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The Effect Of The Drug α-Cyano-4-Hydroxycinnamate (CHC) On Granta Cell Survival

by

Dannicke Chantiloupe

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Under the Supervision of:

Chair: Dr. Jolanta Skalska

Committee Members:

Dr. John D'Angelo

Dr. Geoff Lippa

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List of Abbreviations/ Glossary of Terms

Ag	Silver
ATP	Adenosine Triphosphate
CHC	α-cyano-4-hydroxycinnamate
DI Water	Deionized Water
DLBCL	Diffuse large B cell lymphoma
EDTA	Ethylenediaminetetraacetic acid
EPR	Electron Paramagnetic Resonance
FBS	Fetal Bovine Serum
FCCP	Carbonilcyanide *p*-triflouromethoxyphenylhydrazone
FDA	Food and Drug Administration
Hypoxia	Oxygen deficiency within cells/tissues
HIF-1	Hypoxia-inducible factor-1
KCl	Potassium Chloride
LDH	Lactate Dehydrogenase
MCT	Monocarboxylate Transporter
NAD^+/NADH	Nicotinamide Adenine Dinucleotide
NHL	Non-Hodgkin lymphoma
OXPHOS	Oxidative phosphorylation
PBS	Phosphate-buffered saline
Pt	Platinum
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium

TOMM20	Translocase of the outer mitochondrial membrane 20

Abstract

Cancer can be considered a heterogeneous disease, in which tumor cells are well diversified in the metabolic pathways that they utilize to obtain the energy needed for their proper function and proliferation[1]. However, previous studies have indicated that the hypoxic conditions that normally persist in malignant cells result in an increase in glycolysis[2], and leads to tumors that are more dependent on the glycolytic metabolic pathway. Thus, in this study it was postulated that glycolysis, through the production of lactic acid, is very important in tumor cells, as it serves as a means for maintaining an acidic extracellular environment, as well as promoting tumor metastasis, and proliferation. To test the hypothesis that glycolysis may serve as a source of lactic acid needed for reducing the extracellular environment in cancer cells, Granta cells, which are large B cell lymphomas, were used as the model cell line. An important component of this study included treating the Granta cells with the monocarboxylate transport inhibitor, α -cyano-4-hydroxycinnamate (CHC), which stops the transport of lactic acid outside the cell. Collectively, the findings from this study suggest that Granta cells are not using the glycolytic pathway as a means for obtaining energy or for maintaining an acidic extracellular environment. The research limitations and suggestions for further research are addressed later in this document.

Introduction

Background/Cancer statistics:

Cancer is a group of diseases that is characterized by uncontrolled cell division, invasion/destruction of adjacent tissues, and metastasis—which is the ability of the disease to spread to other tissues via lymph or blood. One of the main problems associated with cancer is the inability of malignant cells to efficiently carry out apoptosis, a programmed self-destructive mechanism which functions to eliminate faulty cells. As a result, harmful cells are able to remain intact in an organism and can continue to multiply and spread to other areas in the body. Cancer can be triggered by many factors which may include external elements such as tobacco use, infectious organisms, and an unhealthy diet[3]. Internal factors associated with cancer include certain hormones, immune conditions, and inherited genetic mutations[3]. However, despite these many factors, a good percentage of cancers can be prevented especially those caused by tobacco use, excessive alcohol consumption, physical inactivity, and poor eating habits which can eventually lead to obesity[3].

Despite the many cancer treatments that are currently available such as radiation, chemotherapy, hormone therapy, immune therapy, surgery, and drugs that specifically interfere with cancer cell growth[3], cancer is still the second leading cause of death in United States, falling behind only heart disease with approximately 584,881 deaths in the year 2014[4]. Approximately 1,658,370 new cancer cases were expected to be diagnosed in the year 2015, of which neither noninvasive carcinomas, nor basal cell or squamous cell skin cancers are included[3]. Therefore, with such an increasingly high number of new cases, and the high mortality rates associated with this disease, research conducted in cancer has continued to be a vital component to progress in

medicine. The importance of cancer research lies in the fact that it is the basis for finding new and better ways to prevent, detect, diagnosis, treat, and even cure cancer[5]. According to Arteaga *et al*., the continual progress being made in cancer research has led the FDA, in the year 2014, to approve new anticancer therapeutics[5]. These newly approved treatments included immunotherapy, which works by preparing the patient's immune system to detect and destroy cancer, and "molecularly targeted therapeutics," which as the name suggests target cancer by aiming at specific molecules involved in its progression[5]. The improvements in therapeutics and the development of more efficient imaging and screening tests, due to advances in cancer research, have allowed for early detections of cancers—before the spread to other areas in the body, and has the potential to increase the curative effects of cancer treatments on a patient[5]. Also, the consistent progress in cancer studies has led to a greater number of individuals surviving longer and leading fuller lives after their cancer diagnosis. For example, approximately 4% of the population in the United States are currently cancer survivors[5], and this is a much higher percentage when compared to cancer survivorship in earlier years. In their report, Arteaga *et al*. further mention that cancer is one of the most costly diseases in the US, with an overall economic estimate of $286 billion in the year 2009[5]. By the year 2030, 21.5 million new cancer cases are expected, and this is estimated to cost $458 billion[5]. Thus, the high economic implications on society with each new occurrence of cancer further highlight the urgency and significance of conducting cancer research.

