Acute Pharmacologic Modulation of Glioblastoma pH Monitored by Chemical Exchange Saturation Transfer Magnetic Resonance Imaging

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Medical Biophysics
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**Abstract**

The response of tumor intracellular pH to a pharmacological challenge could help identify aggressive cancer. pH<sub>i</sub> is important in the maintenance of normal cell function and is normally maintained within a narrow range by the activity of transporters located at the plasma membrane. Modulation of tumor pH<sub>i</sub> may influence proliferation, apoptosis, chemotherapy resistance, and thermosensitivity. Chemical exchange saturation transfer (CEST) is a novel MRI contrast mechanism that is dependent on cellular pH. Amine and amide concentration-independent detection (AACID) is a recently developed CEST contrast method that is intracellular pH (pH<sub>i</sub>) weighted.

The overall goal of this thesis was to maximize the magnitude of intracellular tumor acidification using pharmacologic agents. To this end, a series of studies were performed to quantify the magnitude of acute pH<sub>i</sub> change in glioblastoma after the injection of a single dose of drugs known to interfere with pH regulation. In addition, we attempted to simulatenously block five pH regulatory mechanisms while also providing glucose as an energy substrate to maximize tumor acidification. We hypothesized that this approach would increase the acute pH modulation effect. Using a 9.4T MRI scanner CEST spectra were acquired sensitive to pH<sub>i</sub> using amine / amide concentration independent detection (AACID).

For these experiments a number of different drugs were utilized. Dichloroacetate (DCA) can alter tumor pH<sub>i</sub> by inhibiting the enzyme pyruvate dehydrogenase kinase causing reduced lactate (increasing pH<sub>i</sub>), or by decreasing the expression of monocarboxylate transporters and vacuolar ATPase leading to reduced pH<sub>i</sub> in tumors. The sodium proton exchanger (NHE1) plays a significant role in maintaining pH balance in the tumor microenvironment. Cariporide is a
sodium proton exchange inhibitor and has been shown to suppress the invasion and migration of cancer cells. Quercetin is an inhibitor of monocarboxylate transporters (MCTs). It specifically inhibits MCT1 and MCT2. Pantoprazole is a proton pump inhibitor (PPI) that targets the vacuolar H\(^+\)-ATPase and has been shown to decrease intracellular pH and increase apoptotic cell death in cancer cells. Finally, acetazolamide inhibits carbonic anhydrase activity and decreases expression of the aquaporin-1 (AQP1) water channel.

In the first study, one hour after DCA intravenous injection (200 mg/kg) there was a significant increase in tumor AACID level by 0.04±0.01 corresponding to a 0.16 decrease in pH\(_{i}\), and no change in AACID in contralateral tissue. In a second study, two hours after cariporide intraperitoneal injection there was a significant 0.12±0.03 increase in tumor AACID value corresponding to a 0.48 decrease in pH\(_{i}\), and no change in AACID value in contralateral tissue. In a final study, two hours after combined intraperitoneal injection of all five drugs, there was a significant 0.10±0.03 increase in tumor AACID corresponding to a 0.40 decrease in pH\(_{i}\). After injecting the drug combination with glucose the AACID value increased by 0.18±0.03 corresponding to a 0.72 decrease in pH\(_{i}\). AACID values were unchanged in contralateral tissue.

The combined drug treatment with glucose produced the largest acute CEST MRI contrast indicating tumor acidification. This approach could be used to help localize brain cancer and monitor tumor response to chemotherapy. The use of the approved drugs in combination with pH weighted MRI represents a unique approach to cancer detection does not require injection of an imaging contrast agent.

**Keywords:** Brain cancer; Glioblastoma multiforme(GBM); Apoptosis; pH; MRI; CEST
Co-Authorship


This work was co-authored by: Mohammed Albatany, Alex Li, Susan Meakin, and Robert Bartha.

M.A. and R.B. contributed to overall experimental design. A.L. contributed and performed to the MRI experiments. M.A. analyzed the data and wrote the manuscript. S.M. contributed the brain cancer cell line. R.B. contributed to the manuscript editing.

*In vivo* Detection of Acute Intracellular Acidification in Glioblastoma Multiforme Following a Single Dose of Cariporide (International Journal of Clinical Oncology, Published online: May 2018)

This work was co-authored by: Mohammed Albatany, Alex Li, Susan Meakin, and Robert Bartha.

M.A. and R.B. contributed to overall experimental design. A.L. contributed and performed to the MRI experiments. M.A. analyzed the data, mix the drugs and wrote the manuscript. S.M. contributed the brain cancer cell line. R.B. contributed to the manuscript editing.

Brain Tumor Acidification using Drugs Simultaneously Targeting Multiple pH Regulatory Mechanisms

This work was co-authored by: Mohammed Albatany, Valeriy Ostapchenko, Susan Meakin, and Robert Bartha
M.A. and R.B. contributed to overall experimental design. M.A. contributed and performed to the MRI experiments. M.A. analyzed the data, inject and treat the animals with drugs, also perfuse the mice and slice the brains for the histology and wrote the manuscript. V.O contributed to the histology and caspase3 imaging. S.M. contributed the brain cancer cell line. R.B. contributed to the manuscript editing.
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<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>CEST</td>
<td>Chemical Exchange Saturation Transfer</td>
</tr>
<tr>
<td>AACID</td>
<td>Amine Amide Concentration Independent Detection</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous system</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma Multiforme</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelium Growth Factor</td>
</tr>
<tr>
<td>pH&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Intracellular pH</td>
</tr>
<tr>
<td>pH&lt;sub&gt;e&lt;/sub&gt;</td>
<td>Extracellular pH</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>CA</td>
<td>Carbonic Anhydrase, Contrast Agent</td>
</tr>
<tr>
<td>AQP</td>
<td>Aquaporin</td>
</tr>
<tr>
<td>CAI</td>
<td>Carbonic Anhydrase Inhibitor</td>
</tr>
<tr>
<td>DCE-MRI</td>
<td>Dynamic Contrast Enhanced Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>T1</td>
<td>Longitudinal Relaxation Time</td>
</tr>
<tr>
<td>T2</td>
<td>Transverse Relaxation Time</td>
</tr>
<tr>
<td>PARACEST</td>
<td>Paramagnetic CEST</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>FDG</td>
<td>Fluorodeoxyglucose</td>
</tr>
<tr>
<td>^18F FDG</td>
<td>Fluorine-18 labeled fluorodeoxyglucose</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
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<tr>
<td>MRS</td>
<td>Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>CR</td>
<td>Creatine</td>
</tr>
<tr>
<td>LAC</td>
<td>Lactate</td>
</tr>
<tr>
<td>MT</td>
<td>Magnetization Transfer</td>
</tr>
<tr>
<td>MTR</td>
<td>Magnetization Transfer Ratio</td>
</tr>
<tr>
<td>APT</td>
<td>Amide Proton Transfer</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>LND</td>
<td>Lonidamine</td>
</tr>
<tr>
<td>FSE</td>
<td>Fast spin echo</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>Mz</td>
<td>Longitudinal magnetization</td>
</tr>
<tr>
<td>µs</td>
<td>microsecond</td>
</tr>
<tr>
<td>ms</td>
<td>millisecond</td>
</tr>
<tr>
<td>K</td>
<td>Exchange rate</td>
</tr>
<tr>
<td>K\textsubscript{ws}</td>
<td>Water-solute exchange rate</td>
</tr>
<tr>
<td>K\textsubscript{sw}</td>
<td>Solute-water exchange rate</td>
</tr>
<tr>
<td>M</td>
<td>Moles</td>
</tr>
<tr>
<td>ω</td>
<td>Resonance frequency</td>
</tr>
<tr>
<td>Δω</td>
<td>Frequency difference</td>
</tr>
<tr>
<td>R\textsubscript{1}</td>
<td>Longitudinal relaxation rate</td>
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<tr>
<td>RF</td>
<td>Radio Frequency</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>9.4 T</td>
<td>9.4 Tesla</td>
</tr>
<tr>
<td>B\textsubscript{0}</td>
<td>The static magnetic field</td>
</tr>
<tr>
<td>TR</td>
<td>Repetition Time</td>
</tr>
<tr>
<td>TE</td>
<td>Echo Time</td>
</tr>
<tr>
<td>ETL</td>
<td>Echo Train Length</td>
</tr>
<tr>
<td>FOV</td>
<td>Field- of- View</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered solution</td>
</tr>
<tr>
<td>PTR</td>
<td>Proton transfer ratio</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal to noise ratio</td>
</tr>
<tr>
<td>WASSR</td>
<td>Water saturation shift referencing</td>
</tr>
<tr>
<td>MTR\textsubscript{asym}</td>
<td>Asymmetric magnetization transfer ratio</td>
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</table>
Chapter 1

1 Endogenous Magnetic Resonance Imaging and Spectroscopy
Measurements of Intracellular pH in Cancer

Abstract:

Intracellular pH (pH$_i$) plays an important role in the maintenance of normal cell function and is maintained within a narrow range by the activity of transporters located at the plasma membrane. A common characteristic of cancer cells is their increased production of acidic metabolites and consequently, enhanced acid export to the extracellular space. As a result, cancer cells have relatively basic intracellular pH compared to normal cells. The consequent acidification of the extracellular tumor microenvironment supports tumor growth and increases metastatic potential. Tumor intracellular pH is an informative indicator of the tumor microenvironment as many proteins and peptides have pH-dependent structures and activities. In addition, manipulation of tumor pH$_i$ can influence proliferation, apoptosis, chemotherapy resistance, and thermosensitivity. Therefore, accurate measurement of tumor intracellular pH could have many potential applications. The current review summarizes recent advances in endogenous MRI methods to measure tumor intracellular pH and summarizes studies that use drug treatments to manipulate intracellular pH in cancer.
1. Introduction

Elevated intracellular pH in cancer is regulated by several mechanisms that can be simultaneously blocked by a combination of drugs leading to acute intracellular acidification. The work presented in this thesis applies a technique called chemical exchange saturation transfer (CEST) magnetic resonance imaging (MRI) to study intracellular acidification in brain tumors after drug injection.

This chapter provides an introduction and outline to the presented research on intracellular pH measurement in cancer and the drugs that can modulate intracellular pH in tumors. Section 1.1 outlines the general organization of the thesis. The regulation of pH in normal cells and in cancer cells is presented in Section 1.2. An introduction to CEST theory and mechanisms of producing MRI contrast is presented in Section 1.3. Endogenous MRI measurement of intracellular pH presented in Section 1.4. Endogenous MRI measurement of intracellular pH in cancer is presented in Section 1.5. Finally, a description of various drugs that can be used to modulate pH in cancer is presented in Section 1.6.

1.1 Thesis Overview and Objectives

Anatomical MRI can provide valuable clinical information and enable diagnosis (1-3) in many diseases including cancer. MRI can produce excellent soft-tissue contrast with high-resolution that allows localization and differentiation between different types of soft tissue. Advances in the understanding of cell metabolism have modified our understanding of the pathophysiology of many diseases (1,2). In this regard, the detection of abnormal physiology could help in early disease diagnosis (4,5). Early diagnosis can increase the chance of treatment success. Glioblastoma multiforme (GBM) is one of the most aggressive gliomas and deadliest
brain tumors, with less than half of patients surviving beyond 12–18 months even with optimal treatment (6-9). These tumors represent the second leading cause of death among neurological diseases in the United States and present one of the greatest challenges in the treatment of cancer in the world (6-9).

CEST is a relatively new MRI technique that has emerged to produce contrast related to the presence of metabolites and specific proteins. It can also be made sensitive to biological processes such as apoptosis (10) and angiogenesis (11). However, many factors influence CEST contrast. These factors include the concentration of the target (12), temperature (13), and pH (14). In vivo, the pH and temperature are associated with energy producing metabolic efficiency (14,15). Recently, many applications of CEST in cancer have focused on measuring intra- and extra-cellular pH. Cancer is characterized by significant changes in metabolism resulting in changes in pH that are described in detail in Section 1.2. Therefore, measuring pH is a potentially valuable marker to indirectly probe metabolic changes in cancer.

