumura et al. that both artemisinins and tetraoxanes can oxidise and degrade phosphatidylcholine, but only for DHA the effects are oxygen sensitive [44].

There is evidence that DHA effects involve induction of oxidative stress [13, 21] with increased production of reactive oxygen species (ROS) detected in different cell types,

especially tumor cells [45, 46]. In this study, lipid damage, measured as TBARS production, occurred only with high doses (50μM DHA), and this both in normoxia and hypoxia. So far, artemisinin-induced lipid peroxidation had been demonstrated only on red blood cells and neurons [47, 48]. For comparison, on malaria parasites *in vitro*, DHA at nanomolar concentrations was more effective in normoxia than hypoxia, a finding which was explained by oxidative stress occurring in normoxic conditions [27]. Recent data indicate that artemisinins can disturb the cell redox equilibrium by oxidising susceptible biomolecules such as the reduced forms of flavin cofactors followed by autoxidation in the presence of oxygen [24, 25]. In our experiments, pre-treatment of HMEC-1 with BSO, which deprives the cells of GSH, increased the toxicity of DHA. This could be due to increased level of cell oxidant stress and GSH consumption by DHA. Alternatively, based on the recent observation by Haynes et al., DHA may directly interfere with $FADH_2$/NADPH dependent glutathione reductase that keeps the levels of GSH required for redox homeostasis [25, 49]. Also, the antioxidant ascorbic acid protected the cells from DHA toxicity in normoxia but not in hypoxia, an observation which requires further investigation.

The relative amounts of ROS produced can likely account for the different behavior of DHA in normoxia versus hypoxia [50]. Large amounts of ROS produced after treatment with high-dose DHA in normoxia overcome the antioxidant defenses and induce cell death [51], while with low-dose DHA ROS production is lower (not detectable by the DCF method in our experiments) and apoptosis is absent, although low levels of ROS can still influence cell growth by acting as secondary messenger in different intracellular pathways [52]. ROS may also contribute to the stabilization of the HIF-1α, the most important transcription factor regulating the hypoxic response [53]. In this regard, data in the literature are controversial: an increased expression of HIF-1α has been observed in colon cancer cells treated with 10μM

DHA [54], while a negative modulation of HIF-1α has been reported in tumor cells and embryo bodies (DHA 5-25μM) [46, 55].

In our experiments, apoptosis occurred in cells treated with high-dose DHA (50μM) in normoxia (significantly less in hypoxia). Low doses (1μM) did not induce either apoptosis or cell death, as confirmed by the LDH cytotoxicity assay (not shown) or microscopically (no cell shrinking, blebbing or cell debris observed). These findings are in agreement with data from the literature, indicating that the dose of artemisinin derivatives necessary to induce apoptosis *in vitro* is at least 10μM [36].

In both normoxia and hypoxia, DHA induced a relative increase of cells in G_2 phase and a decrease of cells in G_0/G_1. Similar effects were reported in different cancer cell lines treated with DHA or artesunate [56, 57]. Moreover, DHA decreased S phase and increases G_2 phase in myeloma cells under hypoxia [16]. On the contrary, other authors showed a G_0/G_1 cell cycle arrest for cancer cells of different origin following artemisinin treatment [58, 59]. These inconsistencies are probably due to metabolic features of the cells leading to different growth rates, and/or to the experimental protocol. The G_2 cell cycle arrest could be caused by DNA damage [60], which activates the so called "G_2 checkpoint", preventing cells from proceeding into mitosis, with a consequent accumulation of cells in the G2 phase. It is interesting to note that the anti-angiogenic drugs proposed for anti-cancer therapy alter endothelial cell cycle either inducing a G_0 or a G_2 arrest [61-63], thus confirming the potential for artemisinins as anti-angiogenic drugs.

These results may complement our understanding of artemisinin embryotoxicity. This class of antimalarials is contraindicated in the first trimester of pregnancy [64], based on reproductive studies in experimental animals [65], although no effects have been found in humans. The embryotoxic effects of DHA and artesunate on rodents are caused by discrete areas of embryo cell death induced by anemia resulting from the killing of nucleated red

blood cell precursors. Observed effects are mainly on bones and vessels formation [66]. Using human CD34-derived erythroid precursors it has been found that DHA specifically target the basophilic erythroblasts thus inhibiting erythroid maturation and differentiation [67]. It is therefore possible that artemisinin embryotoxic effects are amplified by the killing of endothelial cells locally in hypoxia.

