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***In Vitro* Oxygen Availability Modulates the Effect of Artesunate on HeLa cells**

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Abstract. *Background/Aim: Hypoxia can affect chemotherapeutic drug efficacy in cancer patients, yet related in vitro assays in oxygen-rich environment remain the norm. Such levels are well beyond normoxic/hypoxic levels typically experienced by normal tissues/tumor masses. The present study evaluated how artesunate anti-tumor efficacy is modulated by oxygen availability in HeLa cells and its implications for future in vitro analyses. Materials and Methods: Real-time cell analysis was employed to evaluate HeLa cell toxicity to artesunate at 21%, 4% or 1% oxygen. Cell count analysis was performed to validate real-time data. Results: An increase in artesunate efficacy was observed when oxygen concentration was reduced from atmospheric levels down to in vivo-relevant levels. Conclusion: Artesunate is more potent than originally reported using standard oxygen conditions during in vitro studies. The inclusion of this long overlooked variable as standard in future in vitro analysis procedures is warranted.*

Chemotherapy, radiation and surgery are the standard methods of cancer treatment today. However, multidrug resistance is a reoccurring drawback with chemotherapy and is displayed by many patients (1). In addition, the majority of chemotherapeutics have many side effects that are associated with high toxicity levels. Therefore, the search is ongoing for novel compounds with potential for more effective chemotherapy.

Tissue hypoxia is defined by clinicians as a decrease in oxygen availability below critical levels, which compromises

the function of cells, tissues or organs (2). This critical value has been identified in a previous clinical study as pO₂ of <10 mm Hg or <1.3%(v/v) oxygen (3). Hypoxia often develops in tumors due to poor blood supply and inadequate oxygen diffusion. More than 50% of solid tumors exhibit areas of hypoxia throughout the tumor mass, with areas of anoxia (no oxygen availability) also occurring (4). Tumor tissue derived from cervical, prostate, breast, head and neck, brain and soft tissue cancers displayed oxygen levels varying from 0.3-2.4% (2-18 mm Hg) (5). Based on this evidence, an oxygen concentration of 1% (v/v) was employed in the present study to mimic hypoxia *in vivo*. Tumor hypoxia is associated with poor prognosis in cancer patients by driving malignant progression. This is achieved through changes in gene expression of growth factors and survival proteins to enable tumor cells to withstand nutrient deprivation and grow in an unregulated manner (2). Apoptosis, cell-cycle arrest, angiogenesis, glycolysis and pH regulation are thought to be regulated by hypoxia-induced genes, usually under the control of hypoxia inducible factor 1 (HIF-1) (6). Hypoxia has also been implicated in conferring resistance to chemotherapy and radiotherapy. Tumor cell oxygenation was first shown to be a key factor in efficacy of irradiation treatment of cancer over 50 years ago (7). Furthermore many chemotherapeutic drugs are dependent on oxygenation of the tumor mass for optimal efficacy. Tumor cells in normoxic conditions were shown to be more sensitive to melphalan, a DNA alkylating chemotherapeutic agent, when compared to cells under hypoxic conditions (8, 9). Hypoxia dramatically alters cell cycle distribution with the majority of cells in G₁/S phase arrest (10). Consequently, chemotherapeutics that target DNA may be susceptible to hypoxia-induced resistance due to a substantial reduction in cell division and DNA replication. Moreover artemisinin was shown to be 30% more effective in an oxygen rich environment (21% O₂, or 152 mm Hg) when compared to a low oxygen environment (1%, 7.6 mm Hg) in treatment of *P. falciparum* strain of malaria.

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Despite evidence suggesting that hypoxia is crucial in chemotherapy outcome, almost all *in vitro* evaluation of novel compounds is performed in standard oxygen-rich environments (20-21% O₂ or 152-160 mm Hg) (11-13). This is markedly different from the normoxic oxygen levels typical of normal cells *in vivo* or indeed from the hypoxic or anoxic regions of a typical tumor mass. Several studies have investigated tissue oxygenation levels *in vivo* both in the case of tumor and non-tumor tissue (5, 14). The oxygen concentration in many non-tumor tissues *in vivo* is approximately 4-5% (38 mmHg) (normoxia) and is maintained within a narrow optimal range. This range however varies between tissue types. For example lung tissue oxygenation can be up to 14% (30-38 mm Hg) whereas liver and bone cartilage is usually within a 2-8% (15-61 mm Hg) range (15). Tumor tissue derived from cervical cancer, prostate cancer, breast cancer, head and neck carcinoma, glioblastoma and soft tissue carcinoma displayed oxygen levels varying from 0.3-2.4% (2-18 mmHg) (5).