Positron emission tomography (PET) is the current standard method to image metabolism in cancer. Specifically, the glucose analog called $^{18}$F-labeled fluorodeoxyglucose ($^{18}$F FDG) can be detected using PET scanners, and provides a measure of glucose uptake in tumors. Measuring tumor uptake of $^{18}$F FDG can help in the prediction of tumor response to treatment and is related to cancer aggressiveness (16,17). Although PET has much greater detection sensitivity than MRI, the development of metabolic imaging by MRI may have some advantages over PET because it is easier to interpret when registered to high-resolution anatomical scans, and it is less expensive and more accessible(18). Thus, scientists are motivated to develop novel techniques to quantify physiological processes using MRI such as tissue acidification, to apply in cancer and
stroke. Some metabolic imaging indicators of disease can be used to increase the accuracy of diagnosis and prognosis compared to anatomical MR imaging (4,19).

1.2 pH Regulation in live tissue

1.2.1 Concept of the pH

The pH is a logarithmic scale that is used to measure the proton (H\(^+\)) concentration of a solution. The pH concept was first introduced by the Danish chemist Søren Peder Lauritz Sørensen at the Carlsberg Laboratory in 1909 (20). A high concentration of protons in solution produces a low pH value, and solutions with a low concentration of protons (H\(^+\)) have a high pH value. There is a tendency for water molecules (H\(_2\)O) naturally to be ionized or dissociate into a hydroxyl ion (OH\(^-\)) and a proton (H\(^+\)) according to equation (1.1).

\[
H_2O \leftrightarrow H^+ + OH^- \quad (1.1)
\]

For example, if an aqueous solution is acidic, the [H\(^+\)] is higher than [OH\(^-\)] and if a solution is alkaline, the [OH\(^-\)] is higher than [H\(^+\)] (21). The pH value is the negative logarithm of [H\(^+\)]:

\[
pH = -\log [H^+] \quad (1.2)
\]

Where the [H\(^+\)] is the molar concentration of protons in the solution (21).

1.2.2 pH regulation in normal tissue

Intracellular pH (pH\(_i\)) is normally maintained within a narrow range by the activity of transporters located at the plasma membrane and has an essential role in the maintenance of normal cell function. Increases in lactate and proton concentration can cause acidification of the tissue microenvironment in many diseases where oxygen supply is less than metabolic demand.
The pH value is an essential marker of disease progression, as many proteins, peptides, and macromolecules have pH-dependent structures and activities (22,23). Normal pH in both the intra-cellular and extra-cellular compartments is maintained through the complex and coordinated interaction between several different physiological systems including cellular metabolism, the cellular membrane potential, intracellular and extracellular buffering systems, cell membrane transporters, delivery of oxygen and glucose, and removal of acid by-products by the blood (24). There are many potential pH regulators involved in this process including Na\(^+/\)HCO\(_3^-\) co-transporters, Na\(^+/\)H\(^+\) exchangers, monocarboxylate transporters, the vacuolar ATPase, carbonic anhydrase, anion exchangers, the Cl\(^-\)/HCO\(_3^-\) exchangers, and ATP synthase (25,26).

A hallmark feature of solid tumors is the maintenance of an alkaline intracellular pH (pH\(_i\)) and an acidic extracellular pH (pH\(_e\)) (22,27). In normal cells, the pH\(_e\) is higher (~7.4) than the pH\(_i\) of ~7.0-7.1 (24,28-32). However, the pH gradient in cancer cells is reversed, with lower pH\(_e\) (~6.7–7.1) than pH\(_i\) ~7.1-7.3 (24,28-32). This altered pH homeostasis is the consequence of increased glycolysis even in the presence of oxygen (aerobic glycolysis), known as the Warburg effect (16). The resulting alkaline pH\(_i\) benefits cancer by supporting increased cell proliferation and evasion of apoptosis (22,33) and increases the resistance of the tumor to chemotherapy and immunotherapy (34). Glucose metabolism plays an important role in acidification or alkalization of intracellular and extracellular spaces (Figure 1.1). In normal cells, glucose is converted to pyruvate, which then enters the mitochondria and oxidized to produce CO\(_2\) and H\(_2\)O and ATP (Figure 1.1, left). In tumor cells, glucose is also converted to pyruvate, but then rapidly converted to lactate and H\(^+\) in the cytosol (Figure 1.1, right). The transformation of glucose to pyruvate via glycolysis occurs in the cytosol and is described in Equation (1.3).
In the process of the breakdown of one glucose molecule, two nicotinamide adenine dinucleotide (NAD\(^+\)) molecules are reduced to form two NADH. In addition, phosphorylation of two inorganic phosphates (Pi) and adenosine diphosphate (ADP) molecules produces two high-energy adenosine triphosphate (ATP) molecules. Two water molecules and two H\(^+\) are also exported to the extracellular space by transporters located on the cell membrane (22,35).

\[
\text{Glucose} + 2\text{NAD}^+ + 2\text{ADP} + 2\text{Pi} \rightarrow 2\text{Pyruvate} + 2\text{NADH} + 2\text{H}^+ + 2\text{ATP} + 2\text{H}_2\text{O} \quad (1.3)
\]

**Figure 1.1** Fate of glucose in normal cell under aerobic respiration (left) and in cancer cell under aerobic glycolysis (right).

1.2.3 pH regulation in cancer

Intracellular pH (pH\(_i\)) has an important role in the maintenance of normal cell function and is normally maintained within a narrow range by the activity of transporters located at the plasma membrane. In cancer, changes in pH\(_i\) have been correlated with both cell proliferation...
and cell death (apoptosis). In cancer tissue, the pH gradient is often reversed. Cancer cells tend to undergo the Warburg effect which is the term given to up-regulated glycolysis under aerobic conditions (Figure 1.1) (16,22,24,36-40).

In tumor advancement, cancer cells proliferate and compose large cellular masses, or tumors, much faster than adequate vasculature can be produced. Cancer cells in the core of the tumor often lack compatible circulation, and the cells that increase glycolysis tend to have an advantage (16,22,41). Cancer cells can increase glycolysis using many control mechanisms such as increased glucose uptake and up-regulation of pro-glycolytic enzymes such as hexokinase (16). The result is an increased population of cancer cells favoring glycolysis. A hallmark feature of most cancer cells is increased glucose uptake, which is commonly detected by positron emission tomography (PET) using the uptake of the glucose analog \(^{18}\text{F}\)-labeled fluorodeoxyglucose (\(^{18}\text{F}\) FDG) (16). Therefore, cancer cells tend to have high rates of glycolysis in both aerobic and anaerobic conditions, although anaerobic glycolysis is an inefficient respiration pathway to produce ATP compared to the aerobic pathway. Also, cancer cells increase the uptake of amino acids for cell proliferation. Therefore, cancer cells increase cellular mass by relying on the Warburg effect to meet the energy demand.

A consequence of up-regulated glycolysis in cancer cells is increased amounts of pyruvate production leading to increased proton and lactate production (16). Contradictorily, there is a tendency for cancer cells to have an alkaline pH\(_i\) compared to normal cells (22). Although the production of lactate by cancer cells is increased, the pH\(_i\) in cancer cells is more alkaline because of increased expression of proton pumps on cellular membranes such as the vacuolar ATPase, monocarboxylate transporters MCTs, and Na\(^+\)/H\(^+\) exchangers (22,23).
Increased expression of lactate and H\(^+\) extruders stimulates glycolysis, which is an important energy source for proliferation and increases cancer cell survival. Also, increased efflux of H\(^+\) and lactate results in increase acidification of the extracellular microenvironment producing a lower pH\(_e\) (~6.7–7.1) (24,28-32). Decreased pH\(_e\) provides selective advantages to cancer cells such as increased proliferation and survival of cancer cells because the surrounding normal cells cannot survive in the low pH extracellular space. Resultant degradation of normal cells provides a significant advantage for evasion and tumor growth (22). Also, the alkaline pH\(_i\) in cancer cells facilitates increased cell proliferation and evasion of apoptosis (22,33) and can decrease the efficacy of chemotherapy and immunotherapy (34). Cancer cells produce significantly more lactate compared to normal cells (16).

Inhibition of pyruvate and lactate transporters (MCT) by drugs such as lonidamine and quercetin impedes oxidative phosphorylation in cancer leading to energy depletion (42,43). Since the metabolism of cancer cells is modulated by increased pH\(_i\) and decreased pH\(_e\), the measurement of pH\(_i\) and pH\(_e\) may improve the reliability of the diagnosis and staging of disease advancement in tumors. Many anti-cancer agents have intracellular and extracellular pH-dependent activity and pharmacokinetics. Also, the measurement of pH change has been used to predict tumor response to chemotherapy and radiation therapy (8,25,44). Therefore pH measurement could help to increase the efficacy of chemotherapy and improve cancer treatment plans (24,45).

Chemotherapy agents preferentially cross the cell membrane when passing through the acidic extracellular environment in cancer tissue. Upon entrance to the basic intracellular space of cancer cells, the weakly acidic agents are trapped by ionization. Previous experimental results support the use of weakly acidic chemotherapy agents when the pH balance is altered in
cancer (24,45). In addition, tumor intracellular acidification could decrease resistance of the tumor to weakly basic drugs such as Doxorubicin and trigger apoptosis in cancer cells (46-49).

1.3 General Overview of CEST contrast

CEST represents a relatively new class of MRI contrast that developed from the field of NMR spectroscopy (50). Consider two separate pools of $^1$H nuclei in the system being measured inside the static magnetic field ($B_0$) of the MRI scanner. One pool is the bulk water resonating at the Larmor frequency, the second pool is a smaller solute pool resonating at a different frequency. A low-power radiofrequency pulse is applied at the specific MR frequency of the solute (S) proton pool. Over time, this radiofrequency pulse will equalize the number of nuclei in the high energy and low energy states. This condition is known as saturation of solute proton pool. The protons are the solute pool undergo chemical exchange with protons in the bulk water (W) pool. There is a net transfer of high energy protons to the bulk water proton pool through the process of chemical exchange, effectively decreasing the population difference between the low energy and high energy states. This reduces the net magnetization of the water pool leading to decreased MR image intensity when detecting the bulk water signal. This chemical exchange process amplifies the detected signal change caused by the low concentration solute. Using standard MRI acquisition methods, the images can be formed from the bulk water magnetization.

To create a CEST spectrum, a radiofrequency saturation pulse is repeatedly applied over a range of frequencies (51) (Figure 1.2). The amplitude of the bulk water signal following application of the saturation pulse is plotted as a function of the saturation frequency (Figure 1.2). The CEST spectrum is usually referenced to the frequency of bulk water at 0 ppm. Direct
saturation of water at 0 ppm causes a large decrease in the bulk water signal observed in the CEST spectrum at this frequency. Importantly, the CEST effect produced by the exchange of solute pool protons with bulk water protons causes a decrease in the bulk water signal measured at saturation frequencies other than 0 ppm. The change in the CEST signal at these specific frequencies is used as the measurement of the CEST effect. There are several parameters can be used to characterize the CEST effect such as the magnitude of the change in water saturation amplitude and the CEST peak width (52-55). The CEST spectrum corresponding to normal mouse brain shows clear CEST peaks at 2.0 ppm and 3.5 ppm (Figure 1.3).