Significance of the project:

The significance of this research project lies in the basis of better understanding the main metabolic pathway of cancerous cells using the cell line Granta cells—large B cell lymphomas that originate from the human blood. Understanding the main metabolic pathway utilized in

malignant cells is a vital component in cancer research and is a critical aspect in developing more efficient anticancer drugs. All living cells carry out metabolism, which involves both anabolic and catabolic reactions. Metabolism is the mechanism by which a cell synthesizes and/or breaks down specific compounds, and some of these reactions may require energy while others may give off energy in the process. Similar to healthy cells, malignant cells need energy, and in order to remain viable they must be able to effectively obtain adequate amounts of energy to sustain their rapid cell proliferation rates. Tumors are characterized by fast cell proliferation and often use glycolysis as their predominant metabolic pathway for obtaining energy in the form of ATP[6]. Glycolysis yields a net production of 2 ATP molecules for each glucose molecule, whereas oxidative phosphorylation with the use of the mitochondrial respiratory chain can produce up to 38 ATP molecules. Hence, oxidative phosphorylation (OXPHOS) is a more efficient means for obtaining energy, and in the presence of oxygen, healthy cells use glycolysis coupled to mitochondrial respiration to obtain the maximum amount of ATP per glucose molecule. It is also important to note that in the presence of oxygen, allosteric effectors such as ATP create a negative feedback and shift energy production toward mitochondrial respiration, an effect known as the Pasteur Effect[7]. However, while healthy cells typically utilize glycolysis only in anaerobic conditions (absence of oxygen), aerobic glycolysis is a characteristic feature in many tumors (Warburg effect)[2].

Additionally, the fast rate by which malignant cells grow results in rapid utilization of oxygen, and this situation consequently leads to hypoxia. To overcome this problem, the hypoxia-inducible factor-1 (HIF-1), which according to Saedeleer *et al*. is the "core machinery" sustaining the glycolytic switch, becomes activated[2]. HIF-1, which is a transcription factor,

alleviates the effects of hypoxia through upregulating genes that encode glycolytic transporters, and activating enzymes that are unaffected by the Pasteur Effect[2].

Glycolysis, not only sustains fast cell growth, and provides tumors with an effective way of obtaining energy under stressful conditions such a hypoxia, but also, regular use of the glycolytic pathway by malignant cells eventually leads to the production of lactic acid which some researchers have identified to be critical in cancer metastasis[8]. Additionally, the acidic extracellular environment produced by the release of lactic acid outside the cell has been suggested to eliminate competition of healthy cells which cannot survive under acidic conditions. Another crucial characteristic for cancer survival involves maintaining plasma proteins in a reduced redox state, and this is suggested to be achieved by glycolysis through the production of by-product, lactic acid. Thus, a shift from glycolysis to OXPHOS could result in the oxidation of surface proteins that can initiate a cascade of reactions leading to cell death. It is also crucial to mention that the utilization of OXPHOS as a metabolic pathway results in the production of reactive oxygen species (ROS), and such reactive molecules can be very cytotoxic to cancer cells[1].

As mentioned previously, there are many distinct enzymes and sequences of events that become activated by the two different metabolic pathways—namely glycolysis and OXPHOS. The activation /deactivation of these enzymes and pathways is critical for tumor cell growth and survival, and further illustrates the importance of understanding the metabolic profile primarily utilized in cancers. Therefore with sufficient research in cancer metabolism, anticancer treatments that effectively target specific enzymes or disrupt sequential events critical for energy production in tumors, can be developed and used by cancer patients.

Description of work performed:

In this study, it was hypothesized that glycolysis in tumor cells, through the production of lactic acid, serves as a means for maintaining an acidic extracellular environment, which may help to keep critical proteins (not yet identified) for cell survival in a reduced state, as well as promote tumor metastasis, and proliferation. Therefore, if lactic acid is prevented from being excreted outside the cell (achieved through inhibiting the lactate transport system—monocarboxylate transporter), extracellular pH will remain at normal levels which should result in less reduced plasma membrane proteins, and also intracellular pH levels should rapidly decrease as the level of lactate production increases inside the cell. Maintaining both intracellular physiological pH homeostasis, and extracellular plasma protein redox state are key components to cancer survival; therefore blocking lactate efflux should induce cell death.

I. The first part of the experiment was aimed at the lactate transport system in the plasma membrane, monocarboxylate transporter 4 (MCT4).

Cells were treated with the drug, α-cyano-4-hydroxycinnamate (CHC). CHC is a drug that blocks the transport of lactic acid outside the cell through inhibiting the lactate transporter—Monocarboxylate transporter 4 (MCT4). It is important to mention that while there are many different MCT isoforms, glycolytic cells such as tumors normally have an upregulation in the MCT4 isoform, which is primarily responsible for lactic acid efflux. Consequently, it was expected that if tumor cells are unable to expel the excess lactic acid outside the cell, intracellular pH will drop to levels that are detrimental for the cell, and result in cell death. Also, if lactic acid transport is blocked, then extracellular pH will not reach acidic levels that may be needed to keep the cell's external environment in a reduced state, and this may impact cell

growth and proliferation. Thus, the higher the concentration of CHC used, the more cell death expected.

