

# Targeting the monocarboxylate transporter MCT2 and lactate dehydrogenase A LDHA in cancer cells with FX-11 and AR-C155858 inhibitors

B. ALOBAIDI<sup>1</sup>, S.M. HASHIMI<sup>2</sup>, A.I. ALOOSAIBI<sup>1</sup>, N. ALQURASHI<sup>3</sup>, S. ALHAZMI<sup>4</sup>

<sup>1</sup>Department of Biology, College of Science, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia

<sup>2</sup>School of Medical Science and Menzies Health Institute Queensland, Griffith University, Parklands, QLD, Australia

<sup>3</sup>Department of Basic Sciences, Deanship of Preparatory Year and Supporting Studies, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia

<sup>4</sup>Department of Biological Sciences, King Abdulaziz University, Jeddah, Saudi Arabia

**Abstract. – OBJECTIVE:** In 1930, Otto Warburg reported that “aerobic glycolysis” is the intrinsic property of all tumor cells’ fermentation of glucose to L-Lactate by lactate dehydrogenase A (LDHA) activity. This only produces per mole of glucose two moles of adenosine triphosphate (ATP), compared with 32 moles of ATP in a normal cell. Thus, tumor cells have to uptake 30 folds more glucose, the resulting accumulated lactate are then transported by a monocarboxylate transporter (MCT) with the participation of a CD147 molecule. Inhibition of MCT1 by RNA interference (RNAi) disrupted the unique metabolism of the tumor and caused tumor cell death. However, the effectiveness of the strategies depends on the targeted delivery of the therapeutics.

**MATERIALS AND METHODS:** In this study, a synergistic approach was used to target LDHA and MCT1 with small molecule inhibitors FX11 and AR-C155858, respectively. Cell cytotoxicity assays (AlamarBlue assay), and Mitochondria Membrane Potential (JC-1) dye assays were performed on human breast cancer cells MCF-7 and colorectal cancer cells HCT116. To achieve this aim, the following objectives were proposed: the effect of metabolic inhibitors on tumor glycolytic metabolite environment, and the efficacy of metabolite inhibitors on human breast and colorectal cancer cells *in vitro*. Then, gene expression analysis was performed using Qiagen RT2 Profiler PCR array for apoptosis. All these assays were performed on human breast cancer cells MCF-7 and colorectal cancer cells HCT116. Normal human fibroblasts were used as control cells under normal and hypoxic culture conditions.

**RESULTS:** In this study, the use of FX-11 inhibitors under normoxia or hypoxia in two or more cancer and normal cell lines has a direct effect on LDHA, whereby it inhibits its production, and this reduces the growth and cell pro-

liferation of tumors. One of the more significant findings to emerge from this study is that using AR-C155858 inhibitor alone has increased the cell proliferation and showed no significant changes compared with the control. The other major finding was that combination of the two inhibitors, FX-11 and AR-C155858, under normoxia or hypoxia in two different cell lines MCF-7 and HCT-116 measured a decrease in the cells proliferative and red/green ratio.

**CONCLUSIONS:** We successfully demonstrated that a combination of MCT1 inhibitor and LDHA inhibitor led to better outcomes. Indeed, this makes LDHA an ideal metabolic therapeutic target.

*Key Words:*

Warburg effect, LDHA, MCT1, Cancer cells MCF-7, Colorectal cancer cells HCT116.

## Introduction

With the rapid evolution of medical technology over the past century, it might seem as though debilitating diseases such as cancer should have been eradicated. Yet, cancer persists, with an expected rise in the incidence of approximately 70% over the next 20 years<sup>1</sup>. Cancer is commonly misconceived as being a singular disease with a single origin; however, cancer is more accurately described as a collection of associated diseases that arise from the uncontrolled division of abnormal cells<sup>2</sup>. Current treatments involve the use of surgery, targeted therapy, radiation therapy, hormone therapy, and first-line immunotherapy<sup>3</sup>. Although there have been

significant advances in anti-cancer therapy, they have not minimized the disease burden, and cancer remains a major global health issue. The discovery of novel treatments is therefore paramount<sup>4</sup>.

The tumor microenvironment plays a crucial role in the transition from precancerous lesions to carcinogenesis by exerting an adaptive pressure that selects cells for their clonal expansion<sup>5</sup>. Cellular energy metabolism is one of the main processes affected during the transition from normal to cancer cells. In particular, Glucose (Glc) metabolism is often altered in tumor cells. Glycolysis is a catabolic process that converts one molecule of Glc to two molecules of pyruvate with the production of two adenosine triphosphate (ATP) molecules and two reduced nicotinamide adenine dinucleotide (NADH) molecules. Pyruvate, in the presence of O<sub>2</sub>, undergoes oxidation to CO<sub>2</sub> and H<sub>2</sub>O *via* the Oxidative Phosphorylation (OXPHOS) pathway, resulting in the production of approximately 36 molecules of ATP. Alternatively, in the absence of O<sub>2</sub>, pyruvate is transformed into lactic acid *via* the anaerobic glycolysis pathway. However, conversion of Glc to lactic acid can occur in the presence of O<sub>2</sub> and this is known as the Warburg effect or aerobic glycolysis. Cancer cells alter their metabolism in order to support their rapid proliferation and expansion across the body. In particular, tumor cells, rather than fueling Glc in the OXPHOS pathway, generally use Glc for aerobic glycolysis<sup>5</sup>.

Glc is metabolized in three different ways; firstly, *via* glycolysis under anaerobic conditions; secondly, by complete oxidation under aerobic conditions; and thirdly, *via* the OXPHOS pathway. Glycolysis under aerobic conditions is the preferred mechanism in cancer cells. Furthermore, in glycolysis, Glc is converted to pyruvate *via* the cytoplasmic metabolic pathway, which then produces ATP<sup>6</sup>.

In mammals, uncontrolled proliferation is prevented as the uptake of nutrients from the environment is regulated through stimulation by growth factors. However, in cancer cells, the dependence on growth factors for the uptake of nutrients from the environment is disrupted by genetic mutations, which functionally alter some of the receptor signaling pathways involved in nutrient uptake. So far, this study<sup>7</sup> has linked these pathways with the uptake and metabolism of nutrients leading to cell growth and survival. Tumor cells undergo metabolic reprogramming, which involves changes in the metabolic fluxes, to satisfy great demands for ATP, NADPH, NADH, and carbon skeletons<sup>8</sup>.

Lactate dehydrogenase (LDH) – which has two main subunits (A and B) and five isozymes (A4, A3B1, A2B2, A1B3, and B4) – catalyzes the reversible conversion of pyruvate to lactate<sup>9</sup>. A recent study<sup>9</sup> confirmed that cancer cells preferentially use LDHA (or LDH-5) to convert pyruvate to lactate when there are high pyruvate levels. Similar to the observations of the Warburg effect in cancer cells, in line with earlier studies<sup>8,9</sup>, LDH inhibitors affect the growth of tumor cells *in vitro*. Therefore, lactate is a good candidate as an essential part of carcinogenesis<sup>9</sup>. The Warburg theory proposes that the mitochondria of cancer cells are unable to provide energy *via* OXPHOS, and therefore rely upon glycolysis for energy production<sup>10</sup>.

Aerobic glycolysis depends on various factors, including oncogene activation, hypoxic micro-environment conditions, absence of tumor suppressors, abnormal expression of metabolic enzymes, and mutations in mitochondrial DNA. Significantly, the high dependence on ATP resources in tumors during glycolysis has led to new strategies for cancer therapy, such as inhibiting glycolysis pharmacologically<sup>11,12</sup>.

It is important to note that both glycolysis and the tricarboxylic acid cycle (TCA) cycle can be used for purposes other than ATP generation<sup>13</sup>. Without free O<sub>2</sub>, glycolysis would be the only source of energy for organisms. However, high levels of O<sub>2</sub> lead cells to depend on OXPHOS and produce significantly more ATP molecules than glycolysis. This mechanism leads the cells to rely on OXPHOS. Nonetheless, glycolysis and OXPHOS are similar, as glycolysis occurs in the cytoplasm and produces two ATP molecules and pyruvate. Hence, pyruvate is then converted to lactate by OXPHOS<sup>14</sup>.