Artemisinin is a sesquiterpene lactone containing a 1,2,4 trioxane ring within its chemical structure. Artemisinin was first isolated from the Chinese herb quinghaosu (*Artemisia annua* or annual wormwood) in 1972 (16). Artemisinin has been used for centuries as an effective Chinese medicine for treatment of fevers, *P. falciparum* and *P. vivax* malaria strains. Naturally-occurring artemisinin has low solubility in oil and water prompting the production and use of several semi-synthetic derivatives such as Artemether, Arteether and Artesunate (ART). All are effective against multidrug-resistant plasmodium strains of malaria. Interestingly, artemisinin and semi-synthetic derivatives have also been shown to have potent anticancer properties both *in vitro* and *in vivo* (17-20). It was reported by Nakase *et al.* (2008) that artemisinin and its derivatives induce apoptosis in -the very aggressive- Kaposi's sarcoma cells. Treatment of human vein endothelial cells (HUVEC) with concentrations above 2.5 μ M resulted in cell growth inhibition, which increased with drug concentration. Fifty-five different cell lines from the Developmental Therapeutics Program of the National Cancer Institute NCI were treated with ART, resulting in growth inhibition of a multitude of cancer cell lines including leukaemia, breast, colon, ovarian, prostate, central nervous system (CNS), melanoma and lung cancer (22). Furthermore ART was effective against 15 out of 16 neuroblastoma cell lines tested in a recent investigation (23). A major advantage of artesunate is the lack of cross resistance. ART and similar derivatives are valuable in the treatment of multidrug-resistant strains of malaria worldwide. Cancer therapy is also frequently adversely affected by the development of resistance by tumors. This has prompted researchers to determine whether multidrug-resistant tumors could be effectively treated with ART. ART was shown to be effective against multidrug-resistant small cell lung cancer (SCLC) cell line H69VP when preloading of cells with

transferrin was performed (24). ART was shown to be just as potent against multidrug-resistant cell lines that over-express MDR1/P glycoprotein, MRP1 and BCRP (25). Additionally, hydroxyurea-resistant CEM/HUR90 cells and methotrexate-resistant CEM/MTX1500LV cells also exhibited no cross resistance to ART. On the evidence of such research, artesunate and similar derivatives could be utilised for treatment of cancers displaying inherent multidrug resistance. However, cancer cells with up-regulated expression of antioxidants such as glutathione transferase have shown resistance to ART by reversal of the ROS-induced damage (25).

In the present study, a novel real-time cell analysis software was employed to investigate HeLa cell sensitivity to ART in varying oxygen environments of atmospheric oxygen, normoxia and hypoxia. This impedance-based, label-free assay was chosen ahead of alternative cellular assays such as MTT due to a potential effect of varying oxygen on cellular metabolism (26). The xCELLigence System measures electrical impedance across micro-electrodes integrated on the bottom of cell culture plates. Impedance data are automatically converted to Cell Index (CI) values by the software. Cell index is defined as a relative change in electrical impedance created by attached cells, which is indicative of cell number, cellular adhesion, cell proliferation, cell size and morphology. As cells detach and die the cell-covered area reduces and CI values decrease. Thus xCELLigence system offers a much more informative and dynamic cell analysis in real time when compared to static endpoint analysis at selected time points.

Materials and Methods

Cell culture. HeLa cervical cancer cells (European Collection of Cell Cultures) were cultured in Dulbecco's Modified Essential Medium (DMEM F12) (Sigma Aldrich, Dorset, UK) supplemented with 5% Foetal Bovine Serum (Sigma Aldrich) and 1% penicillin /streptomycin (Sigma Aldrich) and 1% L-Glutamine (Sigma Aldrich). Cells were maintained at 37°C, 95% humidity, 5% (v/v) CO₂. Cells were also maintained at Oxygen concentrations of either 1% (v/v) (7.6 mmHg), 4% (v/v) (30 mmHg) or 21% (v/v) (160 mm Hg) for a minimum of 24 h before any experiment was commenced and until analysis was completed.

Preparation and addition of artesunate. ART was kindly provided by Dr Sarah Rawe, School of Chemical and Pharmaceutical Sciences, Dublin Institute of Technology. ART was dissolved in dimethyl sulfoxide (DMSO) and further diluted in complete DMEM F12 medium, such that when added to cells, the final concentration of DMSO in all cells was maintained at 0.1% (v/v).