In conclusion, considering both data from the literature and the present work, it appears that DHA at 10μM or more induces EC and tumor cell death through oxidative stress [18, 20, 45], which could be caused by DHA directly through the oxidation of reduced flavin cofactors [25]. However, the mechanisms of toxicity of DHA at low doses, especially under hypoxia, needs to be elucidated further. No apoptosis, cell cycle arrest or induction of oxidative stress were detected on HMEC-1. However, cell proliferation was decreased by low-dose DHA in hypoxia. The expression of the VEGF receptors was reduced by DHA (0.5-1μM) and this is the only anti-angiogenic effect described at the same low doses used in our experiments [15, 18]. Thus, we cannot exclude that this mechanism may contribute, in our model, to the inhibition of HMEC-1 growth induced by low doses of DHA in hypoxia.

Collectively, our results suggest that, depending on the microenvironment in tissues, different doses of artemisinins could achieve different effects. As anti-angiogenic drug DHA may have the advantage that relatively low doses are effective in hypoxic areas of target organs. This could balance the problem of the short half-life, critical for the antitumour therapeutic regimens. Finally, the different behavior of DHA in different oxygen tensions is an unexplored area of research that may help understanding the mechanism of action and emerging resistance of this class of drugs, and may direct future studies for novel antitumour agents.

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Figure legends

Figure 1. Structures of the compounds used in this work.

Figure 2. Growth kinetics of HMEC-1 in normoxia or hypoxia. Cells were cultured in normoxic or hypoxic conditions for 96h and cell growth was measured by MTT assay every 24 h from time 0 h to 96 h.. The equations of the linear regression for the growth curves are displayed for normoxia (entire time range) and hypoxia (<48h and >48h)

Figure 3. Effect of different doses of DHA, DeoxyDHA or tetraoxane on the growth of HMEC-1 cultured in hypoxia or normoxia. HMEC-1 were treated with different doses of the three compounds (0.01-200μM) and cultured in normoxia (continuous line) or hypoxia (dotted line) for 24, 48 or 72 h. Cell growth was measured by the MTT assay and the data are expressed as the percentage of inhibition compared to untreated control cells cultured at the same oxygen tension. Data represent the mean ± S.D. of at least three different experiments run in triplicate.

Figure 4. Effect of DHA on the HMEC-1 cell cycle. Cells were treated with 1μM (dotted bars) or 12.5μM (grey bars) DHA and compared to untreated control cells (black bars). Cells were incubated in normoxia (upper panels) or hypoxia (lower panels) for 24, 48 or 72h. Data are expressed as the percentage of cells in the different phases of cell cycle, measured by flow cytometric analysis. The histograms represent the mean ±S.D of three different experiments (**$p<0.01$; *$p<0.05$ vs. control).

Figure 5. Induction of apoptosis by DHA on HMEC-1 incubated in normoxia (full bars) or hypoxia (striped bars). (A) Phosphatidylserine exposure was evaluated by AnnexinV binding after 24 or 48h treatment with 1 or 50μM DHA (B) Activation of Caspase 3 and 7 after 5h treatment with 1 or 50μM DHA. CPT (4μM) was used as a reference compound. The results are expressed as fold-increase versus control cells cultured in the same oxygen tension. The

histograms represent the mean ± SD of 3 different experiments (**p<0.01; *p<0.05 vs. control).