One recent strategy for cancer therapy has been killing cancer cells without having any impact on normal cells. This approach is called selectively targeted therapy<sup>15</sup>. Another strategy involves targeting the unique metabolism of cancer cells, which is known as metabolic reprogramming. Understanding metabolic pathways opens ways to apply therapeutic strategies during the development of cancer<sup>7</sup>.

In fact, LDHA is a prime target for manipulation for therapeutic purposes, as it has been shown that decreases in Glc fermentation inhibit the growth of tumors<sup>16</sup>. In contrast to normal tissues, tumor tissues are hypoxic and, therefore, cannot activate the regulation gene *MYC*. Studies<sup>16,17</sup> now show that LDHA is responsible for the transformation of tumors, and there are doubts about its link to tumor initiation. The levels of LDH are

important, as high concentrations promote faster tumor growth. However, some researchers have suggested that the inhibition of LDHA leads the cell to bioenergetic and oxidative stress, which results in apoptosis<sup>17</sup>.

Lactate transport in all mammalian cells needs monocarboxylate transporter (MCTs) as proton links. Tumors are highly glycolytic due to the main role of MCTs. Therefore, there is potential for inhibition of MCT1 and MCT2 by AR-C155858, which is an anticancer inhibitor of MCTs<sup>16</sup>.

There are 14 members of the MCT family encoded by the human and mouse genomes<sup>18</sup>. Of these, only MCT1, MCT2, MCT3, and MCT4 have been demonstrated to catalyze the bidirectional proton-linked transport of short-chain monocarboxylates such as L-lactate and pyruvate across the plasma membrane of mammalian cells<sup>19,20</sup>.

Indeed, tumor cells require mitochondrial functions other than energy production to survive, proliferate, and control metabolic fluxes – such as intramitochondrial metabolic pathways and the maintenance of mitochondrial membrane potential ( $\Delta m$ ). Hence, a common idea has emerged that mitochondria are actively involved in cell transformation<sup>21</sup>.

The disruption of the cell death machinery of apoptosis is a hallmark of cancer. This not only causes tumors but also increases the resistance to therapy<sup>22</sup>. Constitutively, the trigger of apoptosis in mammalian cells has been found to involve two main transduction signal pathways, which include extrinsic and intrinsic pathways. The outer death receptor (extrinsic) pathway is associated with signals external to the cell that prompt the inner signal transduction machinery to enter apoptosis. The death receptors on the cell surface are activated by incorporation with the cell membrane, followed by ligation between their cognate ligands. Whereas cellular stresses like growth factor deprivation or high Glc concentration activate the intrinsic pathway<sup>23,24</sup>.

Activation of apoptosis is controlled by members of the BCL-2 family of proteins and ultimately impinges on the function of the caspase family of proteases<sup>25</sup>.

The pathways are highly regulated, so that apoptosis will occur only if signaled. The intrinsic pathway, in particular, is regulated by the B-cell lymphoma-2 (*BCL-2*) protein family, which includes proapoptotic effector proteins, proapoptotic BH3-only proteins, and antiapoptotic *BCL-2* proteins<sup>26</sup>. The antiapoptotic *BCL-2* proteins inhibit apoptosis by inhibiting the proapoptotic *BCL-2* proteins, *BCL-2*-associated X protein

(*BAX*), and *BCL-2* homologous antagonist killer (*BAK*)<sup>27</sup>. BH3 proteins inhibit the antiapoptotic *BCL-2* proteins<sup>28</sup>.

Moreover, doubly deficient cells are resistant to multiple apoptotic stimuli that act through disruption of mitochondrial function, including staurosporine, ultraviolet radiation, growth factor deprivation, etoposide, and the endoplasmic-reticulum stress stimuli thapsigargin and tunicamycin. Thus, activating a ‘multidomain’ proapoptotic member, *BAX* or *BAK*, appears to be an essential gateway to mitochondrial dysfunction required for cell death in response to diverse stimuli<sup>29</sup>.

In tumor cells that have defects in apoptosis, autophagy enables them to survive metabolic stress. As such, autophagy may also be an appropriate therapeutic target for cancer treatment<sup>25</sup>.

## Materials and Methods

### Cell Culture Condition

Human breast cancer cells MCF-7 and human colorectal cancer cells HCT-116 were obtained from the American Type Culture Collection (ATCC). They were routinely cultured as adherent cells in complete media. The cells were incubated under 5% CO<sub>2</sub> and 95 % humidity at 37°C. Routinely, the cells were subcultured when they reached 90% confluency, and different passages from 5-80 were used in the experiments. The remaining cells were stored at -80°C. An automated cell counter (Invitrogen Thermo Fisher Scientific, Waltham, MA, USA) was used to count the cell numbers. The living and dead cells per 1 mL were counted by staining with Trypan blue (MP Biomedicals Inc., Seven Hills, Australia).

### Cell Preparation

Cell lines were seeded in a 96-well plate containing 100  $\mu$ L/well of cell culture medium (approximately 10,000 cells/well) and were incubated overnight until they reached 80% confluency at 10 $\times$  magnification using a light microscope (TS100F-Eclipse, Nikon, New York, NY, USA). The culture medium in each well was then replaced with the same volume of two anticancer inhibitors: FX-11 (100  $\mu$ M), which targets the LDHA enzyme, and AR-C155858 (100  $\mu$ M), which targets MCT1. The inhibitors were used in different concentrations (half serial dilution: 200  $\mu$ M, 100  $\mu$ M, 50  $\mu$ M, 25  $\mu$ M, 12.5  $\mu$ M, and 0  $\mu$ M). Furthermore, the two inhibitors were mixed to determine the IC<sub>50</sub> at a variety of doses.

### **AlamarBlue Assay Viability/Proliferation**

The treated cell lines in a 96-well plate were incubated for 24 h. The mixture in each well was replaced by the cell-viability-determining reagent AlamarBlue (catalog numbers DAL1025 and DAL1100), which is added directly at a volume of 1/10th of the total volume. The cells were incubated for 2 h at 37°C in a cell-culture incubator, and the plate was protected from direct light. The results were recorded using an ELISA BioTek Stnergy neo2 multi-mode reader (Agilent, Santa Clara, CA, USA) at a fluorescence excitation wavelength of 560 nm (excitation range is 540-570 nm) and an emission of 590 nm (emission range is 580-610 nm). A previous assay had been applied under hypoxic conditions in the 96-well plate using the GasPak EZ Anaerobe Pouch System (No. 260683) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

### **Mitochondrial Membrane Potential JC-1 Dye Assay**

The treated cell lines were incubated for 24 h. On the next day, the JC-1 dye (catalog number T3168), which is a sensitive marker for mitochondrial membrane potential, was added at a volume of 1 µL/1 mL complete media. The JC-1 dye was immediately added after it had been diluted. After a further 30 min incubation at 37°C, the cells were washed with PBS ×1 twice, and 100 µL PBS was added to each well. The results were recorded using an ELISA BioTek Stnergy, neo2 multi-mode reader (Agilent, Santa Clara, CA, USA). The results were recorded using a fluorescence endpoint wavelength of 560 nm (with an excitation range of 485/20-530/25 nm) and an emission range of 528/20-590/35 nm. A previous assay had been applied under hypoxic conditions using a 96-well plate using the GasPak EZ Anaerobe Pouch System (No. 260683).

### **Sample Preparation for Gene Expression**

MCF-7 and HCT-116 were added to a six-well plate at a density of  $10^6$ - $10^7$  cells/well (1 mL). After the cells reached 90% confluency, they were exposed to normal and hypoxic conditions for two incubation periods (8 h and 24 h). An inhibitor concentration of 100 µM was chosen for this assay to reach the determined mean  $IC_{50}$ . Subsequently, the cells were harvested by washing in (PBS) ×1, followed by the addition of 500 µL trypsin-EDTA ×10 (0.5%) at a final concentration of 0.05%, and were left to incubate for 5-10 min. Thereafter, 500 µL complete media was added and mixed

with the trypsin, and the cells were centrifuged at  $8,000 \times$  rpm for 5 min. The supernatant was decanted, and the cells were mixed with PBS for a second wash, then centrifuged at  $8,000 \times$  rpm for 5 min. The supernatant was then extracted, and the cell sample was stored at -80°C for use in the following experiment.