**Figure 1.2** Basic CEST acquisition scheme (top) shows the low amplitude saturation pulse (4s) followed by image acquisition. The acquisition is repeated for different saturation frequencies. A series of images are produced (middle) representing the object at each saturation frequency. A CEST spectrum is then produced (bottom) for each pixel in the image.
1.4 Chemical Exchange Saturation Transfer

To describe the production of CEST contrast, consider the Larmor frequency of protons \( \omega_0 \) in a main magnetic field \( B_0 \) given by Equation 1.4 where \( \omega_0 \) is in rad s\(^{-1} \), \( B_0 \) is in Tesla and \( \gamma \) is the gyromagnetic ratio in radians per second Tesla (\( 10^6 \) rad s\(^{-1} \) T\(^{-1} \)).

\[
\omega_0 = \gamma B_0 \tag{1.4}
\]

The Larmor frequency of the bulk water protons in our two pool system is set as the reference frequency \( \omega_0 \) for the CEST experiment. The frequency offset or chemical shift of the solute protons (\( \delta \)) from the bulk water protons is defined in ppm according to Equation 1.5.

\[
\delta = \frac{\omega_s - \omega_0}{\omega_0} \times 10^6 \tag{1.5}
\]

In a typical CEST experiment, solute protons resonating at a frequency that is chemically shifted (\( \delta \)) from the bulk water protons are saturated using an RF pulse. Therefore, the

**Figure 1.3** Normal mouse brain: a) coronal fast spin-echo anatomical image showing a manually defined region of interest (solid white line), b) the CEST spectrum corresponding to the tissue within the ROI shows visible CEST effects at 2 ppm and 3.5 ppm.
application of an RF pulse of magnitude $B_1$ at the frequency offset of the solute protons leads to the excitation of protons in this pool from a lower energy state to higher energy state. The saturation of the solute pool is achieved using RF pulses when the number of protons at each energy level is equal. The process of chemical exchange occurs when a nucleus freely transfers between two different pools that have environments that are chemically different. For CEST applications and experiments, the most common pathways of chemical exchange include molecular exchange, proton exchange, or a combination of both pathways. An example of proton exchange is when a proton an amine group (solute pool) within a specific protein moves to the bulk water pool ($\text{H}_2\text{O}$). The molecular exchange is more commonly associated with CEST contrast agents, when for example a water molecule moves from the bulk water pool to an agent bound pool. The chemical exchange must occur on the slow-to intermediate regime on NMR timescale to produce observable CEST contrast. This is defined by Equation 1.6.

$$\Delta \omega_s \geq K_{sw}$$  \hspace{1cm} (1.6)

Where $\Delta \omega_s$ is the frequency offset between the solute and bulk water protons and $K_{sw}$ represents the chemical exchange rate from the solute to the bulk water pool. The chemical shift is the frequency offset $\Delta \omega_s$ normalized by the reference frequency $\omega_0$. The chemical exchange rate $K_{sw}$ depends on pH and temperature for a specific sample at a fixed concentration of solute. During a long RF saturation pulse (e.g. saturation for several seconds), through the process of chemical exchange, protons from the solute pool will move to the bulk water pool. Since the solute pool protons are saturated, there is a net transfer of high energy protons to the bulk water pool causing an increase in a number of protons antiparallel with $B_0$ (high energy protons) in this pool (partial saturation). This reduces the net magnetization of the bulk water pool leading to a reduction in the recorded signal. During the saturation pulse, as chemical exchange decreases
the net magnetization of the bulk water pool \( (M_{zw}) \), the longitudinal relaxation of bulk water protons restores the net magnetization. This competing process limits the achievable saturation of the bulk water pool and over the duration of the saturation pulse leads the system to a steady state condition. The observed CEST effect is the resulting partial saturation of the bulk water proton pool due to saturation of the solute proton pool. The proton transfer ratio (PTR) \((56)\) can be used to quantify the CEST effect by measuring the reduced bulk water magnetization at steady state \( M_{zw}^{ss} \) relative to the equilibrium magnetization \( M_{zw}^0 \) as shown by:

\[
PTR = 1 - \frac{M_{zw}^{ss}}{M_{zw}^0}
\]  

(1.7)

The reduction in the bulk water MRI signal caused by the saturation of the smaller solute pool and exchange the solute protons with bulk water protons is known as CEST contrast. CEST acts as an amplification mechanism due to the accumulation of bulk water saturated protons over the duration of a long RF saturation pulse.

### 1.4.1 The CEST Spectrum

To create a CEST spectrum (Figure 1.2) the MRI signal from bulk water at steady state \( M_{zw}^{ss} \) is normalized to the equilibrium magnetization \( M_{zw}^0 \) \( (M_{zw}^{ss} / M_{zw}^0) \) and plotted as function of the RF saturation pulse frequency offset (chemical shift from bulk water set to 0 ppm). The MRI signal is obtained from a series of MR images acquired after the radiofrequency pulse is applied at each different saturation frequency. A CEST peak can be visualized in the CEST spectrum as a reduction in the intensity of the MRI signal if suitable experimental parameters are applied (e.g. RF amplitude).

There are many factors that can influence CEST contrast such as: concentration of the metabolites, pH, temperature, \( B_0 \) homogeneity, \( B_1 \) homogeneity, and \( T_1 \) relaxation time. The
amide-proton transfer contrast typically used to measure intracellular pH can be obtained optimally by using a low power and long irradiation pulse. While CEST contrast from the faster exchanging amine proton can be maximized using a higher power and a shorter irradiation pulse (57,58).

### 1.4.2 Factors that Modulate CEST Contrast

One limitation of CEST imaging is the requirement of homogeneous magnetic fields (B₀ and B₁) to ensure consistent RF irradiation across the entire sample. Differences in B₀ field within the sample lead to shifts in the CEST spectrum. Fortunately, these shifts can be corrected on a pixel-by-pixel basis using a separately acquired CEST spectrum and measuring the chemical shift of the on-resonance CEST saturation of water. This water saturation shift referencing (WASSR) approach has been used extensively (59), to account for B₀ variation. Variations in RF transmit B₁ homogeneity are more difficult to correct and require careful calibration of the CEST effect with B₁ along with B₁ mapping (60,61). A better approach is to avoid the need for correction by ensuring a homogeneous B₁ field is produced within the sample of interest. B₁ mapping is required to verify that the B₁ field is homogeneous and to avoid introducing B₁ corrections of the measured CEST effect.

### 1.5 Endogenous MRI Measurement of Intracellular pH

Although pH is an important parameter within the tissue microenvironment that impacts numerous cellular processes and protein behavior, the measurement of *in vivo* intracellular pH has been limited to only two methods: ³¹P MR spectroscopy and CEST.
1.5.1 $^{31}\text{P}$

The phosphorous nucleus ($^{31}\text{P}$) is detectable by nuclear magnetic resonance (NMR) and is also 100% naturally abundant. The sensitivity of $^{31}\text{P}$ is 6.63% that of $^{1}\text{H}$ total sensitivity because the strength of a nuclear signal is proportional to $\gamma^3$, where $\gamma$ is the gyromagnetic ratio. The $^{31}\text{P}$ and the $^{1}\text{H}$ have gyromagnetic ratio 17.24 MHz/T and 42.58 MHz/T respectively. Most phosphorous containing metabolites within cells have concentrations between 0.1 mM and 10 mM. The most important NMR signals from endogenous $^{31}\text{P}$ within tissues arise from inorganic phosphate (Pi), adenosine triphosphate (ATP), and phosphocreatine (PCr). From the chemical shift difference between the Pi and PCr peaks, the intracellular pH of mammalian tissues can be measured accurately and non-invasively (28). The intracellular concentration of Pi is 2–3 mM, but the extracellular concentration of Pi is only 1.0 mM. Therefore the measured chemical shift of Pi reflects predominantly the intracellular pH of tissue. There are many cellular processes such as necrosis that can increase the extracellular inorganic phosphate. Therefore these processes can complicate intracellular pH measurement using $^{31}\text{P}$ MRS (62).

$^{31}\text{P}$ MRS has been used to study in vivo pH$_i$ for many years (28,29,32,63-68). However, because of the low sensitivity of the $^{31}\text{P}$ nucleus, to make reliable pH$_i$ measurements using $^{31}\text{P}$ MRS requires long scan times and the use of large voxels. However, the technique has been highly valuable for monitoring changes in high-energy phosphates, Pi, and pH under hypoxic and ischemic conditions (69-73). $^{31}\text{P}$ MRS has also been used to study brain tumors (74), showing that the intracellular pH is alkaline and not acidic as previously thought and that the intracellular pH of tumors is higher than that of normal tissue (75).
1.5.2 Chemical Exchange Saturation transfer (CEST)

CEST contrast has been used to measure tissue pH, based on the CEST effect observed from amide and amine protons that resonate at chemical shifts of 3.5 ppm and 2.75 ppm respectively downfield from bulk water [33]. The amide protons visible at 3.5 ppm are associated with mobile proteins and peptides. Approximately 90% of proteins and peptides originate from the intracellular compartment, and consequently, pH measured using amide and amine proton CEST effects is heavily weighted to the intracellular space and is referred to as intracellular pH (14,58,76,77). Intracellular pH measurement using amide proton transfer (APT) imaging was first developed by Zhou et al. but the method proposed had several limitations [33]. They used the asymmetric magnetization transfer ratio (MTR$_{\text{asym}}$) to reduce the effect of macromolecule magnetization transfer (MT) effects from the CEST contrast using Equation 1.8.

\[
\text{MTR}_{\text{asym}}(\Delta\omega) = \frac{M_z(-\Delta\omega) - M_z(\Delta\omega)}{M_z(-\Delta\omega)}
\]

where $M_z(\Delta\omega)$ represents the bulk water magnetization after saturation at the frequency offset $\Delta\omega$ and $M_z(-\Delta\omega)$ represents the bulk water magnetization after saturation at the frequency offset $-\Delta\omega$. Quantitative pH measurement using APT imaging is susceptible to error because the amide proton transfer ratio (APTR) is also affected by cellular water content, longitudinal relaxation, and amide proton concentration. Also, nuclear Overhauser effects (NOEs), which are observed between -2.0 and -4.0 ppm, can affect the MTR$_{\text{asym}}$ measurement as NOEs are directly opposite the amide protons at 3.5 ppm. Several other CEST measurements are also sensitive to pH. For example, amine protons associated with glutamate (58,78,79) also have exchange rates sensitive to intracellular pH (12,80). Similarly, the hydroxyl protons associated with glucoCEST measurements have exchange rates that are pH dependent (18,81-84). However, these hydroxyl
protons and the amine protons associated with glutamate are also affected by changes in longitudinal relaxation and the concentration of protons limiting their use for measuring absolute pH in vivo.

Another CEST based method to measure intracellular pH was developed by our group called amine and amide concentration-independent detection (AACID). This technique uses the ratio of the CEST effects from the 3.5 ppm amide protons and the 2.75 ppm amine protons to produce pH$_i$ dependent contrast. Using $B_0$-corrected (166) and smoothed CEST spectra, AACID values were measured on a voxel-by-voxel basis. AACID represents the ratio of the CEST effects of the amine protons resonating at 2.75 ppm and the amide protons at 3.50 ppm, normalized by magnetization transfer (MT) effects measured after saturation at 6.0 ppm. AACID was calculated using equation (1.9) (60).

$$AACID = \frac{M_{Z3.5 \text{ ppm}} \times (M_{Z6.0 \text{ ppm}} - M_{Z2.75 \text{ ppm}})}{M_{Z2.75 \text{ ppm}} \times (M_{Z6.0 \text{ ppm}} - M_{Z3.5 \text{ ppm}})}$$ (1.9)

This approach was shown to be independent of tissue macromolecule concentration, tissue temperature, and bulk water $T_1$ relaxation (60). Importantly, the $B_1$ amplitude of the saturation pulse is set at an intermediate level to provide sensitivity to both amine and amide protons. McVicar et al. (85) used AACID-CEST MRI to evaluate the sensitivity of pH$_i$ for the detection of changes in tumor pH$_i$ induced by a single dose of lonidamine (LND) the monocarboxylate transport inhibitor. Similarly, Marathe et al. (86) demonstrated that a single dose of topiramate, a carbonic anhydrase inhibitor, could induce intracellular acidification in GBM tumors. The focus of this thesis is to identify and evaluate drugs that could inhibit other pH$_i$-regulators and
glycolytic enzymes that modulate pH, since pH modulation by a single agent will likely be compensated by other mechanisms over time.