Real-time cell analysis. E-plates (ACEA Biosciences, San Diego, CA, USA) were seeded with 4.5×10^3 HeLa cells per well, 24 h before artesunate exposure. E-plates were placed within the cradle of an xCELLigence DP Real Time Cell Analyser that remained inside the incubator for the duration of the experiment. 24 h post-seeding, half the medium was removed from each well and replaced

with an equal volume of either fresh complete medium, 0.2% (v/v) DMSO in fresh complete medium, 0.2% (v/v) DMSO + 20 μ M ART in fresh complete medium or 0.2% (v/v) DMSO + 100 μ M ART in fresh complete medium. Cell growth data was captured every 15 min for the duration of the experiment, beginning 24 h before drug addition and continuing for a minimum of 48 h post drug addition and is displayed by the built-in software as “Cell Index”.

Cell count analysis. HeLa cells were seeded on a 6-well plate (Sarstedt, Nümbrecht, Germany) at 100,000-120,000 cells per well (equal density to real time cell analysis). 24 h post-seeding, half the medium was removed from each well and replaced with an equal volume of either fresh complete medium, 0.2% (v/v) DMSO in fresh complete medium, 0.2% (v/v) DMSO + 20 μ M ART in fresh complete medium or 0.2% (v/v) DMSO + 100 μ M ART in fresh complete medium. Cells were harvested and counted 0, 24 or 48 h later using a Z2 Particle Analyser (Beckman Coulter, Miami, FL, USA). Cell counts are expressed as a percentage of control.

Results

In vitro real-time cell analysis was performed at three different oxygen concentrations, 21% (atmospheric), 4% (normoxia) and 1% (hypoxia). Cells were either exposed to no change (DMEM only), vehicle-only, 10 μ M artesunate or 50 μ M artesunate (Figure 1). In atmospheric oxygen conditions, it could be observed that 10 μ M artesunate began to inhibit cell growth 24 h post-treatment, whereas 50 μ M artesunate caused HeLa cell death as early as 12 h after drug treatment. Interestingly, when oxygen availability was reduced to that of normoxic conditions, an increase in artesunate efficacy was observed. This was particularly evident in cells exposed to 10 μ M artesunate, where growth inhibition was observed 12 h post-drug addition and a substantial reduction was observed 48 h post-addition. In normoxia, 50 μ M artesunate resulted in HeLa cell cytotoxicity after 10 h and was fatal to all cells by 48 h.

When artesunate was analysed in hypoxic conditions, a similar increase in HeLa cell sensitivity to the drug was observed; 10 μ M artesunate caused considerable cell growth inhibition when compared to sham exposed control. Furthermore 50 μ M artesunate treatment induced cell cytotoxicity after just 10 h, resulting in almost complete cell death after 48 h.

Cell survival analysis was performed by counting cells in a Beckman Coulter Z2 particle analyser. Experiments were performed at three different oxygen concentrations, 21% (atmospheric), 4% (normoxia) and 1% (hypoxia). Cells were either exposed to no change, 0 μ M, 10 μ M or 50 μ M artesunate 24 h after seeding and cells were counted at 0, 24 or 48 h post exposure. All data are expressed as a percentage of cell numbers at 0 h post-artesunate exposure (Figure 2). Artesunate reduced cell number in a dose-dependent manner at all 3 oxygen environments. However, an enhanced cell sensitivity at lower oxygen concentrations was evident, particularly at the dose of 10 μ M.

Discussion

Hypoxic regions are typically present within solid tumor masses due to inadequate blood supply. Hypoxia is associated with a more aggressive tumor phenotype, which often displays resistance to chemotherapy and radiotherapy (3, 4, 7, 27, 28). Moreover chemotherapeutic drugs are often dependent on oxygenation of the tumor mass for optimal efficacy (8, 9). However, despite the evidence that tissue oxygenation is important in response to chemotherapy, almost all *in vitro* cytotoxic assays to date have been performed in standard oxygen rich environment of 21% (v/v) or 160 mmHg. This is markedly different to tissue oxygen levels *in vivo* with many tissues of the body having 4% oxygenation (normoxia). This range however varies between tissue types. Lung tissue oxygenation can be up to 14% (14), whereas liver and bone cartilage is usually within the 2-8% oxygen range (15). Conversely tumor tissue derived from cervical cancer, prostate cancer, breast cancer, head and neck carcinoma, glioblastoma and soft tissue carcinoma displayed oxygen levels varying from 0.3-2.4% (2-18 mmHg) (5).