### **RNA Isolation**

All steps were performed at room temperature and under centrifugation at  $13,000 \times$  g for 30 seconds unless specified. After exposure to the different conditions (treated with FX-11 at a concentration of 100 µM, incubation for 24 h and 8 h under normoxia and hypoxia), the total RNA was extracted from the treated cells using the ZYMO Quick-RNA™ Miniprep Plus Kit (Zymo Research, Irvine, CA, USA).

The RNA samples were quantified using a Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA).

### **The First-Strand Complementary DNA (cDNA) Synthesis**

First-strand cDNA was synthesized from total RNA with the Sigma, ReadyScript® cDNA Synthesis Mix. The quantity and quality of the cDNA were measured with a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific, USA). The cDNA samples were stored at -20°C until use in the RTqPCR reaction.

### **RTqPCR Reaction Mix Preparation and Thermal Cycling Protocol**

RTqPCR study was conducted to determine the effect of treated cells with (FX-11 drug, or the combination of two drugs AR-C155858 + FX-11) under either hypoxic or normocytic conditions on the expression of *BAX*, *BCL2L1*, and *GPX7* genes. For analysis in a total of 16 groups treated with FX-11 or mixed drugs under hypoxia-normoxia, two cell lines (MCF-7) and (HCT-116) at two-time intervals (8 h and 24 h) were used vs. control no treated cell lines (MCF-7) and (HCT-116) under hypoxia-normoxia. To determine the effect of treated cells with (FX-11 or mixed drugs) under either hypoxic or normocytic conditions with time on *BAX*, *BCL2L1*, and *GPX7* genes expression, mean threshold cycle ( $C_T$ ) values, derived from twice replication of 16 groups for *BAX*, *BCL2L1*, *GPX7*, and *GAPDH* genes for treated (hypoxia-normoxia) vs. control no treated (hypoxia-normoxia), were used for analysis.



### Statistical Analysis

All experiments were replicated at least three times. Data for the test and control groups were statistically analyzed using Excel (Microsoft Office, 2013). GraphPad Prism 7 was used to present data in graphs and charts (GraphPad Software, LaJolla, CA, USA). One-way analysis of variance (ANOVA) was used to statistically compare the test and control groups and to determine the mean half-maximal inhibitory concentration ( $IC_{50}$ ). Data were considered significantly different at  $p \leq 0.05$ . Significance levels in the graphs were indicated as follows: \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , and \*\*\*\* $p \leq 0.0001$ . The  $Rq$  values of each gene were compared across all samples of the four groups using the one-tailed Student's  $t$ -test with unequal variance to calculate the  $p$ -values and identify significant differences in expression. RTqPCR data were analyzed by two methods (i)  $\Delta\Delta C_T$  or Livak method and (ii)  $\Delta C_T$  method. The average  $C_T$  for endogenous control and each experimental gene was calculated from the raw data generated by RT-PCR StepOne System and Data Assist software (Thermo Fisher Scientific, Waltham, MA, USA).

The outliers were either omitted from the analysis or substituted with means. The GraphPad Prism software and MS Excel functions were used to analyze and generate the statistical plots of the data. The  $Rq$  values of each gene were compared across all samples of eight groups using a one-tailed student's  $t$ -test with unequal variance to calculate the  $p$ -value for the identification of significantly expressed genes.

## Results

### Viability/Proliferation – Cytotoxicity in Treated (MCF-7 and HCT-116)

The Alamarblue assay is highly referenced for cytotoxicity and viability assays, and the Alamar-Blue cell viability assay has been used for years in biological and environmental studies<sup>5</sup>. Furthermore, analysis of cell proliferation and cytotoxicity is a vital step in evaluating cellular health as well as in the drug discovery process. AlamarBlue is a proven cell viability indicator that uses the natural reducing power of live cells to convert resazurin to the fluorescent molecule and resorufin<sup>5</sup>.

Our results indicated that FX-11 (50  $\mu$ M to 100  $\mu$ M) revealed a 50% reduction in cell viability of  $IC_{50}$  value of 77  $\mu$ M. So, this inhibitor was effective on MCF-7 cells under normoxia.

Our data demonstrated that exposure MCF-7 and HCT-116 cells with MCT-1 inhibitor AR-C155858 with various concentrations from low to high, and under normoxia or hypoxia result in increased cell proliferation. This finding confirms the association between inhibiting the lactate transporter MCT1, and the metabolic change that would force those cells using lactate to use an alternative fuel, for example, glucose, thus increasing the rate of glycolysis.

### Mitochondrial Membrane Potential JC-1 Dye Assay

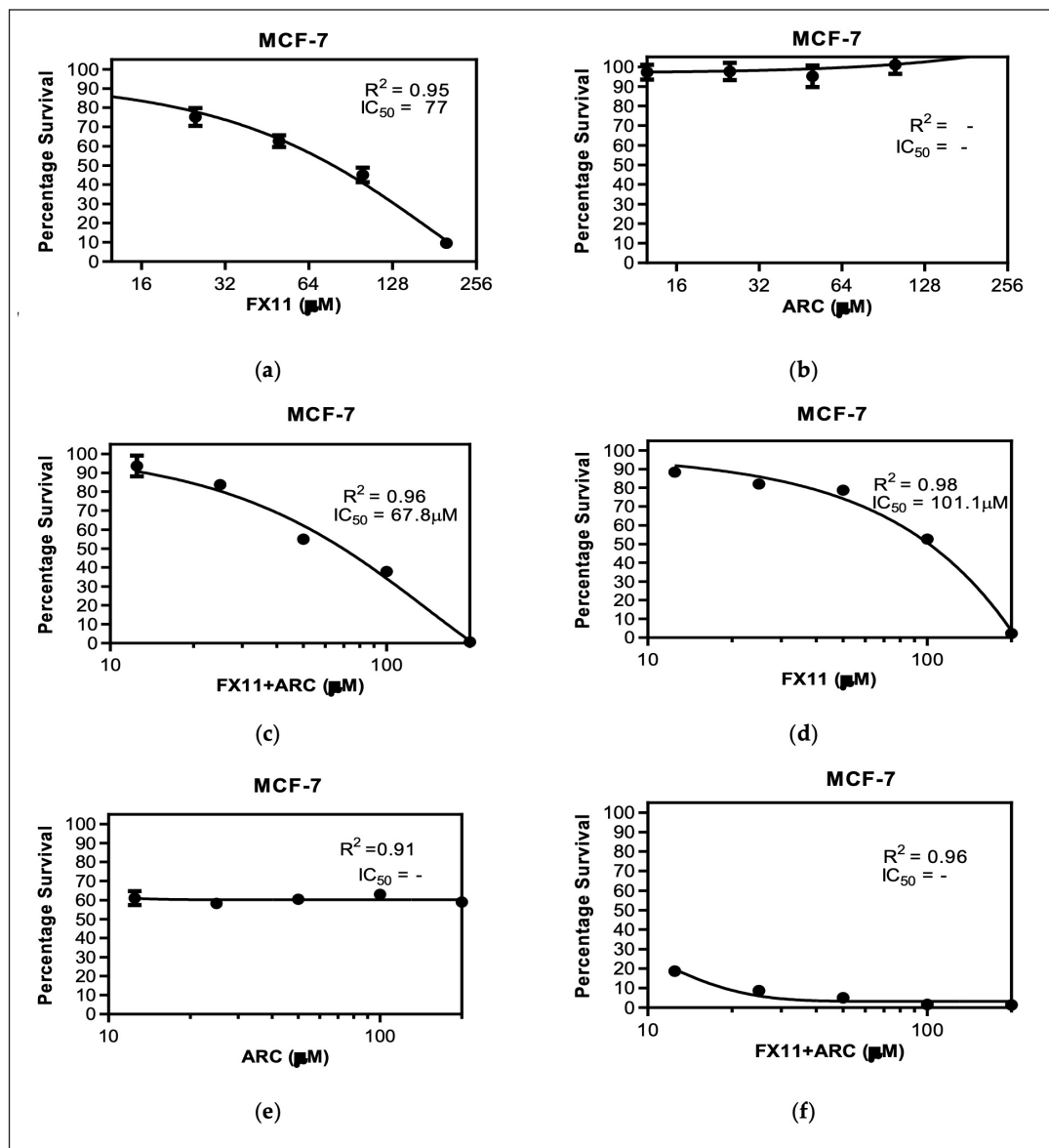
Mitochondrial function is a key indicator of cell health and can be assessed by monitoring changes in mitochondrial membrane potential (MMP). The cells were quantified by fold change in mitochondrial health with JC-1 dye. This exhibits a potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm).