1.6 Endogenous MRI Measurement of Intracellular pH in Cancer

The measurements of intracellular pH changes have been used to evaluate tumor response to treatments such as chemotherapy and radiation therapy (25,44,87). An initial study using $^{31}$P measurement of intracellular pH in brain tumors (74) showed that the intracellular pH of the tumor was alkaline, not acidic. In a different study, it was also found that the intracellular pH of untreated tumors was higher than the intracellular pH of normal tissue (75). Ben-Yoseph et al. also used $^{31}$P MRS to measure tumor pH after drug treatment and found a decrease of 0.45 pH units after injection of 100 mg/kg lonidamine (87). Unfortunately, as described earlier, there are many limitations associated with the use of $^{31}$P to measure pH. Therefore, work in our laboratory has focused on the use of a CEST technique called AACID to quantify the rapid and measurable in vivo tumor acidification after drug injection (85,86,88,89).

1.7 Drugs to modulate pH in cancer:

Cancer cells consume a large amount of glucose, and convert majority of glucose to pyruvate even in the presence of oxygen, a phenomenon known as “aerobic glycolysis” or the “Warburg Effect” (90). Although the Warburg effect leads to higher metabolic acid production, cancer cells also adaptively increase the expression of regulators that extrude lactate and protons that would otherwise accumulate producing a relatively alkaline pH, and consequently increased pyruvate production in the cytoplasm compared to normal cells (91). A hallmark feature of solid
tumors is an alkaline intracellular pH (pH$_i$) and an acidic extracellular pH (pH$_e$) (22,27).

Extracellular acidosis is a hallmark feature of cancer and is an important factor that drives malignant progression and increases metastatic potential and treatment resistance (16,92-97).

The regulation of pH in the intra- and extra-cellular compartments of the tumor is a complex integration of many processes. These pH regulators include Na$^+$/H$^+$ exchangers, monocarboxylate transporters, the vacuolar ATPase, carbonic anhydrase, anion exchangers, the Cl/HCO$_3^-$ exchangers, Na$^+$/HCO$_3^-$ co-transporters, and ATP synthase (25,26). Maintaining an alkaline intracellular pH benefits the cancer cell making it less likely to initiate apoptosis and enhancing cell proliferation (22,33). An alkaline intracellular pH can also increase the resistance of cancer to chemotherapy and immunotherapy (34,40,98-100). Inhibition of pyruvate and lactate transporters (MCT) with drugs such as lonidamine and quercetin impedes oxidative phosphorylation in cancer leading to energy depletion (42,43,101). Since cancer cells produce significantly more lactate compared to normal cells (16,94,102,103), the basic pH of the intracellular compartment is maintained by moving lactate and other acid metabolites to the extracellular space. This causes acidification of the extracellular microenvironment and effectively reverses the intracellular / extracellular pH gradient compared to healthy cells (104-109).

There are many drugs that are capable of interfering with pH regulation in cancer cells. As mentioned above, using $^{31}$P MRS Ben-Yoseph et al. showed that injection of lonidamine at a dose of 50 mg/kg decreased tumor pH$_i$ by 0.25 while a dose of 100 mg/kg decreased tumor pH$_i$ by 0.45 pH units (87). Using AACID-CEST MRI our group also found that a single dose of lonidamine (100 mg/kg) decreased tumor pH$_i$ by 0.45 (85), and that a single dose of topiramate (120 mg/kg) decreased pH by 0.17 (86). The objective of this thesis was to explore the
intracellular acidification efficacy of other drugs and drug combinations. It is important to identify additional drugs that could inhibit other regulators of pH\textsubscript{i} to modulate pH\textsubscript{i} since the effect of a single pharmacologic agent that modulates intracellular pH will likely be compensated by other mechanisms over time. In the following paragraphs, a number of different potential drugs are described that target the most common pH regulatory mechanisms in cancer cells. In all cases, the drugs described are approved for use in humans.

\(\text{Na}^+/\text{H}^+\) exchange inhibitors (e.g. NHE1) play a significant role in maintaining the acidic pH\textsubscript{e} and alkaline pH\textsubscript{i} in malignant tumors, and regulating cell volume (110). Amiloride and cariporide are \(\text{Na}^+/\text{H}^+\) exchange inhibitors. They have been used to decrease intracellular pH in glioblastoma cells in vitro. Amiloride was able to reduce intracellular pH in U87 GBM cells from 7.37 to 7.05 within 50 min (111). Also, cariporide decreased pH\textsubscript{i} by 0.3-0.5 pH units within 2 hours in glioblastoma cells when measured by BCECF, AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester) in vitro (111).

Quercetin is a natural plant product that has been used as an effective chemotherapeutic agent to treat many cancer types such as breast cancer and brain cancer. Quercetin can be given orally and is safe over a wide range of doses (94,112,113). Quercetin is an inhibitor of monocarboxylate transporters (MCTs) (94,114-117), and has also been characterized as an MCT1 and MCT2 specific inhibitor (114). Quercetin produces intracellular acidification in tumors by inhibiting lactate transport (94,114-117). In addition, quercetin enhances the efficiency of chemotherapy, suppresses human glioblastoma cell survival by increasing caspase-3 activation, and induces cell apoptosis in glioblastoma and many other types of cancer cells in vitro (118-124). Quercetin is more potent and effective in an acidic tumor microenvironment (125-127).
Acetazolamide induces intracellular acidification by inhibiting carbonic anhydrase activity and decreasing the expression of the aquaporin-1 (AQP1) (128-136) water channel. Acetazolamide has also been shown to inhibit angiogenesis and endothelial cell proliferation in lung and breast tumor in animal models and human glioblastoma cells (137-140).

Pantoprazole (PPZ) is a proton pump inhibitor (PPI) and induces cancer cell death by inhibiting the V-ATPase. PPIs are considered to have anti-tumor effects due to their ability to induce tumor cell death by reversing H+ homeostasis in the tumor microenvironment by inhibiting the V-ATPase. Pantoprazole has also been shown to be a cancer agent and is effective against many types of cancer cells (141). PPZ can be given alone or concurrent with chemotherapy such as doxorubicin. After PPZ treatment, apoptotic cell death was observed selectively in cancer cells (142,143). Several PPIs (omeprazole, esomeprazole, and pantoprazole) raise lysosomal pH and decrease intracellular pH, a feature associated with their ability to sensitize cancer cells to chemotherapeutics (105). Agents that alter tumor pH homeostasis can decrease drug resistance and inhibit tumor growth (99,144-153).

Dichloroacetate (DCA) is an anti cancer agent. DCA can decrease the expression of monocarboxylate transporters and V-ATPases. It was used as a cancer treatment in two human hepatoma (HCC-LM3 and SMMC-7721) cell lines (154). In addition, DCA has been shown to induce cell death in cancer cell lines including from breast, prostate, lung, medullary thyroid, and endometrial cancers, as well as myeloma and glioblastoma multiforme (47,154,155).
Thesis Overview:

Cancer cells have evolved a high dependence on glycolysis due to the Warburg effect. The overall goal of this thesis is to determine whether different drugs that block various pH regulatory mechanisms can acidify brain tumors and to measure the magnitude of tumor acidification using CEST MRI. In this thesis, specific drugs are used to induce intracellular acidification (lowering $pH_i$) in brain tumors in an attempt to induce a measurable physiologic response. The response of the tumor to each drug could be used to stage the cancer, define the boundary of the tumor, or predict treatment response. The effect of the drug on tumor pH is measured by CEST MRI \textit{in vivo} using a mouse model of brain cancer. Chapter 2 describes the results obtained when using the drug dichloroacetate (DCA), which is approved for human use, to induce intracellular acidification in glioblastoma. This study is the first to show decreased intracellular pH after DCA injection. In chapter 3, the drug cariporide is used to decrease

Figure 1.4 pH control systems in cancer and normal cells. Intracellular alkalisation is a key factor in cancer cells for cells transformation. pH-gradient is reversed in cancer cells compared to the normal cells. $Na^+/H^+$, MCT (monocarboxylate transporter), CA (carbonic anhydrase) and V-ATPase (vacuolar-ATPase) are the most important transporters and the exchangers that cause intracellular alkalisation of the cancer cells.

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>$pH_i$</th>
<th>$pH_e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>$\geq 7.4$</td>
<td>$\sim 7.4$</td>
</tr>
<tr>
<td>Cancer</td>
<td>$\geq 7.4$</td>
<td>$\sim 6.7-7.1$</td>
</tr>
</tbody>
</table>
intracellular pH in a mouse model of glioblastoma. Cariporide is a Na\(^+/\)H\(^+\) exchange inhibitor. Chapter 4, we tested the hypothesis that combined treatment with five drugs that block different pH regulatory mechanisms have a greater effect on tumor acidification than any single drug. We also tested the hypothesis that providing glucose as an energy substrate to the tumor would increase the intracellular acidification. The final chapter of the thesis, summarizes all results obtained and provides a framework to translate this work into humans and to continue further testing of tumor acidification as a treatment strategy, optimized using CEST MRI.
Chapter 2

2 Dichloroacetate Induced Intracellular Acidification in Glioblastoma: In Vivo Detection Using AACID-CEST MRI at 9.4 Tesla

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Abstract:

Intracellular pH (pH$_i$) plays an important role in the maintenance of normal cell function, and is maintained within a narrow range by the activity of transporters located at the plasma membrane. Modulation of tumor pH$_i$ may influence proliferation, apoptosis, chemotherapy resistance, and thermostensitivity. Chemical exchange saturation transfer (CEST) is a novel MRI contrast mechanism that is dependent on cellular pH. Amine and amide concentration-independent detection (AACID) is a recently developed CEST contrast method that is intracellular pH (pH$_i$) weighted. Dichloroacetate (DCA) can alter tumor pH$_i$ by inhibiting the enzyme pyruvate dehydrogenase kinase causing reduced lactate (increasing pH$_i$), or by decreasing the expression of monocarboxylate transporters and vacuolar ATPase leading to reduced pH$_i$. Since the net in vivo effect of DCA on pH$_i$ is difficult to predict, the purpose of this study was to quantify the magnitude of acute pH$_i$ change in glioblastoma after a single DCA injection using AACID CEST MRI. Using a 9.4T MRI scanner, CEST spectra were acquired in six mice approximately 14 days after implanting $10^5$ U87 human glioblastoma multiforme (GBM) cells in the brain, before and after intravenous injection of DCA (dose: 200 mg/kg). Three additional mice received only
phosphate buffered saline (PBS) injection and were studied as controls. Repeated measures t-test was used to compare AACID changes in tumor and contralateral tissue regions of interest. One hour after DCA injection there was a significant increase in tumor AACID level by 0.04±0.01 corresponding to a 0.16 decrease in pH, and no change in AACID in contralateral tissue. Inspection of AACID maps following PBS injection showed no differences. The use of DCA to induce a tumor specific pH change detectable by AACID CEST MRI is consistent with previous studies that have shown similar effects for lonidamine and topiramate. This study demonstrates that a single dose of DCA can be used as a pharmacological challenge to induced rapid tumor intracellular acidification.

2.1 Introduction

Gliomas are the most common primary brain tumors of the central nervous system (CNS). Glioblastoma multiforme (GBM) is one of the most aggressive gliomas and deadliest brain tumors, with less than half of patients surviving beyond 12-18 months even with optimal treatment (6-8,156). These tumors represent the second leading cause of death among neurological diseases in the United States and present one of the greatest challenges in the treatment of cancer in the world (6-9). The current standard of care of GBM tumors includes surgical resection of the primary tumor followed by aggressive concurrent radiation and chemotherapy (157). However, over 90% of GBM tumors recur due to migratory cancer cells that avoid treatment (9).