In the present study novel real-time cell analysis (RTCA) software was employed to investigate the effect of oxygen in potentiating cellular response to anticancer agents. This new impedance based assay is non-invasive and label-free, providing dynamic detection of changes in cell proliferation, cell number, cellular adhesion, cell size and morphology in real time rather than at selected end-points on a bar graph. The resulting data are automatically converted to cell index (CI) by the software (29, 30). As cells detach and die (due to cytotoxic agent) the surface area covered by cells is reduced and CI values decrease. For this study it was necessary to choose a well-studied drug with anticancer activity. Artesunate (ART) is a semi-synthetic derivative of artemisinin with anti-malarial and anti-cancer activity and was chosen for this analysis (22). HeLa cervical cancer cells were employed as a tumor cell line model and are known to be sensitive to artesunate (31, 32) though notably when analyzed in oxygen-rich conditions. In this study we demonstrated that ART potency against HeLa cervical cancer cells is enhanced as oxygen concentration is varied from atmospheric levels to *in vivo*-relevant normoxia and hypoxia. In atmospheric oxygen conditions (Figure 1), it could be observed that ART began to inhibit cell growth in a dose-dependent manner with 50 μ M ART resulting in HeLa cell death as early as 12 h post-drug treatment. However, when oxygen availability was reduced to *in vivo* body relevant oxygen concentrations of 4% (normoxia) and 1% (hypoxia), a significant increase in ART efficacy was observed. This was particularly evident in cells exposed to 10 μ M ART, where growth inhibition was observed as early as 10 h post-treatment. Furthermore, 50 μ M artesunate resulted in HeLa cell cytotoxicity after 10 h and was fatal to all cells by 48 h.

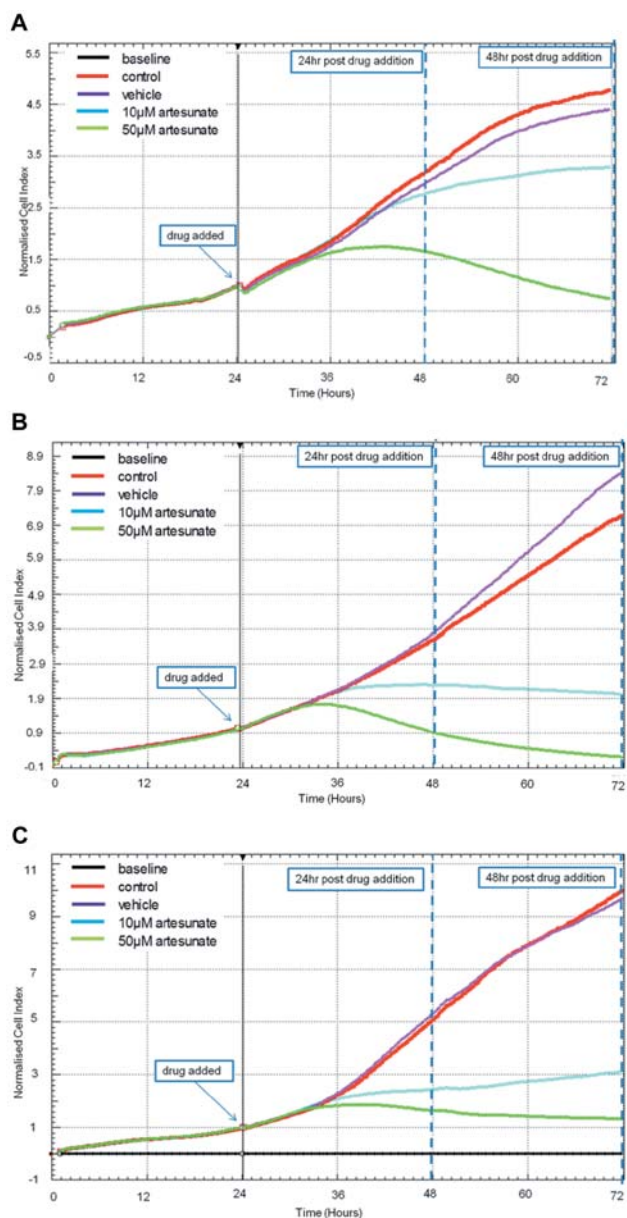


Figure 1. Real-time cell analysis of HeLa cells in (A) 21% oxygen, (B) 4% oxygen or (C) 1% oxygen conditions. HeLa cells seeded at 5×10^3 cells/well, treated with drugs after 24 h growth and monitored using real-time cell analysis until experiment end at 72 h. Cell viability is plotted as cell index (CI) on y axis. Each real time experiment was performed 3 times.