Furthermore, MCF-7 treated with FX-11 under normoxia (Figure 3) shows a significant difference (\*represents  $p \leq 0.05$ ; \*\*represents  $p \leq 0.01$ ; \*\*\*\*represents  $p \leq 0.0001$ ) between the concentration's groups (12.5  $\mu$ M, 25  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M and 200  $\mu$ M)  $p \leq 0.01$ \*\* in 200  $\mu$ M, which was significant when compared to the control 0  $\mu$ M group. As well as the different concentrations of FX-11 under hypoxia on HCT-116 represented significances  $p \leq 0.05$ \* in 50  $\mu$ M and  $p \leq 0.01$ \*\* in 200  $\mu$ M when compared with the control 0  $\mu$ M. Therefore, HCT-116 cells have shown a decrease in the percentage of survival cells, especially with high concentrations. These findings confirm that increasing the concentration of FX-11 ensures a decrease in cell proliferation under normoxia and hypoxia.

### Gene Expression

RTqPCR study was conducted to determine the effect of treated cells with (FX-11 drug, or the combination of two drugs AR-C155858+ FX-11) under either hypoxic or normocytic conditions on expression of *BAX*, *BCL2L1*, and *GPX7* genes.

Rellinger et al<sup>30</sup> demonstrated that FX-11 inhibits aerobic glycolysis at low micromolar concentrations in neuroblastoma cell lines (10  $\mu$ M) that are comparable to its  $K_i$  for LDHA (8  $\mu$ M), suggesting that their experimental conditions selectively inhibited LDHA<sup>30</sup>. These results are consistent with studies<sup>31,32</sup> which found that MCF-7 cells have a much higher glycolytic flux rate. MCF-7 cells have much higher 6-phosphofruc-

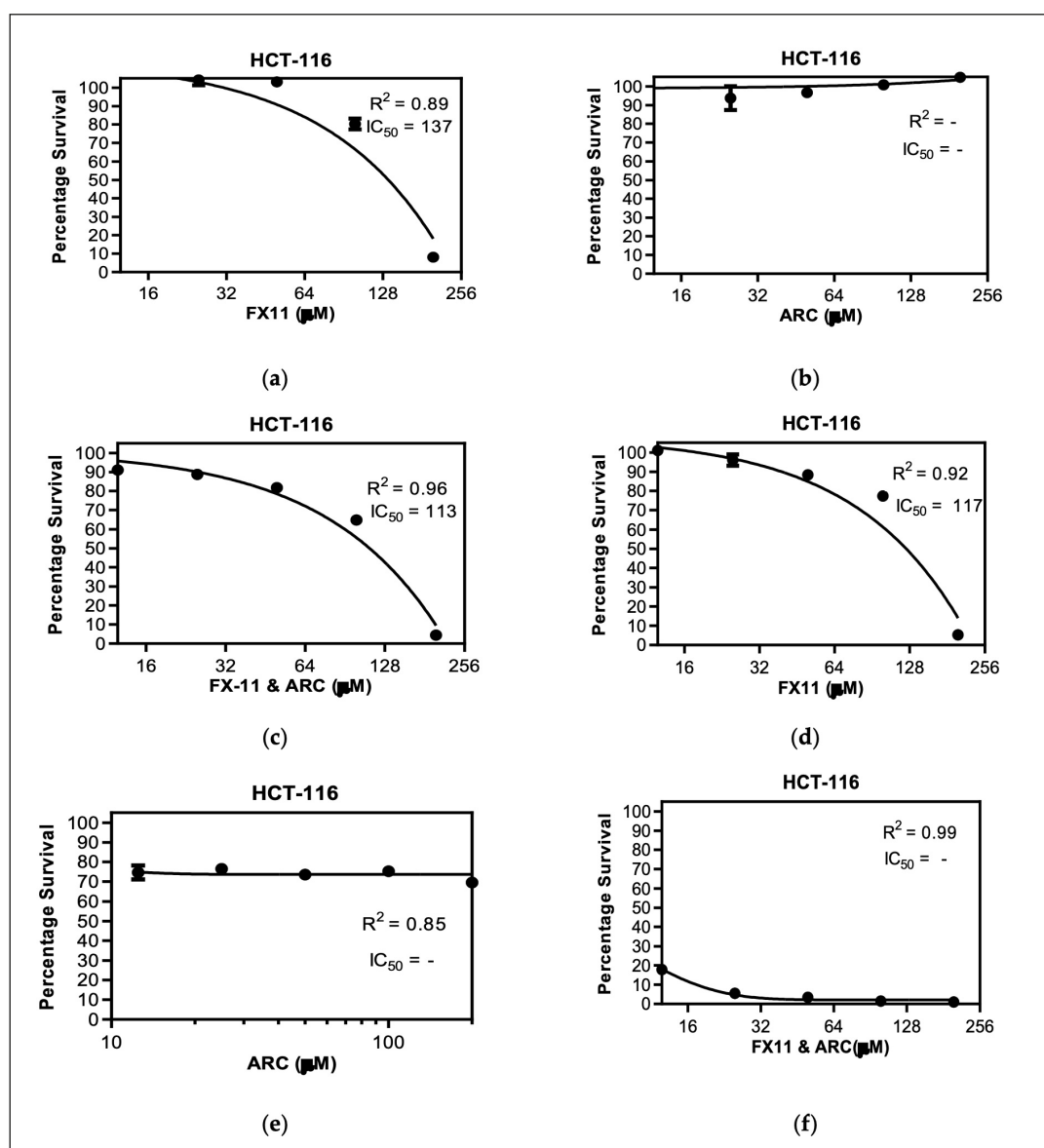


**Figure 1.** Percentage of survival MCF-7 cell using cytotoxicity assay under normoxia and hypoxia, treated with FX-11 and/ or AR-C155858. **a**, MCF-7 cells treated by FX-11 under normal condition resulted in a 50% reduction in cell viability where  $IC_{50}$  value was 77  $\mu M$ . **b**, MCF-7 treated by AR-C155858 under normoxia gave no  $IC_{50}$  value. **c**, Value was 57.8  $\mu M$  for MCF-7 cells treated by the combination of the FX-11+ AR-C155858 in the normal condition of incubation. **d**, MCF-7 cells treated by FX-11 under hypoxia resulted in a 50% reduction in cell viability of  $IC_{50}$  value of 101. **e**, MCF-7 treated with AR-C155858 under hypoxia gave no  $IC_{50}$  value. **f**, MCF-7 cells treated with the combination of the FX-11+ AR-C155858 under hypoxia was mostly killed.

to-1-kinase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase activities. To corroborate these findings further, they studied the glycolytic MCF-7 and the oxidative MDA-MB-453 breast carcinoma cell lines. They confirmed that MCF-7 was more dependent on glucose, whereas MDA-MB-453 was more dependent on glutamine oxidation, such that deprivation of glucose has a more profound growth inhibitory effect on MCF-

7. A dose-dependent study further revealed that MCF-7 is more sensitive to FX-11. Although there are many other differences between these cell lines, the correlation of FX-11 sensitivity and glucose dependency of MCF-7 supports the notion that glycolysis predisposes cancer cells to growth inhibition by FX-11<sup>32</sup>.