After treatment with CHC, the pH of the extracellular environment was measured. It was expected that the controls (cells with no drug) would have the lower extracellular pH, while cells treated with the CHC would have higher extracellular pH levels.

II. The next step of the study was directed towards measuring cell death.

Cell death was assessed through the use of the cytotoxicity test. Damaged cells should have leaky cell membranes that will result in the release of LDH, an enzyme that catalyzes the interconversion of pyruvate and lactate. The presence of LDH in the extracellular environment will be able to react with a given substrate that will then result in the formation of a colored product, which can be measured at an absorbance of 490nm with a spectrophotometer. Cells treated at higher CHC concentrations were expected to have greater release of LDH, and thus should have higher absorbance readings, which indicates level of cell death.

III. In the third stage of the designed experiment, the level of extracellular lactic acid present outside the cells was measured.

If the Granta cells are indeed dependent on glycolysis for metabolism, there will be high levels of lactic acid present outside the cells. As previously stated, lactic acid is a by-product of glycolysis, and excess lactic acid must be transported outside the cell to maintain intracellular pH homeostasis. Thus, if the cell is utilizing the glycolytic pathway, there will be high levels of extracellular lactic acid. The amount of lactic acid present outside the cells was measured based on the oxidation of lactate to pyruvate, which will also result in the formation of the electron donor, NADH. The rate of increase in absorbance of NADH that is detected at 340nm is linearly

proportional to extracellular lactate concentration. Thus, from this measurement the amount of extracellular lactate present can be assessed.

IV. The next step of the experiment was focused on determining the metabolic profile of the Granta cells. Oxygen consumption measurements of these cells were performed with oxygen electrodes.

To measure oxygen consumption, the Apollo 4000 free radical analyzer was used. The apparatus consisted of two electrodes—a platinum (Pt) cathode and a silver (Ag) counter/reference electrode. Oxygen consumption was assessed based on the principle that when oxygen is present within the electrolyte, it will flow to the Pt cathode where it will become electrolytically reduced. The reduction allows a current to flow, and that current creates a potential difference which is recorded on a computer using the Apollo 4000 software. The current flowing is proportional to the oxygen consumed at the cathode provided the solution is constantly stirred to maintain equilibrium.

Reaction occurring:

At Pt cathode: $4e^- + O_2 + 4H^+ \rightarrow 2H_2O$

At Ag anode: $4Ag + 4Cl^- \rightarrow 4AgCl + 4e^-$

Overall: $4Ag + 4Cl^- + 4H^+ + O_2 \rightarrow 4AgCl + 2H_2O$

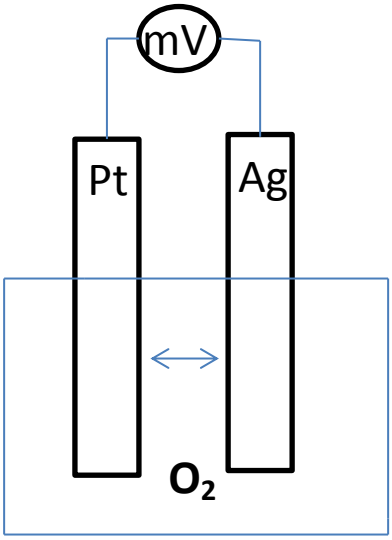
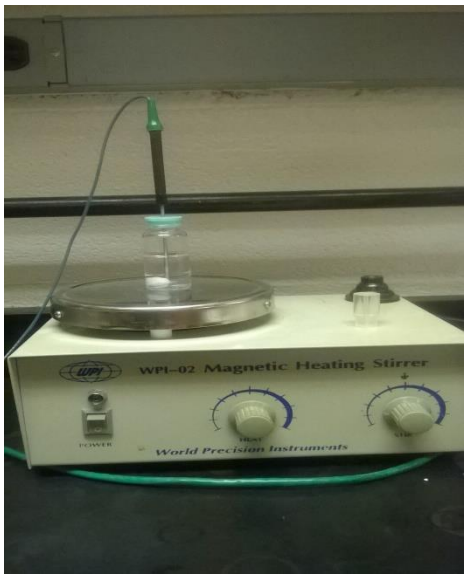


Figure 1: Schematic representation of the oxygen electrodes used. Electrodes were placed in KCl electrolyte.



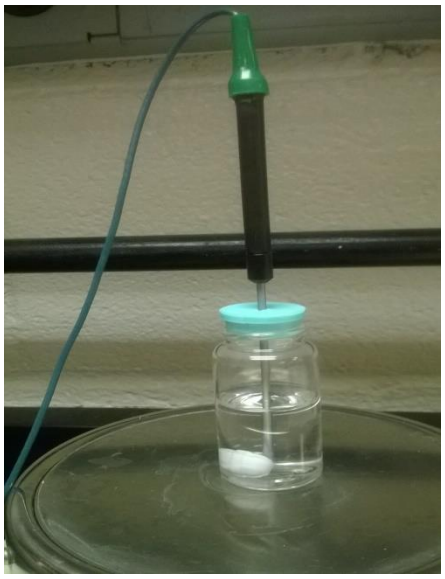


Figure 2: Complete apparatus used to measure oxygen consumption showing the ISO-OXY-2 electrode used. The ISO-OXY-2 electrode consists of a gas permeable membrane

fixed to the tip of an outer stainless steel sleeve. The interior of the probe contains the platinum cathode and silver reference electrode inside the sleeve. After the deposition of the electrolyte inside the sleeve, the interior of the probe, containing the electrodes is slowly inserted into the sleeve and secured by screwing the sleeve cap.