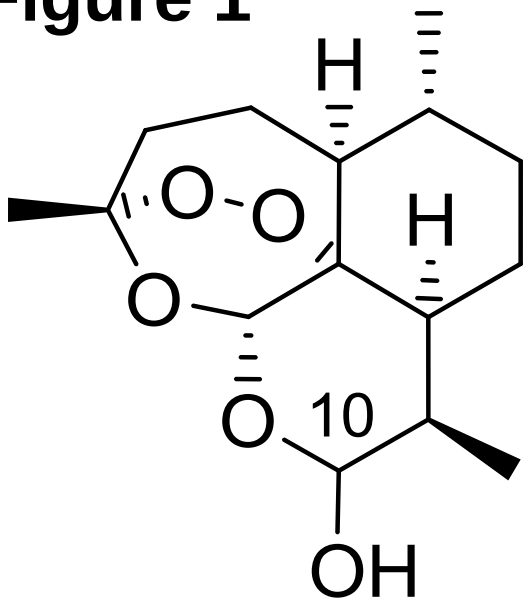
Figure 6. ROS production, membrane lipid peroxidation and effects of antioxidants on HMEC-1 treated with DHA. (A) HMEC-1 were incubated 24h with 0.5μM or 50μM DHA. ROS production was measured using the fluorescent probe DCF-DA and the results are expressed as DCF fluorescence arbitrary units (B) HMEC-1 were treated as in (A) and lipid peroxidation was evaluated as TBARS formation. Results are expressed as pmoles of TBARS per μg of protein. (C) HMEC-1 were pre-treated (4h) with 0.25mM BSO before exposure to 0.5-50μM DHA for 24h (left graph) or 0.5-12.5μM DHA for 72h (right graph) in normoxic or hypoxic conditions. Proliferation was measured by the MTT assay and results are expressed as the percentage of inhibition compared to untreated controls. (D) HMEC-1 were pre-treated (4h) with 1mM Ascorbic Acid (Asc Ac) and then treated with 0.5-50μM DHA for 24h. Proliferation was measured as in (C).Data represent the mean ± S.D. of three different experiments run in triplicate (A, C, D) or duplicate (B) (** p<0.01; *p<0.05 vs. control).

Table 1. Statistic analysis of the effect of DHA, deoxyDHA or tetraoxane on HMEC-1 growth in hypoxia or normoxia at 24, 48 or 72h.

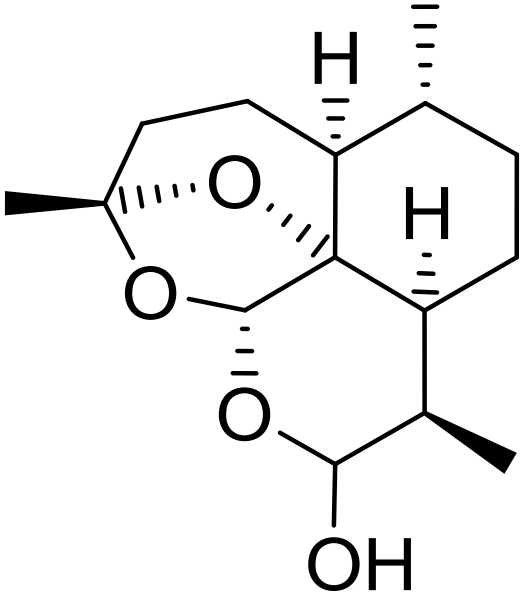
	culture condition (normo- vs. hypoxia)[a]	concentration[a]	interaction condition x concentration[a]
DHA 24h all concentrations	0.9111	**<.0001**	**0.0023**
DHA 48h all concentrations	0.2022	**<.0001**	**0.0007**
DHA 72h all concentrations	0.8993	**<.0001**	**0.0005**
DHA 24h concentrations <=3.1 uM	0.0553	**<.0001**	0.3764
DHA 48h concentrations <=3.1 uM	0.0624	**<.0001**	**0.0468**
DHA 72h concentrations <=3.1 uM	0.0522	**<.0001**	**0.0045**
deoxyDHA 24h all concentrations	0.2664	**<.0001**	0.8568
deoxyDHA 48h all concentrations	0.6586	**<.0001**	0.8225
deoxyDHA 72h all concentrations	0.1309	**<.0001**	**0.0483**
tetraoxane 24h all concentrations	0.2955	**<.0001**	0.6398
tetraoxane 48h all concentrations	0.8613	**<.0001**	0.9934
tetraoxane 72h all concentrations	0.0514	**<.0001**	0.0794

[a] P values express the difference at each time-point between treated and untreated cultures for each compound using a linear model for repeat data with condition (normoxia vs. hypoxia), compound concentration and the interaction between the two as explanatory variables.