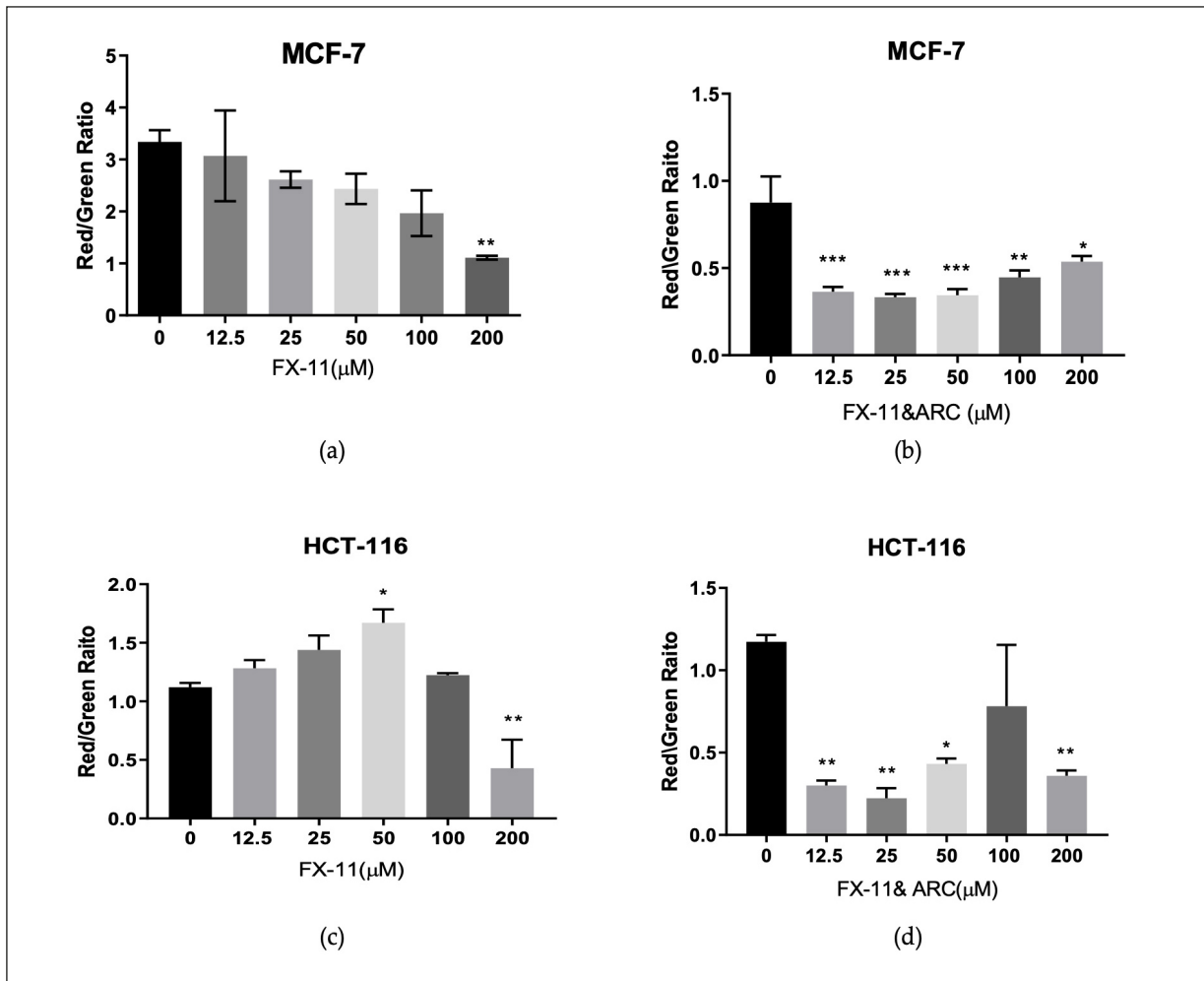
Prior studies<sup>33,34</sup> have noted the importance of MCT1 for therapeutic target intervention of gly-



**Figure 2.** Percentage of survival HCT-116 cell using cytotoxicity assay under normoxia and hypoxia, treated with FX-11 and/or AR-C155858. **a**, HCT-116 cells treated with FX-11 under normal condition resulted in a 50% reduction in cell viability where  $IC_{50}$  value was 137  $\mu M$ . **b**, HCT-116 treated with AR-C155858 under normal condition gave no  $IC_{50}$  value. **c**, Value was 113  $\mu M$  for HCT-116 cells treated with the combination of the FX-11+ AR-C155858 under normal condition. **d**, HCT-116 cells treated with FX-11 under hypoxia resulted in a 50% reduction in cell viability of  $IC_{50}$  value of 117  $\mu M$ . **e**, HCT-116 treated with AR-C155858 under hypoxia gave no  $IC_{50}$  value. **f**, HCT-116 cells treated with the combination of the FX-11+ AR-C155858 under hypoxia gave no  $IC_{50}$  value and the cells were mostly killed.

colytic tumor cells. In this study, they tested the small-molecule MCT1 inhibitor AZD3965 in a panel of SCLC cell lines in anticipation that these cells, derived from highly aggressive, rapidly proliferating, and often hypoxic tumors, will be reliant on glycolysis and thus vulnerable to inhibition of MCT1. Their data demonstrate that some, but not all, SCLC cell lines respond to MCT1 inhibition

and are particularly sensitive when hypoxic and, therefore, obligate glycolytic<sup>35</sup>. According to Bola et al<sup>36</sup>, inhibition of lactate transport resulted in a significant increase in tumor lactate *in vivo*, and this effect was not because of perfusion changes brought about by changes to the blood vessels in the tumor. Moreover, at the end of the treatment period, a significant increase in MCT4 staining