Many tumor cells will utilize the glycolytic pathway, which does not require oxygen, and allows for more rapid cell growth and proliferation. However, if the cells are consuming oxygen then this is an indicator that cellular respiration is occurring.

Carbonilcyanide *p*-triflouromethoxyphenylhydrazone (FCCP), a mitochondrial OXPHOS uncoupler[9] was later added to the reaction mixture in attempt to better investigate the role of the mitochondria in Granta cells cellular respiration. If Granta cells are using OXPHOS as their metabolic pathway then the addition of FCCP was expected to increase oxygen consumption in these cells. FCCP functions to dissolve membrane potential that drives ATP production, and in order to overcome this problem the mitochondria increases its flow of electrons, which results in an increase of oxygen consumption[10].

Materials and Methods:

Cells and Reagents

The cell line that was used for this study was large B cell lymphomas. The cells were grown in the medium RPMI 1640, which contained 10% FBS and antibiotics (1%penicillin/streptomycin), and stored at 37.4 degrees Celsius, with 4.4% CO_2.

Cytotoxicity of CHC on Granta Cells

20,000 Granta cells were plated in 24 different wells. Each well had 500µL of free cultures (Granta cells growing in medium). The concentration of CHC used was, 0.5µM, 1µM, 5µM, 10µM, 30µM, 50µM, 70µM. Of the two plates with treated cells, one was incubated for 12 hours while the other was incubated for 24 hours (time was arbitrarily chosen; however 24hours was the maximum amount of time needed to determine the level of cytotoxicity of CHC on these cells). The toxicity of CHC on the cells was then measured using the cytotoxicity assay, and the change in color produced was measured with a spectrophotometer at 490nm. The results obtained were then used to plot a graph for further analysis.

Lactate Assay using Hydrazine

Background:

Lactate dehydrogenase (LDH) enzymes catalyze the oxidation of lactate to pyruvate[11]. The addition of hydrazine removes pyruvate, and allows for the complete oxidation of all lactate molecules[11]. Measurements are based on the concentration of lactate in the sample, which is proportional to the increase in absorbance as NAD+ is reduced to NADH[11].

Procedure:

To measure lactate concentration, a mixture of NAD+ (0.02g) and DI water (1ml) were added to hydrazine buffer (9ml) in a 150ml beaker (working buffer). RPMI medium (1ml) was removed from 6 well plates and placed into Eppendorf tubes. 3 of the 6 well plates were the control wells (containing 0µM CHC), whereas the other 3 wells were the experimental wells (containing 500µM CHC). There were a total of 7 cuvettes, and each cuvette contained: 700 µL Tris working buffer (0.48mol/L Tris, 0.017 mol/L EDTA and 0.275mol/L hydrazine hydrate at pH 9.8), 100 µL DI water, 20µL of sample from one of the six well plates, and 0.5 µL of the LDH enzyme. N.B. the 7^{th} cuvette contained all of the above named substances; however, instead of containing 20µL of sample from well plate, contained 20µL negative control (fresh medium containing no Granta cells). Each cuvette sample was measured 5 minutes after the addition of the enzyme, using a spectrophotometer set at 340nm.

Oxygen Electrodes

The Apollo 4000, free radical analyzer was used to detect the level of oxygen consumption in Granta cells. 5,000,000 cells (cells counted using the hemocytometer) were suspended in 10 ml of PBS buffer and then added to the calibration bottle along with 9.99 µM of the compound FCCP. The experiment was then run for 20-30 minutes, and results tabulated and then graphed for further analysis.

