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Original Paper

Natural Hypoxia is Not a Limiting Factor in Evaluating the Novel Arylidene Derivative MLT-401 Against an *In Vitro* Colorectal Cancer Model

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Key Words
Colorectal carcinoma • Arylidene derivatives • Natural hypoxia • Anti-cancer drugs • Apoptosis

Abstract
Background/Aims: Cancer cells *in vivo* develop resistance to many anti-tumor drugs. One known factor to influence such drug resistance is hypoxia, which is an important component of the tumor microenvironment. Standard cancer lines mostly do not exhibit a cellular hypoxic microenvironment and there is a paucity of information on the efficacy of lead molecules in both cellular- and environment-induced hypoxic conditions. Therefore, in the present study, we have evaluated the efficacy of the arylidene derivative MLT-401, a lead molecule showing activity against colorectal cancer model using the HCT 116 cell line and CCD-80-C control cells in normoxic and natural (marginal) hypoxic conditions, which is usually observed in high-altitude regions. ***Methods:*** The efficacy of MLT-401 on HCT 116 and CCD-80-C cells were tested in both normoxia and marginal hypoxia conditions. MTT assay was used to evaluate cell proliferation, Annexin V binding assay for apoptotic cell quantification and PI staining for cell cycle were done by flow cytometry. Induction of pro-apoptotic marker BAX and anti-apoptotic Bcl-2 were assessed by western blot. Bcl-2/BAX ratio was calculated based on protein expression by western blotting and bands were quantified by Image J software. ***Results:*** Analysis of cell proliferation showed an average 10-fold reduction in the inhibition of HCT 116 cells in hypoxic conditions with approximately 500 nM MLT-401, while there was no significant change noted in marginal hypoxic conditions. A proportionate increase in the number of apoptotic cells and large M4 fraction of 10.5% and 26.7% of HCT116 against 6.3% of control cells in cell cycle assessment with MLT-401 concentrations ranging from 250 to 500 nM respectively clearly demonstrated anti-cancer activity. A Bcl-2/BAX ratio of <1 showed that the induction of apoptosis was the gross mechanism underlying the inhibition of HCT 116 cells by MLT-401. ***Conclusion:*** Collectively, these results show MLT-401 as an effective anti-colorectal cancer lead molecule irrespective of normoxia or natural hypoxia.

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Introduction

Anti-cancer or anti-tumor drug activity is typically evaluated using *in vitro* screening techniques employing primary cells and commercial cell lines. These cell lines are standardized to laboratory conditions and often do not address the abnormal physiological nature of tissues or cells as observed in *in vivo* conditions [1, 2]. Hypoxia, immune evasion, extracellular matrix composition/organization, inflammation, and immunosuppressive tumor-associated cells are important physiological factors that can affect drug interactions with tumor cells and promote tumor progression [3, 4]. Some of the factors are systemic while few factors are at cellular level influenced by the physiological environment prevailing at the niche. These physiological imbalances result in the formation of a gross cellular catastrophe known as the tumor microenvironment, which has complex characteristics that are not observed in modified cell lines. Moreover, cancer progression is mostly dependent upon such persistent microenvironment and cellular deregulation which forms the basis of most important cancer microenvironment leading to drug resistance.