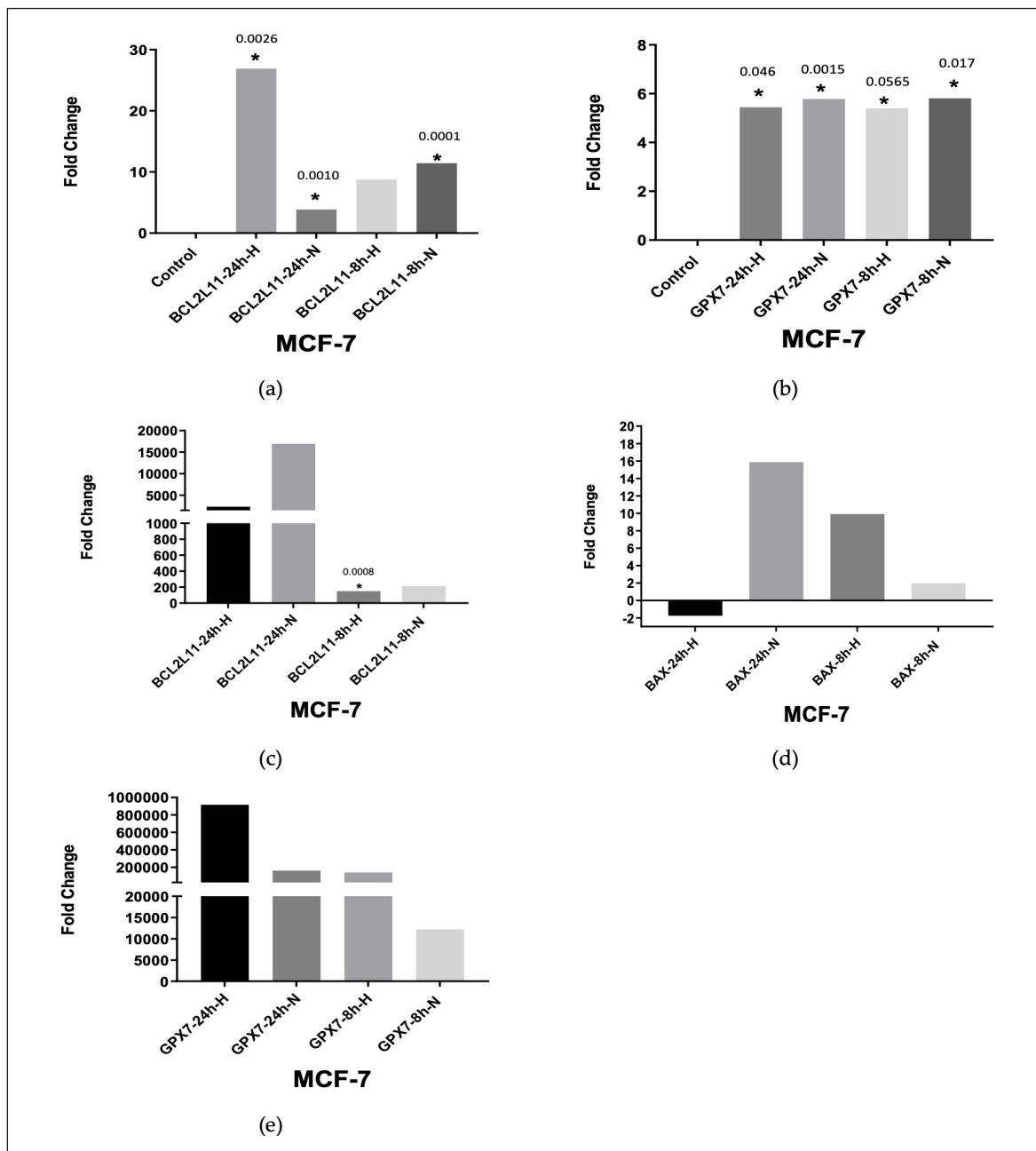


**Figure 3.** Mitochondrial membrane potential assay JC-1 dye, (MCF-7 and HCT-116) treated with FX-11 and/or AR-C155858 under normoxia or Hypoxia. **a**, MCF-7 treated with FX-11 represented\*\* 200 μM  $p \leq 0.01$ . **b**, MCF-7 treated with both AR-C155858 and FX-11 under hypoxia, represented many significances comparing with control 0 μM,  $p \leq 0.001$ \*\*\* in 12.5 μM,  $p \leq 0.001$ \*\*\* in 25 μM,  $p \leq 0.001$ \*\*\* in 50 μM,  $p \leq 0.01$ \*\* in 100 μM and  $p \leq 0.05$ \* in 200 μM. **c**, HCT-116 treated with FX-11 under hypoxia represented significance  $p \leq 0.01$ \*\* in 200 μM and significance  $p \leq 0.05$ \* in 50 μM. **d**, HCT-116 cells treated with FX-11+ AR-C155858 under hypoxia represented many significances  $p \leq 0.01$ \*\* in 12.5 μM,  $p \leq 0.01$ \*\* in 25 μM,  $p \leq 0.05$ \* in 50 μM and  $p \leq 0.01$ \*\* in 200 μM.

was observed; MCT4 is often considered a marker of glycolysis<sup>36</sup>. Another explanation could be a relative resistance of the cell line MCF-7 and HCT-116 for AR-C155858. However, another study<sup>37</sup> demonstrated that the most likely explanation for these results is that the cellosaurus cell line CAL27 already relies on glycolysis as an energy source despite normoxic conditions. Due to the MCT1 inhibition, more glycolysis would be necessary for oxidative metabolisms because pyruvate cannot be used as an energy source anymore. Since the viability/proliferation assay in our study did not show elevated glycolysis when MCT1 was inhibited, the glycolysis might have already been the main

source of energy, and these findings are consistent with the study of Nabeshima et al<sup>37</sup>. The study of Sanli et al<sup>38</sup> confirmed that MCT1 inhibition correlates with lactate metabolism and glutathione synthesis<sup>38,39</sup>. Most studies<sup>36,39</sup> concentrate on the effect of MCT1 inhibition, perhaps because of the possibility of using specific MCT1 inhibitors. The most comparable study of Bola et al<sup>36</sup> tested another specific MCT1 inhibitor (AZD3965) on three different cell lines and measured different metabolic and proliferative parameters *in vitro* and *in vivo*<sup>36</sup>. They successfully demonstrated that a combination of MCT1 inhibition and radiation leads to a better outcome<sup>39</sup>.





**Figure 4.** a, Fold Change bar graph showing expression of BCL2L11 gene in four groups. MCF-7 cell lines treated with FX-11 drug under (hypoxia 24 hr, 8 hr, normoxia 24 hr, 8 hr) FC graph shows the relative expression of the target gene in four groups. b, Fold Change bar graph showing expression of GPX7 gene in four groups. MCF-7 cell lines treated with FX-11 drug under (hypoxia 24 hr, 8 hr, normoxia 24 hr, 8 hr) FC graph shows the relative expression of the target gene in four groups. c, Fold Change bar graph showing expression of BCL2L11 gene in four groups. MCF-7 cell lines treated with the combination of the two drugs, the FC graph shows the relative expression of the target gene in four groups. d, Fold Change bar graph showing expression of BAX gene in four groups. MCF-7 cell lines treated with mix with the combination of the two, FC graph shows the relative expression of the target gene in four groups. e, Fold Change bar graph showing expression of GPX7 gene in four groups. MCF-7 cell lines treated with the combination of the two drugs, the FC graph shows the relative expression of the target gene in four groups.

On the other hand, cells inhibited with AR-C155858 were not expected to proliferate, and this was demonstrated in our study. In addition, we sought AR-C155858 is not cytotoxic to MCF-7 or HCT-116 cells, in the Mitochondrial Membrane Potential assay, we found an increase in the red/green ratio which means more cell proliferation. It is difficult to explain this result, but it might be related to the use of AR-C155858 alone, giving poor outcomes. Whereas treating the cells with FX-11 actually killed the cells, which indicates our data of Proliferation AlamarBlue assay having a decrease in the cell proliferation.

Another important finding was that, under hypoxia, FX-11 100  $\mu$ M resulted in an  $IC_{50}$  value of 101.1  $\mu$ M. So, this inhibitor was effective on MCF-7, especially under normoxia. Comparing our results with Rellinger et al<sup>30</sup>, increasing the dose of FX-11 (20  $\mu$ M) enhanced the blockade of neuroblastoma cell line growth but failed to enhance the blockade of lactate production beyond that observed at lower doses (10  $\mu$ M). These findings suggest that FX-11 (20  $\mu$ M) initiates either off-target or cytotoxic effects with treatments at higher concentrations.

Consistency with those results of blocking lactate transport impairs tumor cell growth through several mechanisms. Some tumor cells rely on lactate as a substrate for oxidative phosphorylation, and in this scenario blocking lactate import inhibits tumor cell growth<sup>34,40</sup>. However, this is the exception, as most tumor cells express high levels of LDH-A, which drives the production of lactate from pyruvate<sup>41</sup>. Lactate uptake in vascular endothelial cells *via* MCT1 appears to promote tumor angiogenesis; thus, blocking this response impairs tumorigenesis<sup>42,43</sup>. Given these effects, a recent AstraZeneca patent application<sup>44</sup> claims the use of MCT1 inhibitors for the treatment of certain cancers. In our current study, the combination of AR-C155858 and FX-11 under normoxia or hypoxia in two different cell lines MCF-7 and HCT-116 revealed a decrease in the cells' proliferative and red/green ratio. They successfully demonstrated that a combination of MCT1 inhibitor and LDHA inhibitor led to better outcomes. Our findings could be comparable with Brandstetter et al<sup>39</sup>, when various cancer cell lines were treated with MCT1 inhibition mediated by AR-C155858 showed a greater impact in combination with irradiation. In addition to their recommendation, the right time for a combination with adjuvant treatments should be examined<sup>39</sup>.