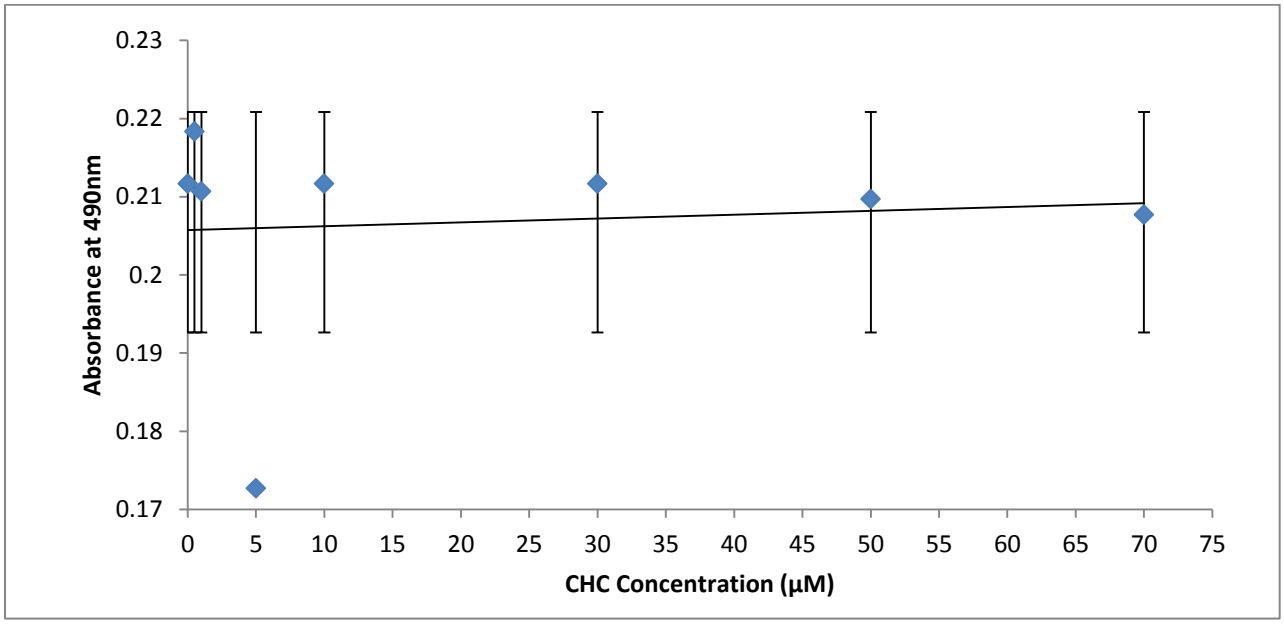
Results

- **Assessment of the effect of MCT4 inhibition by CHC on cell death and extracellular pH.**

The CHC effect on Granta cells survival rate has been measured by the colorimetric method detecting LDH release. As shown in figures 3A & 3B, there is no correlation between the concentration of CHC used and absorbance obtained at 490 nm. Thus, indicating that the incubation of the cells with CHC did not affect cell survival.

Also, the pH level of the extracellular environment in treated cells was measured using pH indicators. From the results obtained (data not shown), the external environment in both the cells treated with CHC, and the cells used as the controls (no drug treatment), the pH level was neutral.

A.



B.

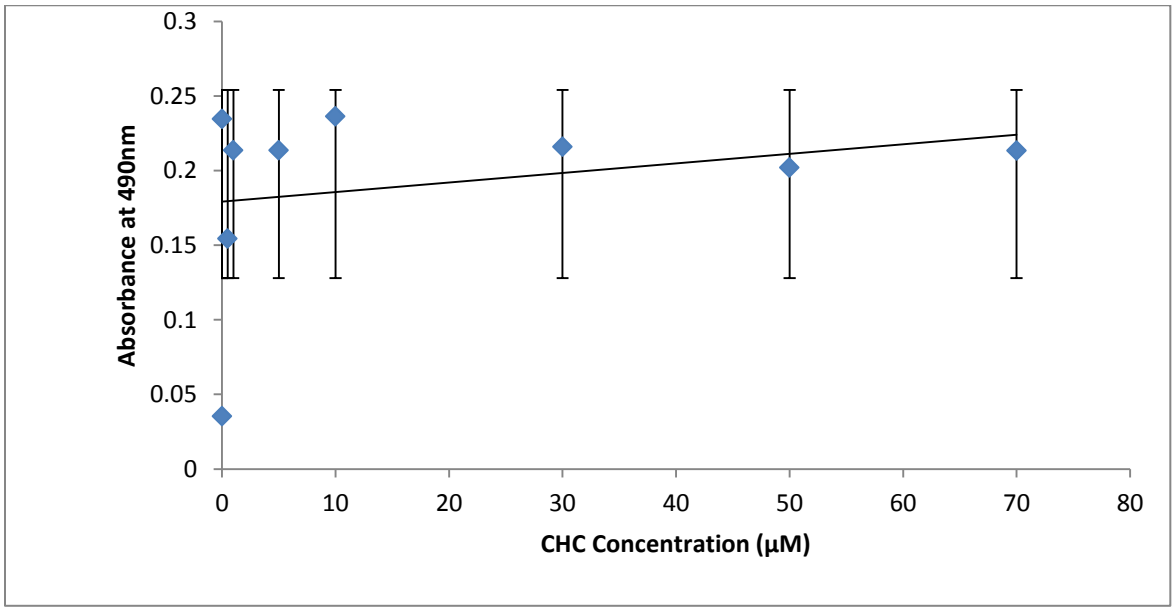


Figure 3: The effect of CHC on LDH release. (A) Cells incubated for 12 hours after treatment with CHC. ± SD; n=5E-05. (B) Cells incubated for 24 hours after treatment with CHC. ± SD; n= 0.0006.

- **Measurement of extracellular lactic acid in Granta cells.**

Due to the fact that lactic acid accumulation inside the cell can disrupt intracellular pH homeostasis, it is important that the cells expel excess lactic acid outside the cell. Therefore malignant cells that are dependent on the glycolytic pathway should have increased levels of lactic acid outside the cell.

As shown in figures 4A & 4B, Granta cells do not have extracellular lactic acid outside the cells, and hence may not be using the glycolytic pathway as their main means for metabolism. If Granta cells were using glycolysis as their major pathway, treatment with CHC should yield

lower levels of extracellular lactate, which should then produce lower absorbance values; however, the controls (no treatment with CHC) should have higher absorbance readings since lactate would be able to freely move outside the cells. Instead, the controls had lower absorbance readings while treated cells had very high absorbance readings, indicating that the cells may not be dependent on glycolysis.