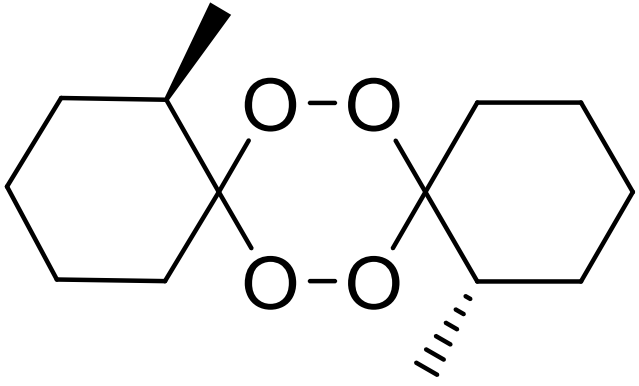
Figure 1



Dihydroartemisinin
(DHA)



Dihydrodeoxy
artemisinin
(deoxyDHA)



1,10-dimethyl-7,8,15,16-
tetraoxadispiro[5.2.5.2]
hexadecane
(tetraoxane)

Figure 2

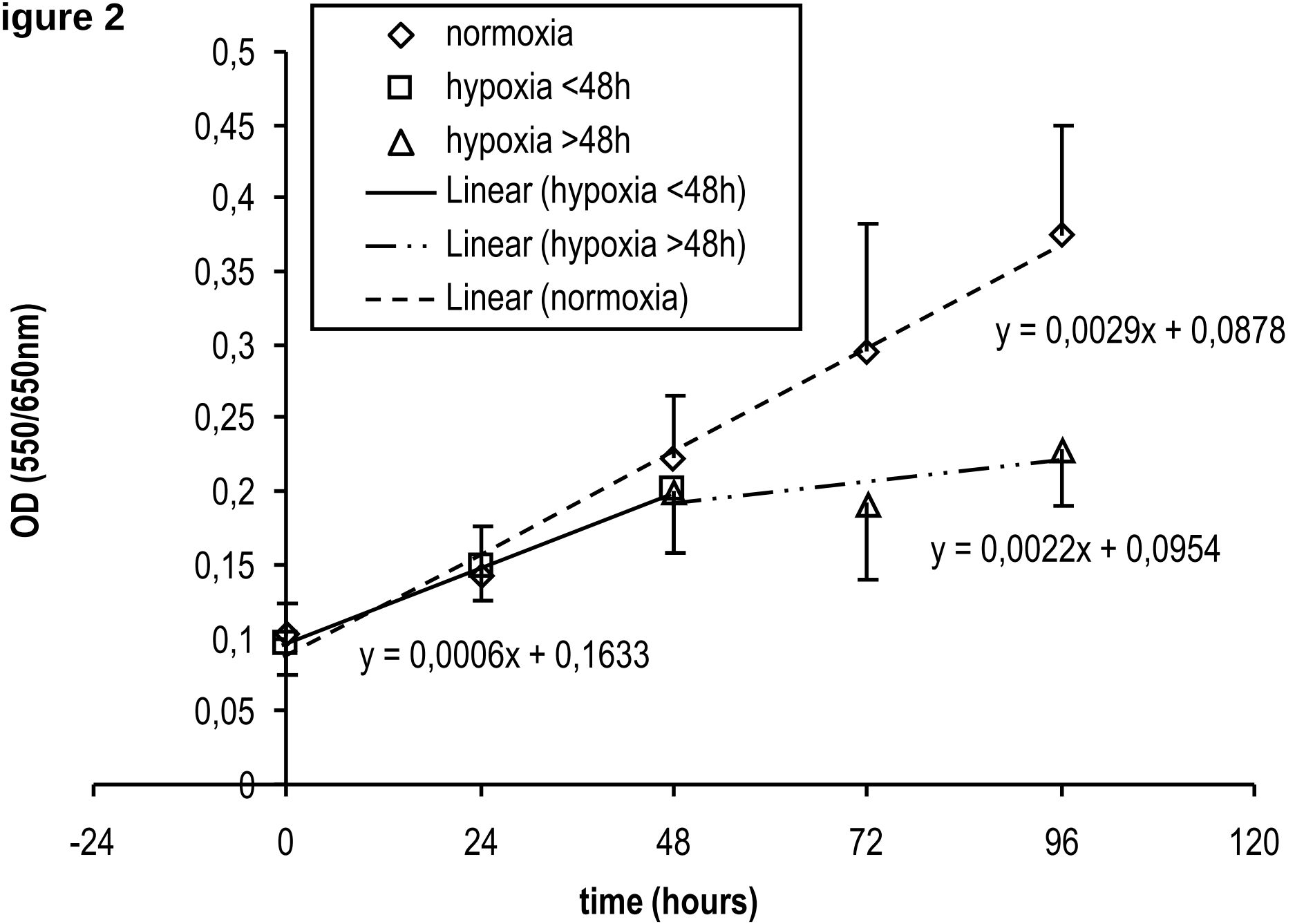

normoxia
hypoxia <48h
hypoxia >48h
Linear (hypoxia <48h)
Linear (hypoxia >48h)
Linear (normoxia)
y = 0,0029x + 0,0878
y = 0,0022x + 0,0954
y = 0,0006x + 0,1633
OD (550/650nm)
0,5
0,45
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
-24
0
24
48
72
96
120
time (hours)