Defective control of apoptosis plays a central role in the pathogenesis of human diseases, in-

cluding cancer, neoplastic, autoimmune, and neurodegenerative diseases. *BCL*-gene and *BAX* family products are intrinsic proteins regulating the decision of a cell to survive or die and executing part of the cell death process itself, respectively, which make *BCL*-gene and *BAX* ideal for therapeutic targets. Schmitt et al<sup>45</sup> demonstrated that the apoptosis proteins are induced under cancer chemotherapy<sup>45</sup>. In human cancer, variant cell lines showing differential expression of the *Bcl-xL* protein, a preventive effect of *Bcl-xL* on cell death induced by various cytotoxic drugs is observed, with greater effects in cells containing the highest level of *Bcl-xL* expression<sup>45</sup>. Indeed, in the cell lines treated with FX-11, our data show that expression of *BAX*, *BCL2L1* and *GPX7* genes were higher in the beginning (8 h) and reduced with time (24 h) in both MCF-7 and HCT-116 cell lines. *BCL2L1* was significantly over-expressed in HCT-116 at 8 and 24 hours, while it was significantly under-expressed in MCF-7 (24 h). *GPX7* was significantly over-expressed in HCT-116 (24 h) only. Expression of *BCL2L1* and *GPX7* genes were increasing over time (8 h) and (24 h) in MCF-7 cell line. Conversely, *BAX* was significantly reduced in expression over time (8 h) and (24 h) in MCF-7 cell line. *BCL2L1*, *BAX*, and *GPX7* were significantly under-expressed in HCT-116 at (8 h) and (24 h). In contrast, *BCL2L1* was significantly over-expressed in MCF-7 cells treated with mixed drugs and under hypoxic conditions at (8 h), while it was significantly under-expressed in HCT-116 cells treated under hypoxic conditions at (8 h). In the current study, *BCL2L1* was significantly over-expressed; an increase on the *BCL2L1* expression indicates that the cells undergo apoptotic and the FX-11, and the combination of the two drugs is efficient. On the other hand, *GPX-7* is an oxidative stress marker that should be upregulated in response to disrupt the mitochondrial membrane potential by the FX-11, and we had a high expression of *GPX7* on MCF-7. Finally, the results of RTqPCR support our results of the mitochondrial membrane potential assay that the combination of the two inhibitors is more effective in inducing apoptosis and oxidative stress.

## Conclusions

One of the main differences between normal cells and tumor cells is their metabolism. In normal cells, there is a metabolic control that ensures the availability of nutrients for proper cellular function.

However, tumor cells have altered metabolism, resulting in accelerated growth rate, high proliferation, and continuous maintenance. The condition in the tumor cells is referred to as the Warburg effect, whereby as a result of the altered cell metabolism, the glucose uptake is increased, and fermentation of glucose to lactate is pronounced. In cancerous cells, lactate dehydrogenase type-A LDH-A or LDH-5 is used, thereby converting pyruvate to lactate with high pyruvate levels. This project has argued the effect of metabolic inhibitors on tumor glycolytic metabolite environment and the efficacy of metabolite inhibitors on human breast and colorectal cancer cells. Our results find that the use of FX-11 inhibitors under normoxia or hypoxia in two or more cancer and normal cell lines has a direct effect on LDHA, whereby it inhibits its production, and this reduces the growth and cell proliferation of tumors. Indeed, this makes LDHA an ideal metabolic therapeutic target. In the present study, we demonstrated that FX-11 inhibits aerobic glycolysis at low micromolar concentrations in MCF-7, HCT-116. Additionally, because of the main role of monocarboxylate transporters (MCTs), tumor cells are highly glycolytic. However, their effect can be reduced by using the molecule inhibitor AR-C155858. In this investigation, the aim was to directly inhibit MCT2 and MCT1 to decrease tumor cell proliferation. One of the more significant findings to emerge from this study is that using AR-C155858 inhibitor alone has increased the cells proliferation and gave no significance. The other major finding was that combination of the two inhibitors FX-11 and AR-C155858 under normoxia or hypoxia in two different cell lines MCF-7 and HCT-116 measured a decrease in the cells proliferative and red/green ratio. We successfully demonstrated that a combination of MCT1 inhibitor and LDHA inhibitor led to better outcomes.

---

### Conflict of Interest

The Authors declare that they have no conflict of interest.

---

### Ethics Approval

Not applicable.

---

### Authors' Contributions

The authors contributed equally and made a significant contribution to the work reported. Authors contributed to the study design, execution, methodology, acquisition of data, software, validation, data analysis, and interpretation, and took part in writing, original draft preparation, reviewing, and editing.

---

### Funding

The authors receive no funds for this work.

---

### Informed Consent

Not applicable in this study.

---

### Acknowledgments

We would like to express our very great appreciation to sincere gratitude and deepest appreciation to Institution of Research and Medical Consultations (IRMC), Imam Abdurrahman Bin Faisal University, and all their members for their time and assistance.

---

### ORCID ID

Bayan Alobaidi: 0000-0003-1062-8697

Saeed Mujahid Hashimi: 0000-0001-5250-0857

Amany I. Alqosaibi: 0000-0001-8348-9451

Naif AlQurashi: 0000-0003-4488-3506

Safiah Alhazmi: 0000-0003-0755-4498

## References

- 1) Arem H, Loftfield E. Cancer Epidemiology: A Survey of Modifiable Risk Factors for Prevention and Survivorship. *Am J Lifestyle Med* 2018; 12: 200-210.
- 2) Hassanpour SH, Dehghani M. Review of cancer from perspective of molecular. *J Can Res Prac* 2017; 4: 127-129.
- 3) Hirsch FR, Scagliotti GV, Mulshine JL, Kwon R, Curran WJ, Wu YL, Paz-Ares L. Lung cancer: current therapies and new targeted treatments. *Lancet (London, England)* 2017; 389: 299-311.
- 4) Prager GW, Braga S, Bystricky B, Qvortrup C, Criscitiello C, Esin E, Sonke GS, Martínez GA, Frenel JS, Karamouzis M, Strijbos M, Yazici O, Bossi P, Banerjee S, Troiani T, Eniu A, Ciardiello F, Tabernero J, Zielinski CC, Casali PG, Cardoso F, Douillard JY, Jezdic S, McGregor K, Briccalli G, Vyas M, Ilbawi A. Global cancer control: responding to the growing burden, rising costs and inequalities in access. *ESMO open* 2018; 3: e000285.
- 5) Annibaldi A, Widmann C. Glucose metabolism in cancer cells. *Curr Opin Clin Nutr Metab Care* 2010; 13: 466-470.
- 6) Li XB, Gu JD, Zhou QH. Review of aerobic glycolysis and its key enzymes - new targets for lung cancer therapy. *Thorac Cancer* 2015; 6: 17-24.
- 7) Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science NY* 2009; 324: 1029-1033.
- 8) Romero-Garcia S, Lopez-Gonzalez JS, B'ez-Viveros JL, Aguilar-Cazares D, Prado-Garcia H. Tumor cell metabolism. *Cancer Biol Ther* 2011; 12: 939-948.