In normal cells, the extracellular pH (pHe) is ~7.4, which is higher than the intracellular pH (pHi) of ~ 7.0-7.1 (24,28-32). A emerging hallmark feature of malignant solid tumors is deregulation of energy metabolism (90) producing an alkaline pHi and acidic pHe; the
intracellular/extracellular pH gradient in cancer cells is reversed such that the pH_e is ~6.7–7.1 and pH_i is ~ 7.1-7.3 (24,28-32). This pH gradient reversal is primarily due to the use of aerobic glycolysis by cancer cells for energy production even in the presence of oxygen, known as the Warburg effect (16). Although the Warburg effect leads to higher metabolic acid production, cancer cells also adaptively increase the expression of regulators that extrude lactate and protons producing a relatively alkaline pH_i (91). These regulators include Na^+\text/HCO}_3^- co-transporters, Na^+\text/H^+ exchangers, monocarboxylate transporters, the vacuolar ATPase, and carbonic anhydrases (25,26). Increased pH_i in cancer promotes proliferation and evasion of apoptosis (22). Conversely, decreasing pH_i in cell culture models has been shown to enhance the effectiveness of hyperthermia, radiation, and chemotherapy treatments in a variety of cancer cell lines (26,33,48,49,158).

However, the response of tumor cell cultures does not necessarily predict the behavior of solid tumors in vivo. Until recently, the study of in vivo intracellular pH was performed by phosphorus (^{31}\text P) magnetic resonance spectroscopy (63). However, ^{31}\text P MRS measurements are limited by low sensitivity, requiring the use of large voxels and/or long scan times to make reliable pH_i measurements. More recently, the use of endogenous chemical exchange saturation transfer (CEST) magnetic resonance imaging (MRI) has been shown to be sensitive to intracellular pH (58,60). One specific CEST based method called amine and amide concentration-independent detection (AACID) uses the ratio of CEST effects from the 3.5 ppm amide protons and the 2.75 ppm amine protons to produce pH_i dependent contrast that is independent of tissue macromolecule concentration, tissue temperature, and bulk water T_1 relaxation (60). The CEST contrast originates from exchangeable amine and amide protons that
are found in tissue proteins and peptides (14,58,77,159). However the AACID CEST measurement of tissue pH is highly weighted to the intracellular compartment (14) because almost 90% of total protein content exists in the intracellular space (77). McVicar et. al. (85), used AACID-CEST MRI to evaluate the sensitivity of pH$_i$-dependent CEST contrast for the detection of changes in tumor pH$_i$ induced by a single dose of the monocarboxylate transport inhibitor lonidamine (LND). Similarly, Marathe et. al. (86) demonstrated GBM specific intracellular acidification following a single dose of the carbonic anhydrase inhibitor topiramate.

Dichloroacetate (DCA) is a small molecule that is orally available for use in humans (160,161), has low toxicity compared to other agents (155), and has been shown to induce cell death in several different types of cancer (160,161). Preclinical work using DCA shows effectiveness as a cancer treatment to induce cell death in different types of tumors. For example, it was used as a cancer treatment in two human hepatoma (HCC-LM3 and SMMC-7721) cell lines (154). In addition, DCA induces cell death in cancer cell lines from breast, prostate, lung, medullary thyroid, and endometrial cancers, as well as myeloma and glioblastoma multiforme (47,154,155). However there are no ongoing clinical trials involving the use of DCA to treat glioblastoma (clinicaltrials.gov). The effect of DCA on intracellular pH is difficult to predict. DCA inhibits pyruvate dehydrogenase kinases (PDKs), which reverses the Warburg effect by activating pyruvate dehydrogenase (PDH) and redirecting pyruvate back into the mitochondria (160,162). This metabolic change is expected to increase intracellular pH by reducing the production of lactate. However, cancer cells also have a higher expression of monocarboxylate transporters and V-ATPases compared to normal cells. Since DCA acts to decrease the expression of these transporters (163) it is expected to have a more pronounced effect in cancer cells compared to normal cells leading to reduced pH$_i$ in tumors. Therefore, it is
unclear whether a single dose of DCA would cause a net increase or decrease in pH$_i$. The goal of the current study was to measure the magnitude of GBM tumor intracellular pH change induced by a single dose of DCA. The change in pH$_i$ was measured by AACID CEST MRI. We hypothesize that a single dose of DCA will not alter pH$_i$ within contralateral tissue, but will cause intracellular acidification within tumor regions within one hour of treatment because the effect of DCA on MCTs and V-ATPases dominates. The combination of DCA treatment and CEST MR imaging may provide a novel method to detect GBM tumors. The targeting of tumor metabolism is also potentially a cancer treatment strategy (47,160) and could change the tumor microenvironment to enhance concurrent anti-cancer treatment.

2.2 Experimental

2.2.1 Subjects

Nine mice were included in the current study. Six NU/NU mice with U87MG brain tumors were used to evaluate the effect of injecting 200 mg/kg of DCA. Three NU/NU mice with U87MG brain tumors were used as control. Control mice received intravenous phosphate buffered saline (PBS) injection only. All animal procedures were performed according to a protocol that was consistent with guidelines established by the Canadian Council on Animal Care and was approved by the University of Western Ontario Animal Use Subcommittee.

2.2.2 Dichloroacetate

Sodium dichloroacetate (DCA) with linear formula (Cl$_2$CHCO$_2$Na) was purchased from Sigma – Aldrich (Canada). The drug was dissolved in PBS (30 mg/mL). DCA was administered
intravenously through a tail vein catheter to achieve a dose of 200 mg/kg over the course of 1 min as previously described (46,164).

2.2.3 Animal tumor preparation

GBM brain tumors were induced in 22-27g, NU/NU mice (N=6) using U87MG glioma cells established from a human GBM (ATCC; Rockville, MD, USA) as described previously (165). Briefly, U87MG cells were grown in Dulbecco’s modified Eagles’s medium supplemented with 10% fetal bovine serum (Wisent Inc., St-Jean-Baptiste, QC, Canada) at 37°C in a humidified incubator with 5% CO₂ and passaged twice a week. On the day of injection, U87MG cells were washed and dissociated with versene solution (phosphate-buffered saline (PBS) plus 0.5 mM EDTA), then washed twice with PBS, counted and re-suspended to a final concentration of 1 x 10⁵ cells in 2 μl PBS. Prior to injection, mice were anesthetized by inhalation of 4% isoflurane and maintained using 1.5% isoflurane mixed with 100% oxygen. The mouse was placed in a stereotactic head frame (Stoelting instruments, Wood Dale, IL, USA). The scalp was swabbed with betadine and an incision was made in the scalp to expose the bregma. A 1 mm diameter hole was drilled at coordinates measured from the bregma (1 mm anterior and 2 mm lateral). U87MG cells (2 μl) were injected at a rate of 0.5 μl/min, at a position 3 mm deep from the bregma into the right frontal lobe using a Hamilton (Reno, NV, USA) syringe with a 27-gauge needle attached.
2.2.4 General mouse preparation for in vivo imaging

Approximately 15 ±1 days after cancer cell injection, the mice were scanned on a 9.4 T small animal MRI equipped with a 30 mm millipede volume coil (Agilent, Santa Clara, CA, USA). Anesthesia was induced using 4% isoflurane in oxygen and maintained with 1.5%–2.5% isoflurane in 100% oxygen. The mouse was secured on a custom-built MRI-compatible stage and the head was secured using a bite bar (86) and surgical tape to limit motion due to respiration. Temperature was monitored with a rectal temperature probe. Body temperature was maintained at 36.9-37.1°C throughout imaging by blowing warm air over the animal using a model 1025 small-animal monitoring and gating system (SA Instruments Inc., Stony Brook, NY, USA). To ensure animals were free breathing throughout the study, respiration was also monitored with a respiratory sensor pad connected to a pressure transducer that was placed on the thoracic region. Following pre-injection imaging, the mouse was injected with DCA inside the MRI by delivering the drug through a fine plastic tube ended with a needle inserted into the animal tail vein. Animals were sacrificed immediately after MR imaging.

2.2.5 In vivo Magnetic Resonance Imaging

Standard anatomical T2-weighted images were used for tumor detection. The T2-weighted images were acquired using a 2-dimensional coronal fast spin echo pulse sequence (FSE) with parameters: TR/TE = 3000/10 ms, ETL = 4, effective TE = 40 ms, FOV = 25.6 x 25.6 mm², matrix size = 128 x 128, slice thickness= 1 mm, acquisition time ≈ 3 minutes. Following initial tumor detection, the two slices from the T2-weighted images with the greatest tumor coverage (representing a total thickness of 2 mm) were selected and the coordinates used to position the coronal CEST image. CEST images were acquired using a fast spin-echo (FSE) pulse sequence
(TR/TE = 7000/7 ms, ETL = 32, effective TE = 7 ms, FOV = 25.6 x 25.6 mm², matrix size = 64 x 64, slice thickness = 2 mm, acquisition time ≈ 18 minutes) preceded by a continuous wave radiofrequency (RF) pulse with amplitude 1.5-µT and 4s duration. The CEST images were acquired at different saturation frequencies (from 1.2 to 4.5 (Δ=0.1) ppm, from 5.4 to 6.6 (Δ=0.1) ppm, and -1000 and 1000 ppm images were acquired as references, total 49 images). A complete series of CEST images were acquired three times before and three times after drug injection to improve signal-to-noise ratio. For B₀ correction, the water saturation shift referencing (WASSR) technique was used (166). A linearly spaced 37-point WASSR CEST spectrum (acquisition time ≈ 9 minutes) with saturation frequencies ranging from -0.6 – 0.6 ppm was acquired using the same pulse sequence except preceded by a short (100 ms) low amplitude (0.2 µT) RF saturation pulse. Each WASSR spectrum (166) and CEST spectrum was interpolated to achieve 1-Hz resolution. Each CEST spectrum was then frequency shifted, using the corresponding WASSR spectrum (166), to account for B₀ variation. B₀ variations were corrected on a pixel-by-pixel basis. The three pre- and three post-injection CEST spectra were summed following B₀ corrections to increase signal-to-noise ratio. A B₁ field map was generated using an actual flip-angle imaging (AFI) pulse sequence (TR = 20 ms, TE = 3.47 ms, echoes = 2, flip-angle = 70°, FOV = 25.6 x 25.6 mm², matrix size= 64 x 64) in a subset of mice (N=3) to verify that the B₁ variation was similar to that found in previous studies using the same experimental setup. The B₁ variation in the CEST slice was less than 5%, so no B₁ correction was necessary (85).

2.2.6 CEST data processing

All acquired CEST MR data were loaded into MATLAB (Mathworks, Natick, MA, USA) for analysis. Using custom MATLAB programs previously described (85,86), B₀ corrections were
performed on a pixel-by-pixel (86) basis and all CEST spectra were smoothed using the ‘smooth’ algorithm from the MATLAB curve fitting toolbox.