Figure 3

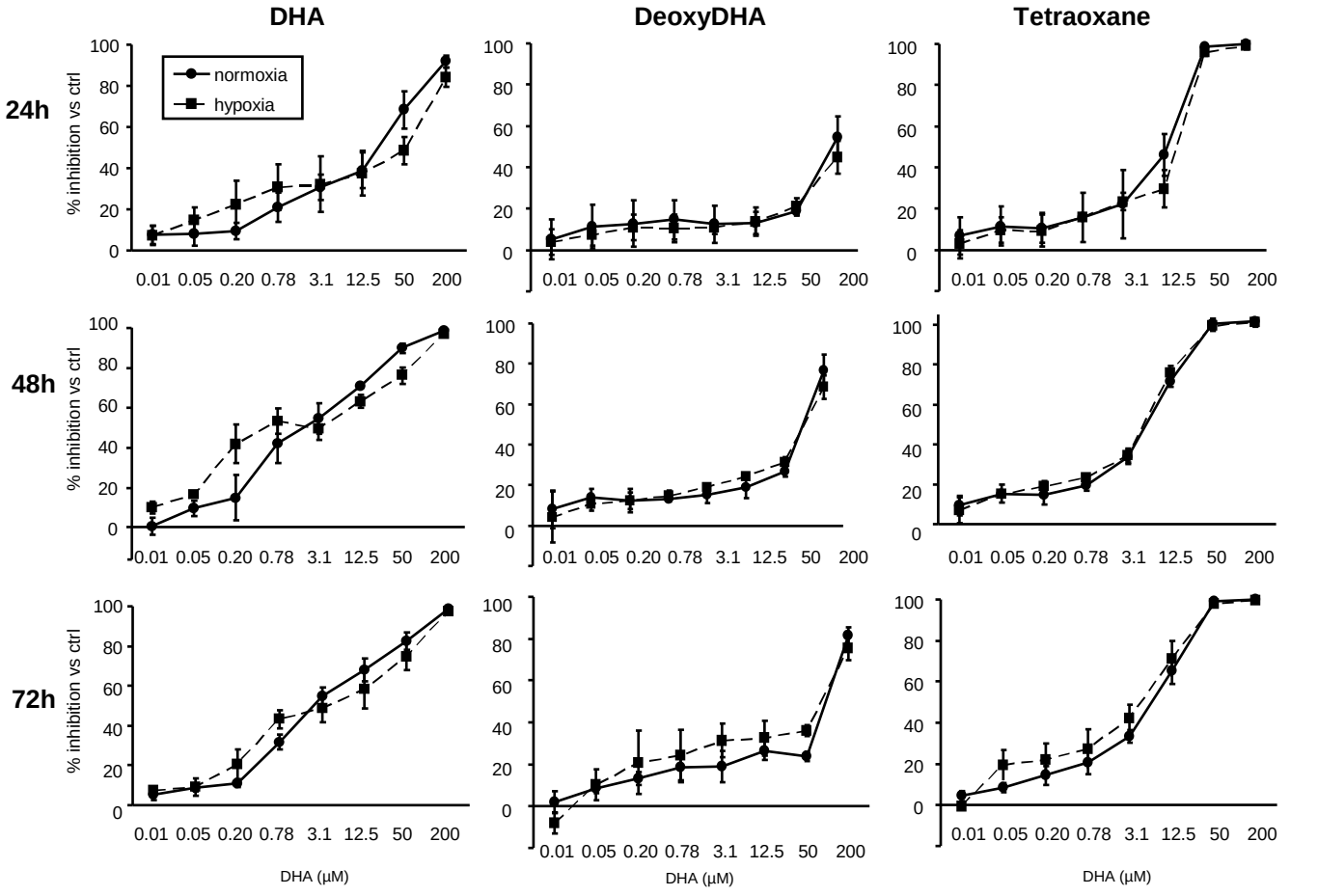

DHA
DeoxyDHA
Tetraoxane
24h
48h
72h
normoxia
hypoxia
% inhibition vs ctrl
0
20
40
60
80
100
0.01 0.05 0.20 0.78 3.1 12.5 50 200
DHA (µM)