A. **B.**

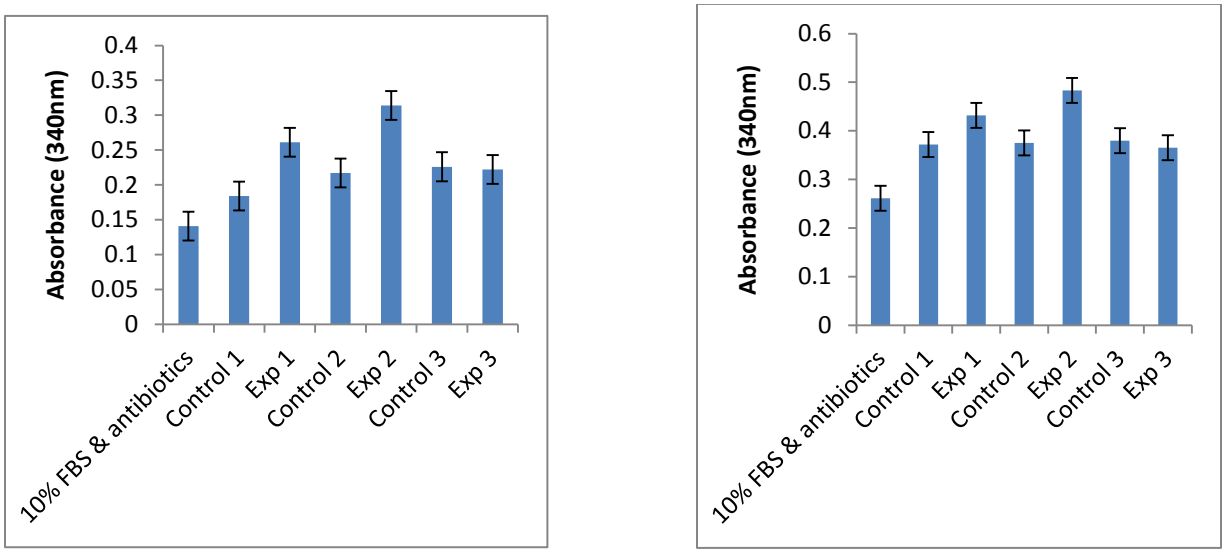


Figure 4: Level of extracellular lactic acid present outside the cells. (A) Absorbance measured 5 minutes after adding enzyme (LDH). ± SEM. (B) Absorbance re-measured after 30 minutes. ± SEM.

- **Metabolic profile of Granta cells**

As shown in figure 5, Granta cells do not use oxidative phosphorylation. The electric current generated between the oxygen electrodes remained consistent throughout the course of the

experiment, and even after the FCCP (compound that disrupts the mitochondrial membrane, and makes it leaky) was added, there were no significant changes in current obtained.

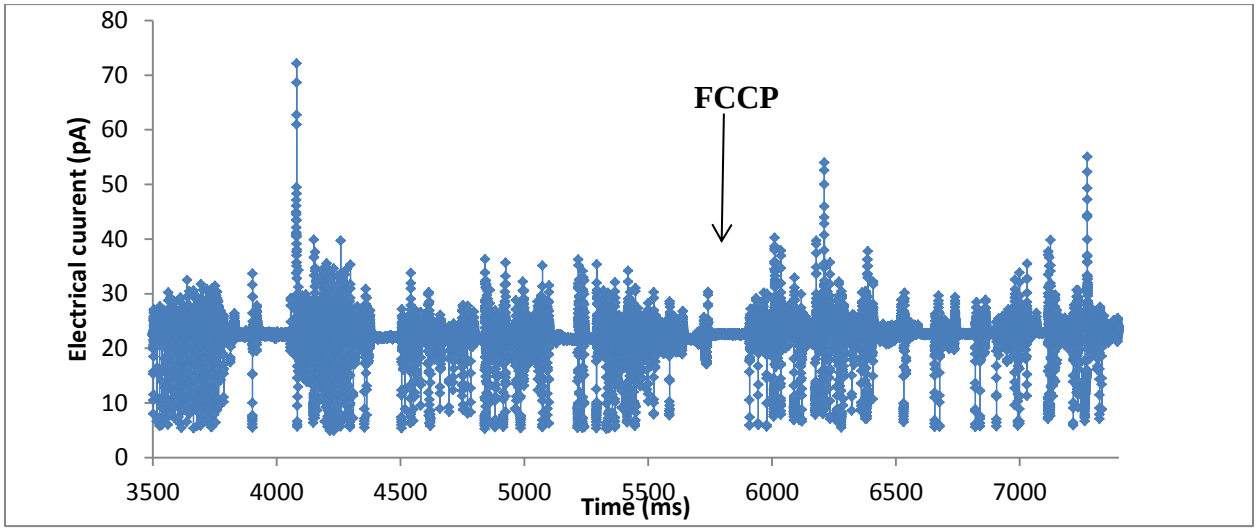


Figure 5: The metabolic profile of Granta cells. Current measurements for oxygen consumption determination.