2.2.7 CEST and contrast calculations

Using $B_0$-corrected (166) and smoothed CEST spectra, AACID values were measured on a voxel-by-voxel basis. AACID represents the ratio of the CEST effects of the amine protons resonating at 2.75 ppm and the amide protons at 3.50 ppm, normalized by magnetization transfer (MT) effects measured after saturation at 6.0 ppm. AACID was calculated using equation (1) (60).

$$AACID = \frac{M_{Z_{3.5\text{ ppm}}} \times (M_{Z_{6.0\text{ ppm}}} - M_{Z_{2.75\text{ ppm}}})}{M_{Z_{2.75\text{ ppm}}} \times (M_{Z_{6.0\text{ ppm}}} - M_{Z_{3.5\text{ ppm}}})}$$  \hspace{1cm} (1)

The change in pH following drug administration was obtained using the calibration provided by Equation (8) in McVicar et al.

$$\Delta pH = -4 \times \Delta AACID$$ \hspace{1cm} (2)

2.2.8 Statistical Analysis

Average AACID values were calculated before and after injection of DCA as well as before and after injection of PBS, within contralateral tissue and tumor regions of interest (ROIs) defined in each mouse brain using MATLAB (‘roipoly’ function). The ROI was drawn manually around the tumor using the hyperintense tissue region observed in the $T_2$-weighted images (Figures 2.1 and
2.2) as a guide. The ROI on the contralateral side was also drawn manually to be similar in size to the ROI used for each tumor and was positioned to mirror the location of the tumor in the opposite hemisphere. A paired t-test was used to calculate differences in mean AACID values measured in the tumor and in contralateral ROIs before and after injection of DCA or PBS (control).

2.3 Results

AACID CEST maps were successfully acquired in all animals. Anatomical FSE MR images acquired 15 ±1 days after U87MG cancer cell implantation were used to identify regions of interest in the tumor and on the contralateral side (Figures 2.1c and 2.2c). AACID CEST maps showed the expected decrease in AACID value in the tumor region compared to the surrounding tissue (Figures 2.1a and 2.2a) indicating a relatively basic intracellular tumor pH. Following injection of PBS only, there was little detectable change in the AACID map (Figures 2.1b and 2.1d). In contrast, an increase in tumor AACID value is clearly visible within the tumor (Figure 2.2b) indicating intracellular acidification within 60 minutes of DCA injection (Figure 2.2d). Pooling all data following PBS injection, no change in AACID value was observed in the tumor or within contralateral tissue (Figure 2.3a). However, one hour after DCA injection there was an average increase in AACID value of 0.04±0.01 (N=6, p=0.02) in the tumor region (Figure 2.1d, 2.3b), but no change in AACID value within contralateral tissue (Figure 2.3b). The measured change in AACID value within the tumor corresponds a 0.16 decrease in intracellular pH.
Fig 2.1 Mouse brain with GBM tumor 14 days after implantation: a) baseline AACID map prior to PBS intravenous injection, b) the AACID map one hour post PBS intravenous injection c) coronal fast spin-echo anatomical image shows regions of interest (ROIs) in the tumor (dashed white line) and on the contralateral side (solid white line) and d) AACID difference maps for the same brain post-pre PBS injection.

Fig 2.2 Mouse brain with GBM tumor 14 days after implantation: a) baseline AACID map prior to DCA intravenous injection, b) the AACID map one hour post DCA intravenous injection c) coronal fast spin-echo anatomical image shows regions of interest (ROIs) in the tumor (dashed white line) and on the contralateral side (solid white line) and d) AACID difference maps for the same brain post-pre DCA (200 mg/kg) intravenous injection.
Fig 2.3 Average AACID values in tumor and contralateral ROIs: a) pre and post intravenous PBS injection as control (N=3). b) pre and post DCA 200 mg/kg intravenous injection (N=6). Error bars represent the standard error of the mean. The asterisks indicated $p<0.05$ in repeated measures t-test.

Tables

Table 2.1: AACID Values Following DCA Injection:

<table>
<thead>
<tr>
<th>Mouse No.</th>
<th>AACID Pre Contralateral</th>
<th>STD Pre</th>
<th>AACID Post Contralateral</th>
<th>STD Post</th>
<th>AACID Pre Tumor</th>
<th>STD Pre</th>
<th>AACID Post Tumor</th>
<th>STD Post</th>
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<td>1.229</td>
<td>0.029</td>
<td>1.242</td>
<td>0.013</td>
<td>1.079</td>
<td>0.018</td>
<td>1.096</td>
<td>0.020</td>
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<td>2</td>
<td>1.327</td>
<td>0.010</td>
<td>1.334</td>
<td>0.013</td>
<td>1.205</td>
<td>0.011</td>
<td>1.246</td>
<td>0.010</td>
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<td>0.013</td>
<td>1.266</td>
<td>0.011</td>
</tr>
<tr>
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<td>1.320</td>
<td>0.011</td>
<td>1.157</td>
<td>0.008</td>
<td>1.205</td>
<td>0.006</td>
</tr>
<tr>
<td>5</td>
<td>1.266</td>
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<td>1.218</td>
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<td>1.212</td>
<td>0.015</td>
<td>1.195</td>
<td>0.018</td>
</tr>
<tr>
<td>6</td>
<td>1.273</td>
<td>0.017</td>
<td>1.263</td>
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<td>1.127</td>
<td>0.013</td>
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<td>0.012</td>
<td>1.163</td>
<td>0.013</td>
<td>1.203</td>
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</table>
Table 2.2: AACID Values Following PBS Injection:

<table>
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<th>Mouse</th>
<th>AACID Pre Contralateral</th>
<th>STD Pre</th>
<th>AACID Post Contralateral</th>
<th>STD Post</th>
<th>AACID Pre Tumor</th>
<th>STD Pre</th>
<th>AACID Post Tumor</th>
<th>STD Post</th>
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<tbody>
<tr>
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<td>0.006</td>
<td>1.246</td>
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<td>0.015</td>
<td>1.181</td>
<td>0.012</td>
</tr>
<tr>
<td>Average</td>
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<td>0.011</td>
<td>1.176</td>
<td>0.014</td>
<td>1.175</td>
<td>0.011</td>
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</table>

2.4 Discussion

This study demonstrates a tumor selective increase in the AACID CEST MRI signal suggesting intracellular acidification in a GBM mouse brain tumor model during the first hour after intravenous injection of a single dose of DCA (200 mg/kg). The AACID CEST MRI signal change was estimated to be the result of a ~0.16 pH decrease based on previous calibrations. In contrast, there was no change in the AACID CEST MRI signal on the contralateral side after DCA injection, or within tumors or contralateral tissue following PBS injection. DCA is approved for human consumption, is generally well tolerated (160), and has been tested as treatment in a variety of cancers including glioblastoma (167).

DCA is an anticancer agent that alters metabolism and pH (161,168). Previous studies have suggested that intracellular pH may increase in cancer cells following DCA treatment as a direct consequence of the downregulation of glycolysis by DCA (161,168). Specifically, an increase in intracellular pH is possible because DCA is expected to increase glucose oxidation and therefore decrease the production of lactic acid within the tumor (161,168). However, the
current *in vivo* study contradicts these previous in-vitro studies. One potential explanation for these unexpected results is that DCA has also been shown to decrease the expression of monocarboxylate transporters and V-ATPases in tumor cells but not in normal cells (163). Cancer cells also have a higher expression of monocarboxylate transporters and V-ATPases compared to normal cells. Since DCA acts to decrease the expression of these transporters it is expected to have a more pronounced effect in cancer cells compared to normal cells.

The contribution of amide, amine, and aliphatic signals to different tissue types and cancers is an area of active research (169). The use of CEST MRI contrast to monitor intracellular pH has many potential applications in cancer detection and treatment optimization including the differentiation of recurrent glioma from radiation necrosis (14). The AACID CEST measurement of tissue pH used in the current study has been shown to be independent of protein concentration, tissue $T_1$ relaxation, and temperature (60). Although the absolute value of intracellular pH measurement in tumors with AACID remains to be verified, in the current study, AACID was used to measure acute pH change following pharmacologic challenge. The AACID CEST measurement of tissue pH used in the current study incorporates contributions from the extracellular and intracellular compartments but is predominantly weighted to the intracellular space due to the distribution of amine and amide protons. The paradigm of using a drug designed to manipulate intracellular pH monitored by AACID CEST MRI is akin to pharmacologic MRI using the blood oxygen level dependent (BOLD) contrast (170). Such methods have several potential advantages over conventional gadolinium based contrast agents. First, the contrast produced is based not simply on accumulation of a contrast agent, but rather is related directly to the physiology of the tissue, or tumor. Second, use of different drugs approved in humans provides an opportunity to test the effect of blocking different biological
mechanisms of pH control. Different cancers may respond differently to each drug. Finally, gadolinium-based contrast agents have the potential for side effects that are different from those produced by drugs like DCA and must be used with caution in patients with kidney disease. Gadolinium is also expensive compared to drugs such as DCA. The physiological information obtained from measurement of intracellular pH changes may also provide information regarding the response of a tumor to specific a treatment. This idea is supported by Sagiyama et. al. (8) who found that amide proton CEST could detect temozolomide treatment effects one week after treatment.

In previous studies using the same methodology to evaluate the magnitude of tumor acidification we found that lonidamine, a lactate transport inhibitor, decreased intracellular tumor pH by 0.25 (dose: 50 mg/kg), and by 0.45 (dose: 100 mg/kg) (85). In addition, topiramate, a carbonic anhydrase inhibitor, decreased intracellular pH by 0.17 (86) at a dose of 120 mg/kg. Topiramate is approved for use in humans as an anti-seizure medication, but lonidamine is not currently approved in humans. At the dose used in the current study (200 mg/kg), DCA was not as effective as lonidamine but was similar to topiramate with regard to tumor acidification. The DCA dose (200 mg/kg) studied is commonly used in the literature for animal studies (46,47). Pharmacologic agents that maximize cancer cell acidification would provide the greatest image contrast for pharmacological AACID CEST MRI. Modulation of tumor metabolism and acidity also represent an interesting and emerging target for anti-cancer treatment, either alone or in combination with other existing treatments. Drugs that interfere with tumor metabolism could help enhance chemotherapy and radiation therapy. In addition, tumor intracellular acidification could decrease resistance of the tumor to weakly basic drugs such as Doxorubicin and trigger
apoptosis in cancer cells (46-49). Acidification of the intracellular environment by regulation of the intracellular pH is known to induce apoptosis in cancer cells even in a neutral extracellular pH (pH_e) (48). An acidic pH_i may also be responsible for the activation of caspases in cancer cells and induce DNA fragmentation (49). An acidic intracellular tumor environment may significantly disturb tumor cell proliferation and growth (49).

The current study has several limitations that should be considered. First, the number of animals used was small. However, the effect sizes are large and the ROI based pH measurements have low variability. In addition, the test-retest design eliminates inter-subject variation. Therefore the animal numbers used were sufficient to determine whether DCA produced a measurable pH effect. Second, the AACID CEST MRI measurement of pH is based on changes in amine and amide proton exchange rate. The decrease in intracellular pH detected in the current study using the AACID CEST method could be verified in a future study using 31P magnetic resonance spectroscopy. Third, we did not optimize the dose of DCA used in the current study. Previous studies have used doses ranging from 25-200 mg/kg (47). The high dose chosen in the current study was attempted to increase the likelihood that we would observe an effect based on previous work (46,164). Future studies could consider higher doses of DCA since limited side effects are expected after a single dose. Finally, DCA has multiple mechanisms of action that could alter tissue pH. In the current study, we did not examine the effect of chronic administration of DCA on tumor pH. Future studies should examine whether repeat exposures would lead to decreased or increased tumor pH_i.