Figure 4 Normoxia

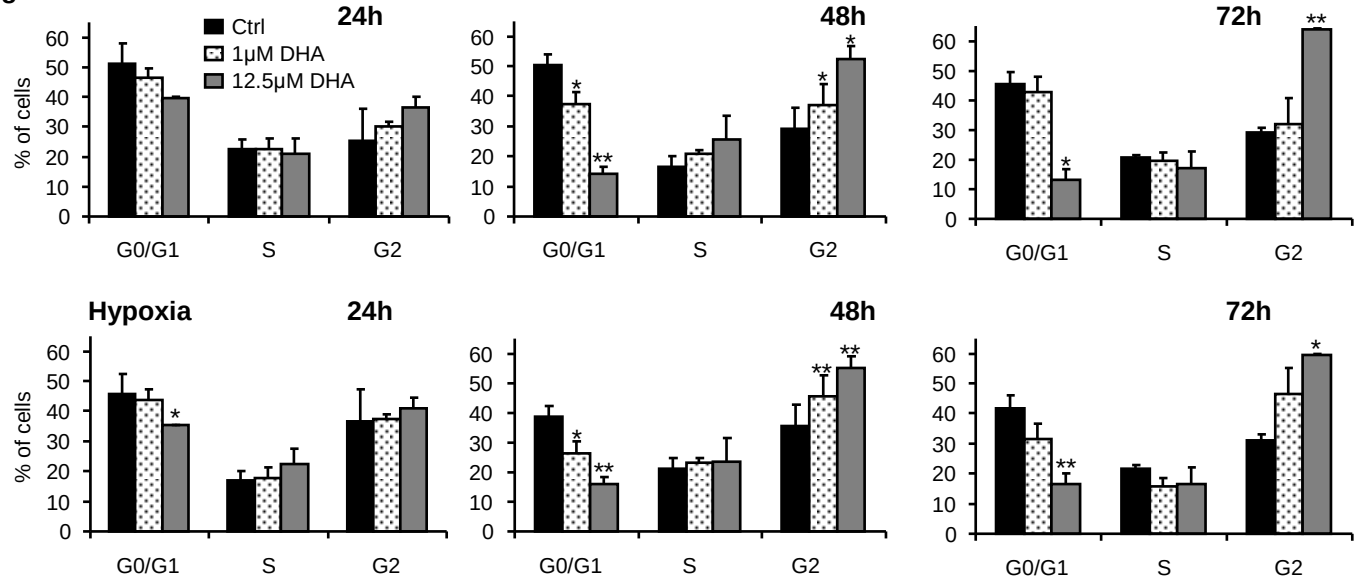
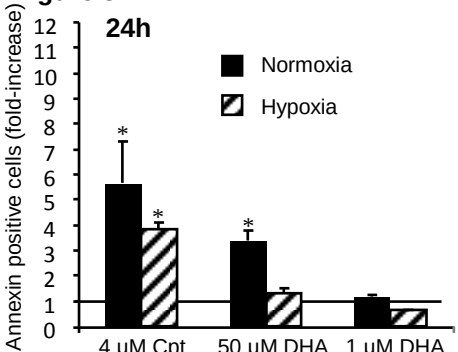
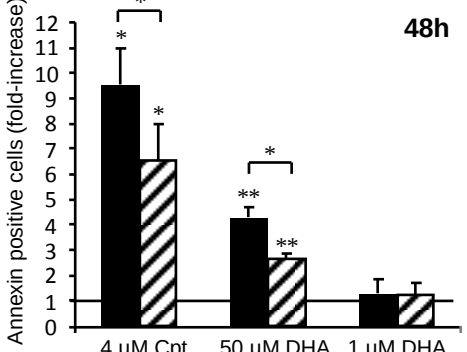
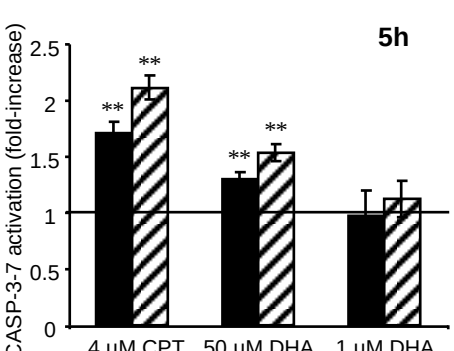