Lead molecules with anticancer/tumor activity are usually evaluated using standard cancerous lines that do not represent real cancer models. Although these lines do not exhibit the exact tumor microenvironment as observed *in vivo*, ease to use and high throughput adaptations makes it comfortable for many drug screening laboratories. In many cases, lead molecules with positive results against cancer or tumor cell lines do not translate into successful animal or clinical trials [5]. One of the limiting factors affecting drug efficacy, for *in vivo* tumors, is hypoxia, which is a common phenomenon observed in the tumor microenvironment and is known to influence tumor metastasis and confer drug resistance [6]. Theoretically, low oxygen availability is an important factor that can alter the therapeutic effectiveness of a drug. Further, hypoxia can induce or mitigate drug-induced toxicity and alter cellular metabolism, enzyme kinetics, and overall bioenergetic turnover of cells [7]. However, in the case of cancer drug development, the process is reversed, as the tumor microenvironment itself is hypoxic and environmental hypoxia may have its own effect on drug action [8]. Further, *in vivo* hypoxia is known to promote tumor cell immune evasion, a strategy that allows tumor cells to escape immune surveillance and survive. Hence, most of the lead drug molecules *in vitro* largely do not work in the microenvironment of a tumor. The traditional understanding of hypoxia and the cancer microenvironment is that hypoxic stress impairs tumor cell killing mediated by both innate and adaptive immune cells, which have altogether different drug interactions [9, 10]. It is also known that hypoxic stress can also play a major role in the acquisition of anti-tumor drug resistance, which is not clearly understood. The known mechanism is that tumor cells become adapted to hypoxic microenvironment by transcriptional regulation of the hypoxia-inducible factor (HIF) family of proteins [11]. Further, the hypoxia-mediated nuclear translocation of HIF heterodimers results in the transcriptional activation of downstream targets like glucose transporter 1 (GLUT1), and CAIX that are involved in chemoresistance [12]. Hypoxia-induced chemoresistance has been discussed in the context of various molecular signals such as nuclear factor kappa-B, cyclooxygenase-2, and apoptosis inhibitory protein. Further, environmental or physiological hypoxia might alter gross cellular metabolism and may be one of the factors underlying the inductionof drug resistance [13]. As an example, hypoxia-mediated HIF proteins induce some drug-metabolizing enzymes of the cytochrome P-450 class, rendering themselves as important cofactors in cancer expression and metastatic potential [14].

To overcome these limitations of anti-tumor drugs, especially in the industrial screening where large arrays of chemicals, semisynthetic molecules and natural products are used the activity of many promising lead molecules needs to be assessed in hypoxic conditions. To address these physiological concerns, the current study was designed to use the arylidene derivative MLT-401, an indanone compound known for its biologic activity, with special reference to its anti-tumor/cancer efficacy on various cancer models [15, 16]. The selection of MLT-401 was based on its previously reported structural similarity with arylidene indanone molecules having profound anti-tumor activity [17]. Further, these indanone compounds

have not been affected by the drug resistance as observed with many other cancer drugs. Considering these facts, the anti-tumor activity of MLT-401 was evaluated in the standard colorectal cancer cell line HCT 116 and control cells CCD-80-Co. This study will also throw light on the benefits and disadvantages of natural hypoxia on the efficacy of indanone-based drugs. The preliminary results of this study were presented previously in an abstract form by the author, as a recipient of the American Physiological Society Travel Award, at the 38th Congress of the International Union of Physiological Sciences, which was hosted by the Brazilian Physiological Society in Rio de Janeiro, Brazil [18].

Materials and Methods

Cell culture

HCT 116 and CCD-18-Co cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin. All cells (passages 3–12), were maintained at 37°C in a 5% CO_2 environment in a humidified atmosphere until the required confluence (70%) was attained. The medium was replaced every 2 days and maintenance was strictly in accordance with the standard methods. All experiments were performed after the due approval of the Biomedical Research Ethics Committee at King Khalid University, Abha, Saudi Arabia (REC. No. 2017-01-01).

Induction of transient hypoxia

To induce hypoxia, cell culture medium was bubbled for 30 min with 100% N_2 followed by the addition of a freshly prepared sodium dithionite solution to give a final concentration of 0.25 mM, which was sufficient to reduce pO_2 to low levels. The tissue culture plates were sealed with Vaseline to avoid the influence of the regular 5% CO_2 atmosphere of the incubator. This method was modified from the standard cited literature to suit our study purpose [19].

Cell proliferation assay

Cell proliferation was assessed as *per* our standard protocol described elsewhere [20]. Briefly, 5000 cells/well in 100 µL RPMI medium supplemented with 10% FBS and 1% penicillin/streptomycin were plated in triplicates in a 96-well plate. After an overnight incubation, the medium was replenished with either fresh growth medium for normoxic conditions or low-oxygen medium for marginal hypoxic conditions containing 50 µL MLT-401 to the desired final concentration. The hypoxic medium plates were sealed to avoid the external influence of gases. Dimethyl sulfoxide (DMSO) and blank controls were also included simultaneously. The medium was changed to regular fresh medium and the plates were incubated at 37°C and 5% CO_2 for 48 h. Then, 15 µL of 5mg/mL MTT was added and incubated for 3.5 h. The medium was aspirated and MTT was dissolved in 150 µL DMSO and absorbance was read at 560 nm with reference at 640 nm. Percentage inhibition was calculated after subtracting the value for Day 0 MTT. The results were analyzed using GraphPad Prism software.