Discussion

Tumors are heterogeneous diseases, in which cancerous cells efficiently use aerobic glycolysis, anaerobic glycolysis or oxidative phosphorylation (OXPHOS)[1]. Earlier studies done on cancer have proposed that malignant cells have defects in their mitochondrial OXPHOS, which then results in great dependence of these cells on the glycolytic pathway[1]. However, more recent studies have shown that tumor cells have intact OXPHOS, which is suppressed in these cells due to an increase in the glycolytic pathway. For example, Fantin *et al*. detected that when lactate dehydrogenase-A (LDH-A) is inhibited in tumors, OXPHOS function could be boosted[1]. Additionally, certain cancers such as HL60, HeLa, 143B and U937 rely primarily on OXPHOS to support the growth of cells; however this metabolic pathway can be altered in hypoxic conditions[1].

In this study, it was shown that Granta cells are not dependent on glycolysis. Based on this finding, it is important to note that cancer can be divided into two main types: blood-borne cancers and solid cancers. Granta cells are blood-borne (liquid) cancers, which originate from the bone marrow, and disrupt proper functioning of the blood, and the immune system. On the other hand, solid cancers are the ones that actually form tumors—which are anomalous clumps of cells, and normally overcrowd healthy cells preventing them from carrying out their functions. Thus, although Granta cells (blood-borne cancers) are not using glycolysis, the hypothesis might hold true for solid tumors.

The rationale for the hypothesis presented in this study was based off the fact that tumors are highly proliferating cells that require energy at very fast rates. The rapid energy demands in these fast proliferating cells favor glycolysis to supply the required energy[6]. It is important to note that although glycolysis yields less ATP, it is a much more rapid process for producing ATP

and is thus more suitable for very rapid proliferating cells such as cancer[1]. Additionally, fast growing cells eventually become hypoxic, which is overcome primarily through the "glycolytic switch," where glycolysis is uncoupled from respiration and becomes the primary source of ATP production[12].

Furthermore, previous studies have shown that the lactic acid end product of glycolysis produces an acidic microenvironment that gives cancer cells a growth advantage when compared to other normal cells, since normal cells find it difficult to cope in an acidic environment, it is suggested that malignant cells eliminate competition from other cells due to their ability to survive in acidic environments[1]. Besides providing a growth advantage, lactate secretions have been identified to promote metastasis, which is an important feature in all cancer types, allowing them to invade and spread to other cells and tissues in the body. It is also important to mention that unlike glycolysis, mitochondrial OXPHOS generates reactive oxygen species (ROS) which can be cytotoxic to tumors[1]. Therefore, based on the many advantages associated with using the glycolytic pathway, it was postulated that Granta cells (large B cell lymphomas) are also using this metabolic pathway.

Diffuse large B cell lymphomas (DLBCL), which are another type of large B cell lymphoma, are the most common type of non-Hodgkin lymphomas (NHL) and account for up to 30% of new cancer cases in the United States[13]. Due to the extensive research done on DLBCL compared to Granta cells, the findings on these cells were used as a reference for Granta cells which are also large B cell lymphomas. DLBCL are genetically diverse tumors, with some subgroups containing an up-regulation in genes that code for mitochondrial oxidative phosphorylation (Oxphos-DLBCL)[14], and others containing genes that are upregulated in B-cell receptor signaling (BCR- DLBCL) and are chiefly dependent on aerobic glycolysis[14]. Also, it is

important to mention that while B-cell receptor signaling is a vital growth and survival pathway for BCR- DLBCL, this survival pathway is nonfunctional in the Oxphos-DLBCL subgroup, and suggests that Oxphos-DLBCL are dependent on alternative survival pathways[14]. Thus, based on the two subsets of DLBCL previously mentioned, large B-cell lymphomas are heterogeneous tumors, and are not limited to aerobic glycolysis, but can utilize anaerobic glycolysis, and/or oxidative phosphorylation depending on the nutrients readily available[15].

The first set of data obtained from this experiment confirms that Granta cells are not dependent on aerobic glycolysis. The results obtained from the cytotoxicity test, using the drug CHC (MCT4 inhibitor), did not have any effect on the survival rate of the cells (figure 3A & 3B). According to Hamdan *et al*, monocarboxylate transporters (MCTs) transport lactate and pyruvate, and are very important in tumor progression[16]. The drug, CHC has been observed to inhibit tumor growth *in vitro* through its ability to block lactate efflux[16]. Recent studies have revealed that CHC significantly induces necrosis in multiple cancers including, glioblastomas and tumors of the prostate by increasing lactic acid production and inhibiting plasma membrane MCT activity[16]. Also, further studies have identified that MCT inhibitors can reduce tumor size and can sensitize hypoxic tumor regions to radiotherapy[16].

Thus, based on the results obtained from the cytotoxicity test in this study, it was then proposed that if indeed Granta cells were utilizing glycolysis, then the cells may have been using another lactate transporter that may be resistant to the drug CHC. However, this postulation was soon dismissed after pH measurements of the extracellular environment of both the cells treated with CHC and the controls (no CHC treatment) yielded a neutral pH. As stated previously, the up-regulation of glycolysis in malignant cells results in acid production (lactic acid), which must be successfully transported out of the cells, and consequently gives rise to an acidic extracellular

environment[17]. Thus, the neutral pH measurements that were obtained in this study further verified that Granta cells are not dependent on glycolysis.