When ART was analyzed in hypoxic conditions, a similar increase in HeLa cell sensitivity to the drug was observed compared to that of atmospheric oxygen. This is significant, since many aggressive malignant tumors exhibit areas of hypoxic tissue (33).

Therefore artesunate, and indeed many other chemotherapeutic agents assessed at atmospheric oxygen, may be more

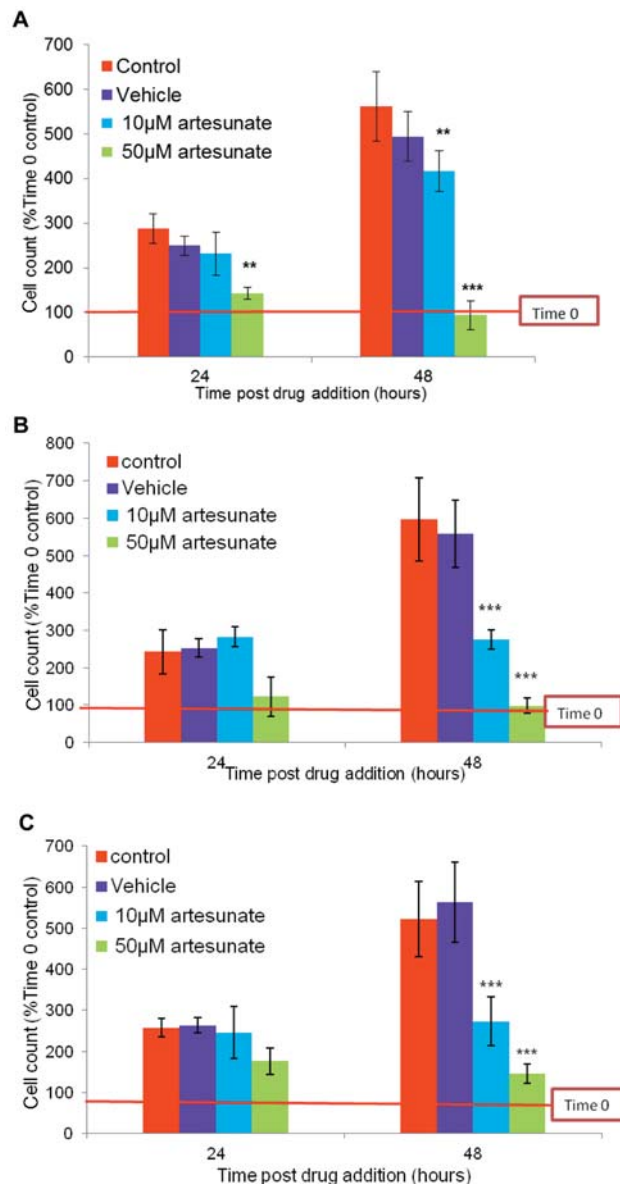


Figure 2. HeLa survival assay: HeLa cells maintained in either (A) 21% oxygen, (B) 4% oxygen or (C) 1% oxygen conditions. HeLa cells seeded in 6-well plates were counted to measure relative cell survival and are expressed as a percentage of cell numbers at time zero drug addition. All data is representative of triplicate experiments (\pm SD). Differences between treated/exposed and sham exposed/control cell populations were analyzed using one-way ANOVA, with Tukey post hoc test using $p < 0.05$ as the criterion for significance (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

potent against cancer cells *in vivo* than originally thought. Cell count analysis was also performed (Figure 2) to corroborate with RTCA as this is a relatively new technique. By investigating cell numbers at 0, 24 and 48 h post-drug exposure, a similar dose-dependent response of HeLa to Artesunate can be observed. A significant increase in drug

efficacy can be observed in 10 μ M artesunate when oxygen availability is lowered below 4% oxygen, further supporting real-time data.

Treatment of HeLa cervical cancer cells with artesunate at varying oxygen conditions of atmospheric, normoxia and hypoxia revealed an increase in ART efficacy when oxygen levels were lowered to *in vivo* levels. ART is even more potent than originally reported during *in vitro* studies. Additionally, static end-point assays such as MTT, which rely on mitochondria, may be far more active at atmospheric conditions, than would otherwise be at normoxia. Consequently there is a need to factor this long overlooked variable as standard procedure for future *in vitro* analyses. Oxygen concentration is a parameter that can no longer be overlooked for future design of *in vitro* analyses.

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