In summary, we found that 200 mg/kg of DCA could induce acute (within 1h) tumor AACID CEST MRI signal change likely resulting from intracellular acidification (corresponding
to a pH decreases of 0.16) in a mouse model of glioblastoma. Since DCA is safe for use in humans, further evaluation of AACID CEST MRI in glioblastoma patients could incorporate the use of this drug to induce a measurable signal change.
Chapter 3

3 In vivo Detection of Acute Intracellular Acidification in Glioblastoma Multiforme Following a Single Dose of Cariporide

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Abstract:

Glioblastoma is an aggressive brain cancer that is very difficult to treat. Clinically, it is important to be able to distinguish aggressive from non-aggressive brain tumors. Previous studies have shown that some drugs can induce a rapid change in intracellular pH that could help to identify aggressive cancer. The sodium proton exchanger (NHE1) plays a significant role in maintaining pH balance in the tumor microenvironment. Cariporide is a sodium proton exchange inhibitor. We hypothesized that cariporide could selectively acidify brain tumors. The purpose of this study was to determine whether amine/amide concentration independent detection (AACID) chemical exchange saturation transfer (CEST) MRI measurement of tumor pH\textsubscript{i} could detect acidification after cariporide injection. Using a 9.4T MRI scanner, CEST spectra were acquired in six mice approximately 14 days after implanting $10^5$ U87 human glioblastoma multiforme cells in the brain, before and after administration of cariporide (dose: 6 mg/kg) by intraperitoneal injection. Three additional mice were studied as controls and received only vehicle injection (DMSO+PBS). Repeated measures t-test was used to examine changes in tumor and contralateral tissue regions of interest. Two hours after cariporide injection there was a significant 0.12±0.03 increase in tumor AACID value corresponding to a 0.48 decrease in pH\textsubscript{i}, and no change in
AACID value in contralateral tissue. A small but significant increase of 0.04±0.017 in tumor AACID value was also observed following vehicle injection. This study demonstrates that acute CEST MRI contrast changes indicative of intracellular acidification after administration of cariporide could help localize glioblastoma.

3.1 Introduction

Gliomas are the most common primary brain tumors of the central nervous system (CNS). Glioblastoma multiforme (GBM) is one of the most aggressive and deadliest gliomas. The treatment for GBM tumors usually includes surgical resection of the primary tumor followed by aggressive concurrent radiation and chemotherapy (6-8,171). However, these cancer cells often migrate away from the primary tumor and avoid treatment, resulting in recurrence in more than 90% of cases (171). Although new treatment strategies continue to be tested, people with GBM typically survive only 12-18 months following diagnosis (7,8,171). Due to their aggressive nature and high rate of recurrence, glioblastomas are considered to be one of the greatest challenges in the treatment of cancer [1-4].

An emerging hallmark feature of solid tumors is the maintenance of an alkaline intracellular pH (pHᵢ) and an acidic extracellular pH (pHₑ) (22,27). In normal cells the pHₑ is higher (~7.4) than the pHᵢ of ~ 7.0-7.1 (24,28-32). However, the pH gradient in cancer cells is reversed with lower pHₑ (~6.7–7.1) than pHᵢ ~7.1-7.3 (24,28-32). This altered pH homeostasis is the consequence of increased aerobic glycolysis even in the presence of oxygen, known as the Warburg effect (16). To remove acid metabolites that would accumulate due to the Warburg effect, cancer cells increase their expression of regulators that extrude H⁺ and lactate. However
cancer cells overcompensate, producing a relatively alkaline pH. There are many potential pH regulators involved in this process including: Na+/HCO\textsubscript{3} co-transporters, Na\textsuperscript{+}/H\textsuperscript{+} exchangers, monocarboxylate transporters, the vacuolar ATPase, carbonic anhydrase, anion exchangers, the Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchangers, and ATP synthase (25,26). The resulting alkaline pH benefits the cancer by supporting increased cell proliferation and evasion of apoptosis (22,33), and can increase the resistance of the tumor to chemotherapy and immunotherapy (34). Acidification of the tumor intracellular pH has the potential to decreased cell proliferation and induce apoptosis resulting in cell death (26,158).

The purposeful modulation of tumor intracellular pH combined with measurement of the pH change has been used to predict tumor response to chemotherapy and radiation therapy (8,25,44). One method that is particularly well suited to monitoring tumor pH is chemical exchange saturation transfer (CEST) magnetic resonance imaging (MRI), which provides high-resolution pH maps in a reasonable scan time (60). Using this approach, we have previously shown that lonidamine a monocarboxylate transport inhibitor, topiramate a carbonic anhydrase inhibitor, and dichloroacetate, which decreases the expression of monocarboxylate transporters and V-ATPases, all selectively cause rapid and measurable in-vivo acidification of glioblastoma. To make these measurements, we used a CEST technique called amine and amide concentration-independent detection (AACID) that uses the ratio of CEST effects of amide (Δω = 3.5ppm) and amine (Δω = 2.75ppm) protons to generate pH dependent contrast independent of tissue macromolecule concentration and temperature (60). Using AACID-CEST MRI we previously found a single dose of lonidamine (LND) decreased tumor pH by 0.45 (85), a single dose of topiramate decreased pH by 0.17 (86), and a single dose of dichloroacetate decreased pH by 0.16 (88) approximately 1-1.5 hours after injection. Since pH modulation by a single pharmacologic
agent will likely be compensated by other mechanisms over time, it is important to identify additional drugs that could inhibit other key pH<sub>i</sub>-regulators and glycolytic enzymes to modulate pH<sub>i</sub>.

Cariporide is an anticancer agent that alters metabolism and pH<sub>i</sub> by selectively inhibiting the sodium proton (Na<sup>+</sup>/H<sup>+</sup>) exchange isoform 1 (NHE1) (172) with little effect on other ion transport systems (173). NHE1 plays a significant role in maintaining the acidic pH<sub>e</sub> and alkaline pH<sub>i</sub> in malignant tumors, and regulating cell volume (110). It is activated by growth factors and cellular proliferation processes (174). In cancer cells, cariporide effectively inhibits NHE1 and has been shown to suppress the invasion and migration of cancer cells (175). Cariporide has already been tested in vitro in U87 and C6 glioma models. Cariporide was able to decrease intracellular pH of U87 and C6 glioma cells each by 0.3 units within 120 min (111,176). Furthermore, in a study of human tongue carcinoma cells (Tca8113) under hypoxic conditions, treatment of cells with cariporide for 24h decreased pH<sub>i</sub> in a dose-dependent manner (175).

Cariporide has also been shown to decrease proliferation and migration of human umbilical vein endothelial cells in vitro (177). In addition, cariporide was shown to have anti-angiogenetic effects in vitro and in vivo (177). Specifically, cariporide decreased intracellular pH and down-regulated vascular endothelial growth factor (VEGF) secretion in K562 leukemia and inhibited K562 tumor growth reducing the density of microvessels in mice. These in vitro studies motivate the current work to examine the in vivo effectiveness of cariporide in decreasing glioblastoma pH<sub>i</sub>. An orthotopic U87MG mouse model of glioblastoma was used for the current work because this model is known to recapitulate many of the physiological features of the human disease and is ideally suited for the application of ultra high field AAGIC CEST MRI for pH measurement. The purpose of this study was to quantify the in vivo acidification of glioblastoma following
cariporide treatment by mapping tumor pH\textsubscript{i} using AACID CEST MRI. We hypothesized that blockage of the sodium proton (Na\textsuperscript{+}/H\textsuperscript{+}) exchange isoform 1 (NHE1) by cariporide would produce acute intracellular acidification detectable by AACID CEST MRI. The measurement of single-dose drug induced acidification could help predict tumor response to treatment or potentially provide information on tumor extent and aggressiveness.

3.2 Experimental

3.2.1 Subjects

 Twelve female Crl:Nu-Foxn\textsuperscript{1}\textsuperscript{Nu} (NU/NU) mice (Charles River Laboratories, Canada) adult mice were included in the current study in three groups. Six NU/NU mice with U87MG brain tumors were used to evaluate the effect of cariporide on tumor pH\textsubscript{i}, three NU/NU mice with U87MG brain tumors were injected with phosphate buffered saline (PBS) and dimethyl sulfoxide (DMSO) as controls, and three NU/NU mice without tumors were used to study the effect of cariporide on normal brain. One additional mouse with tumor was used for hematoxylin and eosin (H&E) staining. Mice were group housed in ventilated racks on a 12h/12h light/dark cycle. All animal procedures were performed according to a protocol that was consistent with guidelines established by the Canadian Council on Animal Care and was approved by the University of Western Ontario Animal Use Subcommittee.

3.2.2 Cariporide

 Cariporide is a selective Na\textsuperscript{+}/H\textsuperscript{+} exchange inhibitor with linear formula C\textsubscript{12}H\textsubscript{17}N\textsubscript{3}O\textsubscript{3}S. The drug was purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). The drug was dissolved in DMSO and diluted with PBS (1:19). The DMSO was purchased from Sigma
Aldrich Canada. Cariporide was administered by intraperitoneal (i.p.) injection at a dose of 6 mg/kg in 1 ml over the course of 2 minutes. Control mice with brain tumors received an i.p. injection of PBS+DMSO only.

3.2.3 Animal model of glioblastoma

U87MG glioma cells established from a human GBM (ATCC; Rockville, MD, USA) were used to induce GBM brain tumors in 22-27g, NU/NU mice (N=6) as described previously in detail (86,88,165). Briefly, Dulbecco’s modified Eagles’s medium supplemented with 10% fetal bovine serum (Wisent Inc., St-Jean-Baptiste, QC, Canada) was used to grow the U87MG cells at 37°C in a humidified incubator with 5% CO₂. Cells were passaged twice a week. On the same day as the injection into the brain, the U87MG cells were washed and dissociated with versene solution (phosphate-buffered saline (PBS) plus 0.5 mM EDTA). Cells were then washed twice with PBS, counted and re-suspended to achieve a final concentration of 1 x 10⁵ cells in 2 μl PBS. For injection of the cells, mice were anesthetized using 4% inhaled isoflurane and maintained using 1.5% isoflurane mixed with 100% oxygen. A stereotactic head frame (Stoelting instruments, Wood Dale, IL, USA) was used to guide injections through a hole drilled 1 mm anterior and 2 mm lateral from the bregma. Using a Hamilton (Reno, NV, USA) syringe with a 27-gauge needle attached, U87MG cells (2 μl) were injected at a position 3 mm deep from the bregma into the right frontal lobe at a rate of 0.5 μl/min.
3.2.4 General mouse preparation for in-vivo imaging

The mice were scanned using a 9.4 T small animal MRI system equipped with a 30 mm millipede volume coil (Agilent, Palo Alto, CA, USA) 15 ±1 days after cancer cell injection as previously described (86,88). Briefly, mice were anesthetized using 4% isoflurane in oxygen and maintained during scanning with 1.5%–2.5% isoflurane in oxygen. A custom-built MRI-compatible stage and a bite bar were used to secure the head. Surgical tape was also used to limit motion due to respiration. A respiratory sensor pad connected to a pressure transducer that was placed on the thoracic region was used to monitor respiration rate. Warm air blown over the animal using a model 1025 small-animal monitoring and gating system (SA Instruments Inc., Stony Brook, NY, USA) was used to maintain body temperature at 36.9-37.1°C throughout imaging, monitored by a rectal probe. After the baseline pre-injection images were acquired, the mouse was injected with cariporide inside the MRI by delivering the drug through fine plastic tube ended with a needle positioned to achieve intraperitoneal injection. Animals were sacrificed immediately after MR imaging.