Annexin V assay

An annexin V binding assay was performed using an Annexin V Detection Kit (e-Bioscience, San Diego, CA) as *per* the manufacturer's instructions. Briefly, 0.5×10^6 cells were grown in 6-well tissue culture plates for 24 h until the cell sheets were semi-confluent. The cells were incubated with medium containing the final concentrations of MLT-401 used in this study. The plates were incubated in a CO_2 incubator for 72 h. Post-incubation, the cells were harvested, washed twice with wash buffer, and incubated with 0.25 µg/mL annexin V reagent in 1× binding buffer for 15 min. The cells were washed twice to remove excess annexin V and were re-suspended in binding buffer containing 0.5 µg/mL propidium iodide. A flow cytometer was used to acquire 10, 000 events. Early and late phase apoptotic cells were segregated with a quadri plot graph and the total percentage of apoptotic cells was presented using a bar chart.

Cell cycle analysis

Briefly, 0.5×10^6 cells *per* well were added to a 6-well tissue culture plate. MLT-401 was added at the desired concentration to the medium and the cells were incubated for 72 h at 37°C in a 5% CO_2 incubator.

After 72 h, the cells were fixed in 70% ethanol and stored at -20°C till acquisition using a flowcytometer; 10, 000 events were acquired and % percentage of cell population in different cell cycle stages with respect to control was calculated [21].

Western immunoblotting

HCT 116 and CCD-18-Co cells were cultured with various concentrations of MLT-401 for 48 h. The cells were scraped and lysed in RIPA buffer, as described elsewhere. The insoluble materials were removed by centrifugation at 14, 000 × *g* for 10 min at 4°C. Protein quantification was performed using a standard Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, IL). Cell lysate (20 µg/lane) was resolved by 8–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by polyvinylidene difluoride membrane transfer. The transferred membrane was probed with anti-BAX, anti-Bcl-2, and anti-β-actin primary antibodies (Cell Signaling Technologies, Beverly, MA), followed by the addition of the corresponding horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized by using ECL reagents (Amersham Bioscience, Piscataway, NJ) and exposed to Kodak X-Omat Blue XB-1 films (Rochester, NY). Bands were quantified using ImageJ (Ver. 1.46; NIH, Bethesda, MD) and normalized to actin and the ratio was determined.

Statistical analysis

Data from multiple repeat experiments were quantified and displayed as the mean ± standard error of the mean. The difference between two groups was analyzed using a two-tailed Student's t test. A P-value < 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism 6.0 (La Jolla, CA).

Results

MLT-401 is a derivative of arylidene indanone compounds. MLT-401 has a molecular weight of 248.32 with a formula $C_{18}H_{16}O$. Its structure is similar to other arylidene molecules with modifications at the positions near the arylidene ring (Fig. 1). The nontoxic dose of arylidene in the normal control CCD-18-Co cells was determined in both normoxic and marginal hypoxic conditions. The nontoxic dose of the solvent DMSO has been determined previously and is not shown here. The nontoxic concentration of MLT-401 used in the current investigation ranged from 250 to 500nM for HCT116 cells, while the normal CCD-18-Co cells tolerated approximately 5000 nMMLT-401 (Fig. 2a and b).

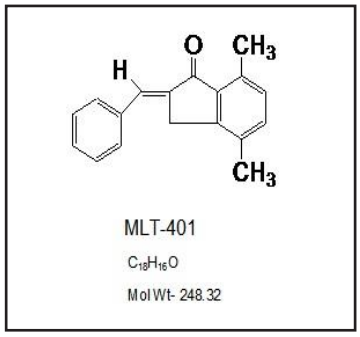

Fig. 1. Structure, molecular formula, and molecular weight of MLT-401.

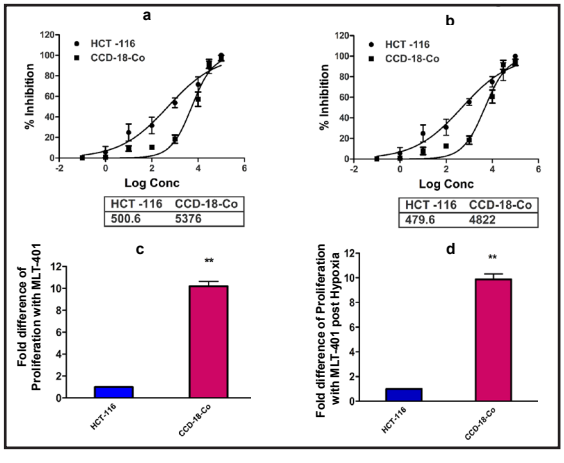

Fig. 2. Proliferation of CCD-18-Co and HCT 116 cells under (a) normoxic and (b) hypoxic conditions in the presence of MLT-401. Fold difference in the inhibition of proliferation by MLT-401 under (c) normoxic and (d) hypoxic conditions.