Figure 5


24h
Normoxia
Hypoxia
Annexin positive cells (fold-increase)
0
1
2
3
4
5
6
7
8
9
10
11
12
4 µM Cpt
50 µM DHA
1 µM DHA

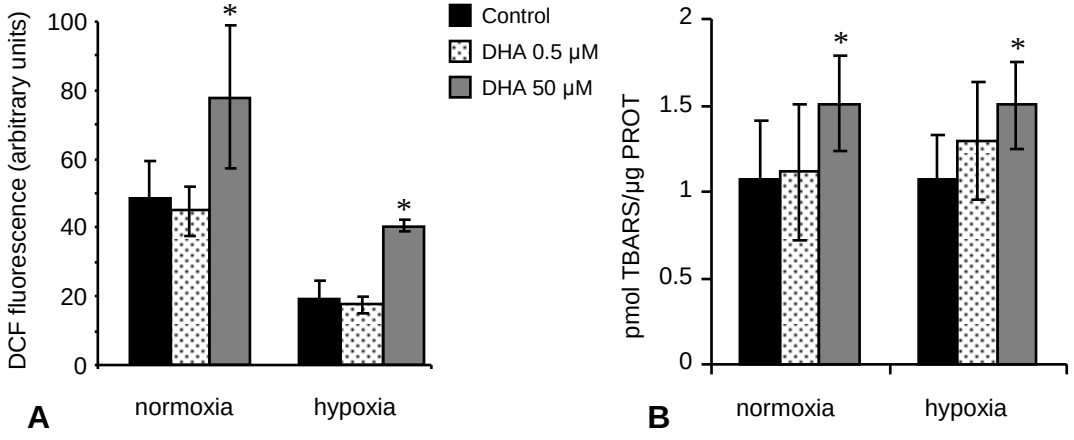

48h
Annexin positive cells (fold-increase)
0
1
2
3
4
5
6
7
8
9
10
11
12
4 µM Cpt
50 µM DHA
1 µM DHA