Additionally, Ullah *et al.* mentions that MCT4 is the most strongly expressed lactate transporter in glycolytic cells which is triggered as a result of hypoxia through a HIF-1α-mediated mechanism[18]. MCT4 functions as the primary lactate efflux transporter system, and although it has a lower affinity for its substrate[19] when compared to the isoform MCT1, it is very crucial in glycolytic cells where the removal of lactate from the cell is critical for cell survival. MCT1, on the other hand, is more predominant in cells and muscles that use OXPHOS, and is most commonly involved in the transport of lactate inside the cell, where lactate can then be used as a fuel for respiration or for gluconeogenesis[18,19]. Therefore, according to the study presented by Ullah *et al.*, and the results obtained from this study, if Granta cells were using glycolysis, MCT4 would be the most likely transporter for the efflux of lactate, and the inhibition of this transporter by CHC would have had an unfavorable impact on the cells' survival.

To maintain intracellular physiological pH, the lactic acid formed from glycolysis must be excreted via the MCT4 system in the plasma membrane. Therefore, Granta cells using glycolysis should have higher than normal levels of lactate outside the cell. However, the lactic acid measurements obtained from this study indicated that there is no extracellular lactic acid outside the Granta cells (figures 4A & 4B). This data further illustrates that Granta cells may not be using the glycolytic pathway as their main means for metabolism. Studies conducted by Gooptu *et al.*, suggest that diffuse large B cell lymphomas do not express MCT4, but instead strongly express TOMM20—translocase of the outer mitochondrial membrane 20, which is a biomarker for the oxidative phosphorylative phenotype[20]. Thus, the high expression of TOMM20 in these large B cell lymphomas is indicative that oxidative phosphorylation, rather than

glycolysis is the main metabolic pathway, and therefore high levels of extracellular lactic acid is not expected in such tumor cells.

Based on all the data obtained from this study, there is clear evidence that Granta cells may not be utilizing glycolysis. However, the results acquired from measuring oxygen consumption in Granta cells indicated that these cells are not using oxidative phosphorylation either. Smolkova *et al.* state that there are four basic waves of carcinogenesis[1]. In waves 1 and 2, cell metabolism highly favors glycolysis; while in wave 3, mitochondrial OXPHOS is moderately restored, and in wave 4 there is revival in the mitochondria where OXPHOS is fully restored[1]. From this, it shows that not only glycolysis, but also OXPHOS is an important metabolic pathway in tumors, and thus cancerous cells, like normal cells must use at least one of the metabolic pathways to obtain energy.

Implications and Further work

Based on the data obtained in this study, Granta cells may not be using the glycolytic pathway; however the results obtained from measuring oxygen consumption in these cells were inconclusive. Nonetheless, many different research projects have indicated that large B cell lymphomas are heterogeneous tumors and are thus able to utilize many different metabolic pathways[13,14,15]. Also, Gooptu *et al.* have shown that large B lymphomas do not express MCT4, but rather TOMM20[20], which further supports the findings in this study, where three of the experiments conducted in this research project lead to the conclusion that Granta cells are not using glycolysis, and thus may not have an up-regulation in MCT4.

It is important, therefore, to run additional experiments to further test oxygen consumption in these cells, as the results obtained are inconsistent. The Apollo 4000 free radical analyzer was used to assess the level of dissolved oxygen present in the PBS buffer containing the Granta cells. However, additional experiments can be done to test the level of oxygen consumption in these cells by modifying the buffer used to better match the conditions that the cells are typically grown in. That is, for further testing of oxygen consumption, glucose may be added to PBS buffer as a means to enhance the environment of the Granta cells.

Additionally, apart from the Apollo 4000 free radical analyzer, there are many other techniques that can be utilized in assessing oxygen consumption in cancerous cells. Other methods may include the electron paramagnetic resonance (EPR) oximetry, and the MitoXpress fluorescent assay[21]. The EPR technique uses high-tech instrumentation that allows for continuous monitoring of oxygenation within tissues[21]. The MitoXpress assay is based on a phosphorescent oxygen-sensitive probe, to which the oxygen molecules are able to react[21]. As the oxygen

diminishes in the surrounding solution, there is an increase in the probe phosphorescence signal[21]. Thus changes in oxygen consumption are measured as changes in the MitoXpress probe signal over time[21].

Additionally, to confirm that Granta cells do not have reduced plasma membrane proteins, further research can be performed. One method that can be used in such a study is the proteomic approach, which can lead to the identification of proteins and protein modifications such as phosphorylation, mutations, glycosylations or oxidations/reductions.

Conclusion

Based on the findings obtained from this study, Granta cells are not using glycolysis. These cells seem to be relying on OXPHOS for maintaining their energy demands. As previously discussed, further research needs to be carried out to better understand the oxygen consumption/mitochondrial activity within Granta cells. Also, since Granta cells are strictly blood-borne cancers, conducting further experiments that investigate the metabolic profiles of both solid and blood-borne tumors, treated under similar conditions, could yield pertinent data that might better explain the results from this study.

It was hypothesized that Granta cells are using glycolysis not only as means for obtaining energy, but also, it was hypothesized that glycolysis through the production of lactic acid, is used as a mechanism to maintain tumor viability. However, based on the results obtained, the mechanism of these cells malignancies may not be related to the presence of lactic acid or the redox state on the plasma membrane.

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