The results of the initial cell proliferation inhibition assays with various concentrations of MLT-401 showed a dose-dependent reduction in the proliferation of HCT116 colorectal carcinoma cells, while the normal CCD-18-Co cells were not inhibited (Fig. 2a). Drug activity under the influence of marginal hypoxia also did not affect the inhibition of HCT116 (Fig. 2b). An average 10-fold reduction in proliferation was observed

with a concentration of 500 nM MLT-401 in both normal and marginal hypoxic conditions. We next investigated the basic mechanism of cell death. Our cell cycle assessment showed the accumulation of early and late phase apoptotic cells (Fig. 3a and b). These results clearly indicate that MLT-401 has profound activity in inhibiting cancer in a classical manner with early and late phase apoptosis. We did not observe a similar effect with the CCD-18-Co cells. To substantiate the above observation, classical annexin V versus propidium iodide staining was used to assess the cell cycle. A large accumulation of M4 fraction about 10.5% to 26.7% in HCT 116 cells against 5.9 % to 6.3% of control cells was observed at a concentration ranging from 250 to 500 nM MLT-401, respectively (Fig. 3c). Quantitation of the M4 fraction showed a dose-dependent increase of apoptotic cells in the HCT 116 cell line compared with the control CCD-18-Co cells.

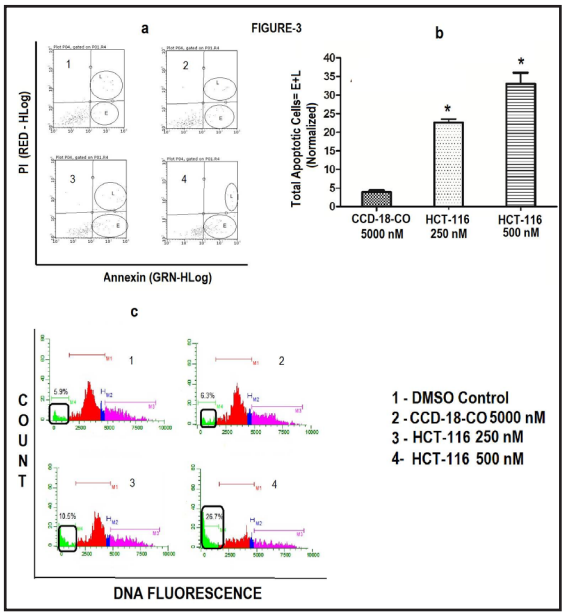

Fig. 3. (a) Annexin V staining showing the accumulation of early and late apoptotic HCT116 cells and CCD-18-Co cells after treatment with MLT-401. (b) Quantitation of the early and late apoptotic cells. (c) Cell cycle assessment of HCT116 cells showing the accumulation of the M4 phase.

Next, we investigated whether MLT-401 inhibition of cancer cells is through the upregulation of pro-apoptotic factors. This was in lieu of the accumulation of the M4 fraction observed previously (Fig. 3a, b, and c). A marginal increase of Bax expression was observed in HCT 116 cells treated with 250 and 500 nM MLT-401, while a drastic reduction in Bcl-2 expression confirmed the induction of apoptosis (Fig. 4a). Further, a Bcl-2/Bax ratio of < 1 (Fig. 4b) indicated apoptosis as the mode of cell death.

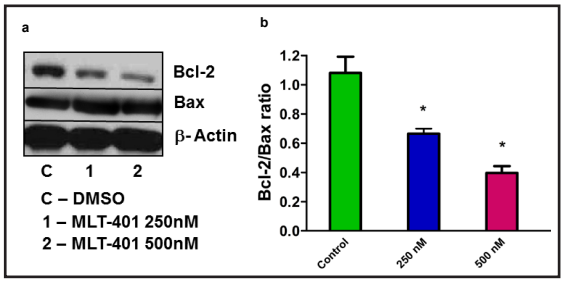

Fig. 4. (a) Western blot showing the reduction of the expression of the anti-apoptotic marker Bcl-2 following treatment with MLT-401 in HCT116 cells. (b) Quantitation of the bands showing the reduced expression of Bcl-2.