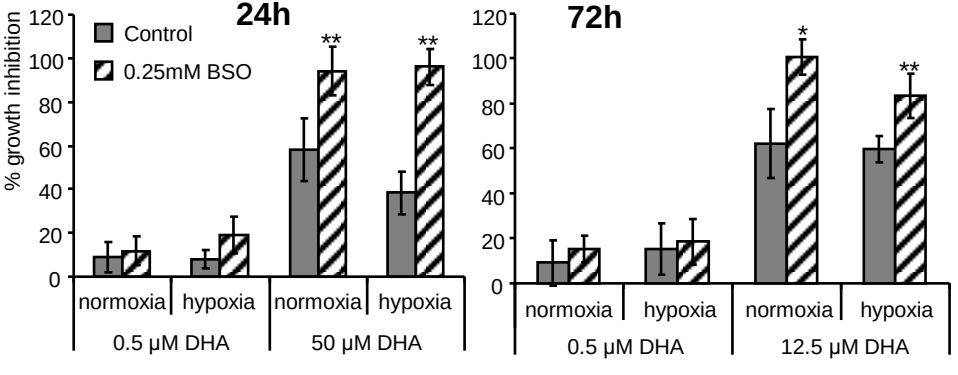
A

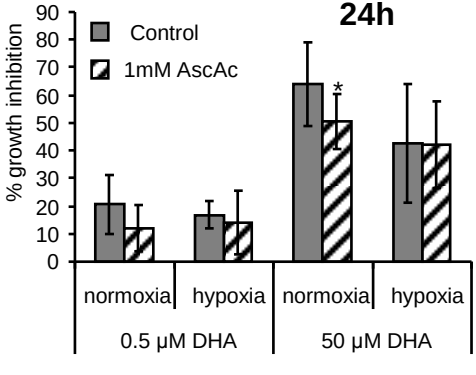

5h
CASP-3-7 activation (fold-increase)
0
0.5
1
1.5
2
2.5
4 µM CPT
50 µM DHA
1 µM DHA

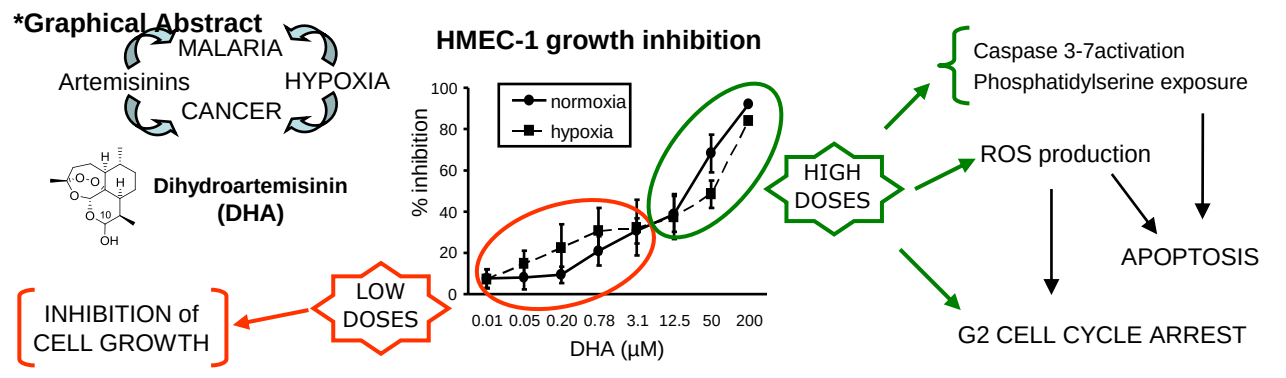
B

Figure 6


DCF fluorescence (arbitrary units)
100
80
60
40
20
0
*
*
Control
DHA 0.5 µM
DHA 50 µM
normoxia
hypoxia
A
pmol TBARS/µg PROT
2
1.5
1
0.5
0
*
*
normoxia
hypoxia
B


% growth inhibition
24h
Control
0.25mM BSO
**
**
normoxia
hypoxia
normoxia
hypoxia
0.5 µM DHA
50 µM DHA
72h
*
**
normoxia
hypoxia
normoxia
hypoxia
0.5 µM DHA
12.5 µM DHA
C


% growth inhibition
24h
Control
1mM AscAc
*
normoxia
hypoxia
normoxia
hypoxia
0.5 µM DHA
50 µM DHA
D


*Graphical Abstract
MALARIA
Artemisinins
HYPOXIA
CANCER
Dihydroartemisinin
(DHA)
HMEC-1 growth inhibition
normoxia
hypoxia
% inhibition
100
80
60
40
20
0
0.01 0.05 0.20 0.78 3.1 12.5 50 200
DHA (μM)
LOW
DOSES
INHIBITION of
CELL GROWTH
HIGH
DOSES
Caspase 3-7activation
Phosphatidylserine exposure
ROS production
APOPTOSIS
G2 CELL CYCLE ARREST