- 9) Pennington Z, Goodwin ML, Westbroek EM, Cottrill E, Ahmed AK, Sciubba DM. Lactate and cancer: spinal metastases and potential therapeutic targets (part 2). *Ann Transl Med* 2019; 7: 221-221.
- 10) Koppenol WH, Bounds PL, Dang CV. Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer* 2011; 11: 325-337.
- 11) Pelicano H, Martin DS, Xu RH, Huang P. Glycolysis inhibition for anticancer treatment. *Oncogene* 2006; 7: 4633-4646.
- 12) Im SA, Lu YS, Bardia A, Harbeck N, Colleoni M, Franke F, Chow L, Sohn J, Lee KS, Campos-Gomez S, Villanueva-Vazquez R, Jung KH, Chakravarty A, Hughes G, Gounaris I, Rodriguez-Lorenc K, Taran T, Hurvitz S, Tripathy D. Overall Survival with Ribociclib plus Endocrine Therapy in Breast Cancer. *N Engl J Med* 2019; 381: 307-316.
- 13) Ferreira LMR, Li AM, Serafim TL, Sobral MC, Alpoim MC, Urbano AM. Intermediary metabolism: An intricate network at the crossroads of cell fate and function. *Biochim Biophys Acta Mol Basis Dis* 2020; 1866: 165887.
- 14) Zheng J. Energy metabolism of cancer: Glycolysis versus oxidative phosphorylation (Review). *Oncol Lett* 2012; 4: 1151-1157.
- 15) Wang G, Wang JJ, Yin PH, Xu K, Wang YZ, Shi F, Gao J, Fu XL. New strategies for targeting glucose metabolism-mediated acidosis for colorectal cancer therapy. *J Cell Physiol* 2018; 234: 348-368.
- 16) Rajeshkumar NV, Dutta P, Yabuuchi S, De Wilde RF, Martinez GV, Le A, Kamphorst JJ, Rabinowitz JD, Jain SK, Hidalgo M, Dang CV, Gillies RJ, Maitra A. Therapeutic targeting of the warburg effect in pancreatic cancer relies on an absence of p53 function. *Cancer Res* 2015; 75: 3355-3364.
- 17) Im DK, Cheong H, Lee J S, Oh MK, Yang KM. Protein kinase CK2-dependent aerobic glycolysis-induced lactate dehydrogenase A enhances the migration and invasion of cancer cells. *Sci Rep* 2019; 9: 5337.
- 18) Halestrap AP, Meredith D. The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. *Pflugers Arch* 2004; 447: 619-628.
- 19) Bröer S, Schneider HP, Bröer A, Rahman B, Hamprecht B, Deitmer JW. Characterization of the monocarboxylate transporter 1 expressed in *Xenopus laevis* oocytes by changes in cytosolic pH. *Biochem J* 1998; 333: 167-174.
- 20) Manning Fox J E, Meredith D, Halestrap AP. Characterization of human monocarboxylate transporter 4 substantiates its role in lactic acid efflux from skeletal muscle. *J Physiol* 2000; 529: 285-293.
- 21) Le A, Cooper CR, Gouw AM, Dinavahi R, Maitra A, Deck LM, Royer RE, Vander Jagt DL, Semenza GL, Dang CV. Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. *Proc Natl Acad Sci U S A* 2010; 107: 2037-2042.
- 22) Pistrutto G, Trisciuglio D, Ceci C, Garufi A, D'Orazi G. Apoptosis as anticancer mechanism: function and dysfunction of its modulators and targeted therapeutic strategies. *Aging* 2016; 8: 603-619.
- 23) Ashkenazi A. Targeting the extrinsic apoptosis pathway in cancer. *Cytokine Growth Factor Rev* 2008; 19: 325-331.
- 24) Fulda S. Targeting apoptosis for anticancer therapy. *Semin Cancer Biol* 2015; 31: 84-88.
- 25) Jin S, DiPaola RS, Mathew R, White E. Metabolic catastrophe as a means to cancer cell death. *J Cell Sci* 2007; 120: 379-383.
- 26) Lopez J, Tait SWG. Mitochondrial apoptosis: killing cancer using the enemy within. *Br J Cancer* 2015; 112: 957-962.
- 27) Zaman S, Wang R, Gandhi V. Targeting the apoptosis pathway in hematologic malignancies *Leuk Lymphoma* 2014; 55: 1980-1992.
- 28) Pfeffer CM, Singh ATK. Apoptosis: A target for anticancer therapy. *Int J Mol Sci* 2018; 19: 448.
- 29) Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science NY* 2001; 292: 727-730.
- 30) Rellinger EJ, Craig BT, Alvarez AL, Dusek HL, Kim KW, Qiao J, Chung DH. FX11 inhibits aerobic glycolysis and growth of neuroblastoma cells. *Surgery* 2017; 161: 747-752.
- 31) Mazurek S, Michel A, Eigenbrodt E. Effect of extracellular AMP on cell proliferation and metabolism of breast cancer cell lines with high and low glycolytic rates. *J Biol Chem* 1997; 272: 4941-4952.
- 32) Le A, Cooper CR, Gouw AM, Dinavahi R, Maitra A, Deck LM, Royer RE, Vander Jagt DL, Semenza GL, Dang CV. Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. *Proc Natl Acad Sci U S A* 2010; 107: 2037-2042.
- 33) Mathupala SP, Parajuli P, Sloan AE. Silencing of Monocarboxylate Transporters via Small Interfering Ribonucleic Acid Inhibits Glycolysis and Induces Cell Death in Malignant Glioma: An in Vitro Study. *Neurosurgery* 2004; 55: 1410-1419.
- 34) Sonveaux P, Végran F, Schroeder T, Wergin MC, Verrax J, Rabbani ZN, De Saedeleer CJ, Kennedy KM, Diepart C, Jordan BF, Kelley MJ, Gallez B, Wahl ML, Feron O, Dewhirst MW. Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. *J Clin Invest* 2008; 118: 3930-3942.
- 35) Polański R, Hodgkinson CL, Fusi A, Nonaka D, Priest L, Kelly P, Trapani F, Bishop PW, White A, Critchlow SE, Smith PD, Blackhall F, Dive C, Morrow CJ. Activity of the monocarboxylate transporter 1 inhibitor AZD3965 in small cell lung cancer. *Clin Cancer Res* 2014; 20: 926-937.
- 36) Bola BM, Chadwick AL, Michopoulos F, Blount KG, Telfer BA, Williams KJ, Smith PD, Critchlow SE, Stratford IJ. Inhibition of monocarboxylate transporter-1 (MCT1) by AZD3965 enhances radiosensitivity by reducing lactate transport. *Mol Cancer Ther* 2014; 13: 2805-2816.

- 37) Nabeshima K, Iwasaki H, Koga K, Hojo H, Sumiyama J, Kikuchi M. Emmprin (basigin/CD147): matrix metalloproteinase modulator and multifunctional cell recognition molecule that plays a critical role in cancer progression. *Pathol Int* 2006; 56: 359-367.
- 38) Sanli T, Rashid A, Liu C, Harding S, Bristow RG, Cutz JC, Singh G, Wright J, Tsakiridis T. Ionizing radiation activates AMP-activated kinase (AMPK): a target for radiosensitization of human cancer cells. *Int J Radiat Oncol Biol Phys* 2010; 78: 221-229.
- 39) Brandstetter G, Blatt S, Goldschmitt J, Taylor L, Heymann P, Al-Nawas B, Ziebart T. Targeted sensitization of tumor cells for radiation through monocarboxylate transporters 1 and 4 inhibitions in vitro. *Clin Oral Investig* 2021; 25: 295-310.
- 40) Fiaschi T, Marini A, Giannoni E, Taddei ML, Gandellini P, De Donatis A, Lanciotti M, Serni S, Cirri P, Chiarugi P. Reciprocal metabolic reprogramming through lactate shuttle coordinately influences tumor-stroma interplay. *Cancer Res* 2012; 72: 5130-5140.
- 41) Dawson DM, Goodfriend TL, Kaplan NO. Lactic Dehydrogenases: Functions of The Two Types Rates of Synthesis of The Two Major Forms Can be Correlated with Metabolic Differentiation. *Science* 1964; 143: 929-933.
- 42) Végran F, Boidot R, Michiels C, Sonveaux P, Feron O. Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF- $\kappa$ B/IL-8 pathway that drives tumor angiogenesis. *Cancer Res* 2011; 71: 2550-2560.
- 43) Sonveaux P, Copetti T, De Saedeleer CJ, Végran F, Verrax J. Targeting the Lactate Transporter MCT1 in Endothelial Cells Inhibits Lactate-Induced HIF-1 Activation and Tumor Angiogenesis. *Plos One* 2012; 7: e33418.
- 44) Doherty JR, Yang C, Scott KE, Cameron MD, Fallahi M, Li W, Hall MA, Amelio AL, Mishra JK, Li F, Tortosa M, Genau HM, Rounbehler RJ, Lu Y, Dang CV, Kumar KG, Butler AA, Bannister TD, Hooper AT, Unsal-Kacmaz K, Roush WR, Cleveland JL. Blocking lactate export by inhibiting the Myc target MCT1 Disables glycolysis and glutathione synthesis. *Cancer Res* 2014; 74: 908-920.
- 45) Schmitt E, Sané AT, Steyaert A, Cimoli G, Bertrand R. The Bcl-xL and Bax-alpha control points: modulation of apoptosis induced by cancer chemotherapy and relation to TPCK-sensitive protease and caspase activation. *Biochem Cell Biol* 1997; 75: 301-314.