From the above observations, it is evident that MLT-401 inhibits the proliferation of cancer cells through the induction of apoptosis and cell cycle regulation. Further, from the results obtained, it can be hypothesized that the Bcl-1/Bax ratio of <1 not only indicates apoptosis but also the down-regulation of anti-apoptotic factors.

Discussion

The use of arylidene and its derivatives as candidate molecules for anti-cancer or anti-tumor therapy has been examined for a few decades. Arylidene rings are reported to confer stability to the molecule and its biological activity [22]. Many studies have assessed various other arylidene derivatives exhibiting antibacterial [23], antifungal [24], or anticancer [25, 26] activities. Other members of our study group have also reported anti-cancer properties [17, 21] for a few derivatives of this family.

The results of the current study show that a low concentration of MLT-401 inhibited the proliferation of HCT116 cells. Generally, a low concentration of drug inhibiting cancer cell

proliferation may be a positive signal for a wide therapeutic window with a speculation that this might be less toxic to the normal surrounding tissues *in vivo*. This is evident from the results of CCD-16-Co cells that tolerated approximately 5000 nM MLT-401. Similar effects were observed with other arylidene derivatives studied using various cancer models [9, 27]. The selective inhibition of cancerous cells over normal cells both in normoxic and marginal hypoxic conditions shows that the molecule works downstream of the hypoxic conditions. Some arylidene derivatives have been shown previously to work on upstream pathways, such as the involvement of the Akt pathways ras and p53 [17, 28, 29]. It may be further noted that the involvement of various upstream and downstream nodal pathways is characteristic of the source of cancer models [30, 31]. For the first time, we have taken marginal hypoxia, which further mimics the tumor microenvironment into consideration to check its effect on an anti-cancer drug. None of the other published literature has examined environmental hypoxia, which may be detrimental or influential in the mechanism of action, especially with cancer targets. Hypoxia inducing resistance to drugs might be working though upregulation of many other pathways too. However established cell culture system with varied cancer characteristics and upon environmental selection pressure may impact our understanding of the drug targets. Henceforth, true mechanism or effect of the drug may not be studied appropriately. Consequently, progression of a potent drug or lead molecule into pre-clinical investigations may be halted.

Next, we investigated the mechanism behind the inhibition of proliferation. Apoptosis is known to be the most common method of cancer cell death [32]. Many pathways with different stimulations, stress conditions, and drugs lead cancerous cells to undergo apoptotic death [33-36]. The accumulation of early and late phaseapoptotic cells shows the efficacy of a compound to stimulate the process of apoptosis irrespective of the proliferative nature of cancer cells. Our results were in agreement and another way of confirming the induction of apoptosis by MLT-401. It is well established from the literature [37] and our own previous investigations [17, 21] have shown that the cells which get accumulated in the M4 fraction were apoptotic cells.

Although there are many pathways leading to apoptosis, the up-regulation of pro-apoptotic factors and down-regulation of anti-apoptotic factors are usually investigated to confirm the end stage apoptotic death of cancer cells. In the current investigation, MLT-401 down-regulated Bcl-2 expression, while there was not much of an up-regulation of Bax; however the ratio of Bcl-2/Bax was less than 1, which was in agreement with some standard anticancer drug evaluation studies [38-40]. Many other studies have shown that apoptosis is regulated by the anti-apoptotic molecule Bcl-2 and relative Bax expression in different cancer models. However, the dose-dependent selective toxicity of cancer cells to MLT-401 clearly shows that this lead molecule is not affected by the hypoxic microenvironment observed in many tumor/cancer tissues. Collectively, our results indicate the mechanism of apoptotic cells death by MLT-401 and show it to be an ideal candidate lead molecule for the treatment of colorectal cancer.

Conclusion

From the above investigations, MLT-401, a novel arylidene derivative, inhibits HCT 116 colorectal cancer cells in a dose-dependent manner. Marginal hypoxia does not interfere with the anti-tumor activity of MLT-401. Apoptotic cell death is evidenced as the mechanism of cell death. A low concentration of the lead molecule is sufficient for the inhibition of cell proliferation. In conclusion, MLT-401 is an ideal candidate molecule exhibiting anticancer/tumor activity.

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Disclosure Statement

No conflict of interests exists.

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