3.2.5 In-vivo Magnetic Resonance Imaging

The tumor was initially localized using T2-weighted images. The T2-weighted images were acquired using a 2-dimensional fast spin echo pulse sequence (FSE) with parameters: TR/TE = 3000/10 ms, echo train length (ETL) = 4, effective TE = 40 ms, FOV = 25.6 x 25.6 mm², matrix size = 128 x 128, slice thickness= 1 mm. Two slices from the stack of T2-weighted images were selected to provide maximum tumor coverage (2 mm thickness) for CEST imaging. The CEST images were acquired using the same methodology as previously used to study the effects of lonidamine, topiramate, and dichloroacetate (85,86,88). Briefly, a fast spin-echo (FSE) pulse
sequence was used (TR/TE = 7000/7 ms, ETL = 32, effective TE = 7 ms, FOV = 25.6 x 25.6 mm², matrix size = 64 x 64, slice thickness = 2 mm, acquisition time ≈ 18 minutes) preceded by a continuous wave radiofrequency (RF) pulse with amplitude 1.5-µT and 4s duration. Saturation frequencies were varied from 1.2 to 4.5 ppm and from 5.4 to 6.6 ppm with step size of 0.1 ppm. Images were also acquired with saturation frequencies of -1000 and 1000 ppm as references, producing a total of 49 images. A complete series of CEST images were acquired three times before and three times after drug injection to improve signal-to-noise ratio. The water saturation shift referencing (WASSR) technique was used for B₀ correction (166) also described previously (85,86,88). A linearly spaced 37-point WASSR CEST spectrum (acquisition time ≈ 9 minutes) with saturation frequencies ranging from -0.6 – 0.6 ppm was acquired using the same pulse sequence except using a short (100 ms) low amplitude (0.2 µT) RF saturation pulse. Each WASSR spectrum (166) and CEST spectrum was interpolated to achieve a 1-Hz resolution. Each CEST spectrum was then frequency shifted, using the corresponding WASSR spectrum (166), to account for B₀ variation. B₀ variations were corrected on a pixel-by-pixel basis. Following B₀ corrections the three pre- and three post-injection CEST spectra were summed to increase signal to noise ratio.

### 3.2.6 CEST data processing

All CEST image processing was performed on a pixel-by-pixel basis using custom MATLAB (Mathworks, Natick, MA, USA) code. All CEST spectra were smoothed using the ‘smooth’ algorithm from the MATLAB curve fitting toolbox. Each WASSR spectrum and CEST spectrum was interpolated to a resolution of 1-Hz. Each CEST spectrum was then frequency shifted, using
the corresponding WASSR spectrum, to account for $B_0$ variation. The three pre- and three post-injection CEST spectra were summed following $B_0$ corrections to increase signal to noise ratio.

### 3.2.7 CEST and contrast calculations

AACID values were calculated on a pixel-by-pixel basis using the associated $B_0$-corrected and smoothed CEST spectra. The AACID value represents the ratio of the CEST effects of the amine protons that resonate at 2.75 ppm and the amide protons that resonate at 3.50 ppm. Macromolecule MT effects were minimized by measuring the CEST effect relative to the saturation observed at 6.0 ppm. The AACID value was calculated using Equation (1) (60).

$$AACID = \frac{M_{Z3.5\ ppm} \times (M_{Z6.0\ ppm} - M_{Z2.75\ ppm})}{M_{Z2.75\ ppm} \times (M_{Z6.0\ ppm} - M_{Z3.5\ ppm})}$$

(1)

Following drug administration, the change in pH was estimated by Equation (2) obtained using the calibration provided by Equation 8 in McVicar et. al. (60).

$$\Delta pH = -4 \times \Delta AACID$$

(2)

### 3.2.8 Statistical Analysis

Regions of interest (ROIs) were manually defined within tumor tissue and contralateral tissue in each mouse brain using MATLAB (‘roipoly’ function). The ROIs containing tumor tissue were drawn manually based on the signal changes observed in the T$_2$-weighted images. The ROIs drawn on the contralateral side were chosen to be similar in size and position to the tumor ROI
for each mouse. An average AACID value was calculated before and after injection of cariporide or vehicle within each tissue ROI. Differences in mean AACID values measured in the tumor and in contralateral ROIs before and after drug or vehicle injection were assessed using a paired t-test with p<0.05 considered statistically significant. A similar comparison was made in control mice after drug injection using the AACID values from the whole brain. For control animals a whole brain ROI was drawn manually using the standard T2-weighted anatomical images.

### 3.3 Results

Animals were scanned 15 ±1 days after U87MG cancer cell implantation. The anatomical FSE MR images were used to visualize and draw ROIs in the tumor and on the contralateral side (Figures 3.1a and 3.2a). The average weight of the mice at the time of imaging is 22-25g. Prior to drug injection AACID CEST maps showed a lower AACID value in the tumor region compared to the surrounding tissue as expected (Figures 3.1b and 3.2b) indicating a relatively basic intracellular tumor pH. Following injection of vehicle only (PBS+DMSO), there was a small but significant average increase (Figure 3.1c, 3.3a, 3.4a) in the AACID value within the tumor but no significant change within contralateral tissue. The post-baseline difference image (Fig 3.3a) shows a relatively constant change throughout the brain and tumor region. Following injection of cariporide in PBS+DMSO, a significant increase in tumor AACID value of 0.21±0.02 (N=6, p<0.05) is visible within the tumor (Figure 3.2c) indicating intracellular acidification within 2 hours of cariporide injection. There was no change in AACID value within contralateral tissue (Figure 3.4b). The post-baseline difference image (Figure 3.3b) shows a larger effect (Figure 3.4b) compared to vehicle alone. The measured change in AACID value within the tumor after cariporide injection corresponds to a 0.48 decrease in intracellular pH, estimated using Equation
2. In further control experiments, there was no change in AACID value in normal brain following injection of cariporide in PBS+DMSO (Figures 3.5 and 3.6).

Figure 3.7 shows H&E staining in a mouse with a typical brain tumor that demonstrates the size, localization, and homogeneity of the tumor tissue.

**Figure 3.1** Mouse brain with GBM tumor 15 ±1 days after implantation: a) coronal fast spin-echo anatomical image showing regions of interest (ROIs) in the tumor (dashed white line) and on the contralateral side (solid white line), b) the corresponding baseline AACID map prior to PBS+DMSO intraperitoneal injection, and c) the corresponding AACID map two hours post PBS+DMSO intraperitoneal injection.
**Figure 3.2** Mouse brain with GBM tumor 15 ±1 days after implantation: a) coronal fast spin-echo anatomical image showing regions of interest (ROIs) in the tumor (dashed white line) and on the contralateral side (solid white line), b) the corresponding baseline AACID map prior to cariporide injection, and c) the corresponding AACID map two hours post cariporide (6 mg/kg) intraperitoneal injection.

**Figure 3.3** Mouse brain AACID difference maps for the same brains: a) post PBS+DMSO injection - baseline, b) post cariporide (6 mg/kg) injection - baseline. Intraperitoneal injection.

**Figure 3.4** Average AACID value in tumor and contralateral ROIs: a) pre and post intraperitoneal PBS+DMSO injection as control (N=3). b) pre and post cariporide (6 mg/kg) intraperitoneal injection (N=6). Error bars represent the standard error of the mean. The asterisks indicated $p<0.05$ in repeated measures t-test.
**Figure 3.5** Normal mouse brain: a) T2-weighted image b) AACID map prior to cariporide injection c) AACID map post cariporide intraperitoneal injection.

**Figure 3.6** Average AACID value in normal brain ROIs on the left side, right side and whole brain: a) pre and post cariporide (6 mg/kg) intraperitoneal injection N=3.

**Figure 3.7** H&E stained brain section from a representative mouse with U87 glioblastoma multiforme (GBM) brain tumor. (a) Whole brain section. (b) Magnified region corresponding to the black box in (a) showing the boundary of the tumor within the brain.
3.4 Discussion

This study is the first to demonstrate changes in AACID CEST MRI indicative of intracellular acidification in orthotopic U87MG brain tumors within the first two hours after i.p. injection of a single dose of cariporide (6 mg/kg). The side contralateral to the tumor showed no change in the AACID value after cariporide injection. It is also noteworthy that the vehicle (PBS+DMSO) alone produced a small but measurable increase in AACID value within tumors.

Cariporide is an anticancer agent (172) that alters pH$_i$ by inhibiting the Na$^+$/H$^+$ exchange isoform-1 (NHE-1). Cariporide is not expected to cross an intact blood-brain barrier (178,179), however under ischemic conditions (178), and in cancer (180), it has been shown to enter the affected tissue. The results of the current study are consistent with several previous reports that have shown that the inhibition of Na$^+$/H$^+$ exchange using cariporide decreased intracellular pH in human umbilical cord derived mesenchymal stem cells (hUC-MSCs) (181) as well as human tongue carcinoma (175) and K562 leukemia cells (182).

We have previously used the same methodology to evaluate the magnitude of tumor acidification following administration of the drugs lonidamine, topiramate, and DCA. Lonidamine, a monocarboxylate transport inhibitor, decreased intracellular pH by 0.25 at a dose of 50 mg/kg and decreased intracellular pH by 0.45 at a dose of 100 mg/kg (85). Topiramate, a carbonic anhydrase inhibitor, decreased intracellular pH by 0.17 (86) at a dose of 120 mg/kg. More recently we also showed that giving 200 mg/kg of dichloroacetate which decreases the expression of monocarboxylate transporters and V-ATPases, decreased intracellular pH by 0.16 (88). At the dose studied in the current work, cariporide had a similar effect on intracellular pH as 100 mg/kg lonidamine. However, cariporide has been tested in humans for cardiac applications, but at the doses studied showed some toxicity and is not currently approved for
humans. Further studies are needed to determine the utility of combing a pharmacologic challenge using cariporide in combination with CEST MRI for cancer detection. It should be noted that the modification of tumor intracellular pH is emerging as a novel target for anti-cancer treatment. The use of drugs like cariporide to acidify tumors could enhance the effectiveness of existing chemotherapies, and could also be used directly to reduce cancer cell proliferation and tumor growth (183).

There are several limitations to the current work that should be considered when interpreting the results. First, cariporide at the dose studied (6 mg/kg) cannot be dissolved in distilled water or PBS. Therefore, it was dissolved in DMSO. DMSO alone caused a small increase in AACID value suggesting a decrease in intracellular pH. However, this could be an advantage for studies aimed at lowering intracellular pH, as long as the concentration of DMSO is sufficiently small to limit any toxic effects. Second, in the current study, only one dose of cariporide was examined. Future studies should determine whether higher doses of cariporide could increase tumor acidification, and whether the effect is repeatable after multiple exposures. Third, the number of animals included in the current study was small. However due to the large change induced by the drug, more animals were not needed in this proof of principle study. It should also be noted that the use of AACID CEST to measure absolute intracellular pH requires further calibration. In the current study, an estimate of the change in pH induced by cariporide was made using a previously published calibration performed in the brain under normoxic and ischemic conditions (60).

In conclusion, we found an increase in AACID CEST MRI signal within 2 hours following a single dose of cariporide indicating tumor acidification. The results of the current study demonstrate that acute CEST MRI contrast changes after administration of cariporide
could help localize brain cancer by rapidly and selectively inducing a shift in intracellular pH. Since humans can tolerate cariporide, future studies in people with glioblastoma may be warranted to determine whether pharmacologic modulation of tumor pH would aid in cancer localization.
Abstract:

Non-invasively distinguishing aggressive from non-aggressive brain tumors is an important clinical challenge. Intracellular pH (pH$_i$) regulation is essential for normal cell function and is normally maintained within a narrow range. Cancer cells are characterized by a reversed intracellular to extracellular pH gradient, compared to healthy cells that is maintained by several distinct mechanisms. Previous studies have demonstrated acute pH modulation in glioblastoma detectable by chemical exchange saturation transfer (CEST) magnetic resonance imaging (MRI) after blocking individual pH regulatory mechanisms. The purpose of the current study was to simultaneously block five pH regulatory mechanisms while also providing glucose as an energy substrate. We hypothesized that this approach would increase the acute pH modulation effect allowing the identification of aggressive cancer. Using a 9.4T MRI scanner, CEST spectra were acquired sensitive to pH$_i$ using amine / amide concentration independent detection (AACID).

Twelve mice were scanned approximately 11±1 days after implanting $10^5$ U87 human glioblastoma multiforme cells in the brain, before and after intraperitoneal injection of a combination of five drugs (quercetin, cariporide, dichloroacetate, acetazolamide, and pantoprazole) with and without glucose. Two hours after combination drug injection there was a
significant 0.1±0.03 increase in tumor AACID corresponding to a 0.4 decrease in pH_i. After injecting the drug combination with glucose the AACID value decreased by 0.18±0.03 corresponding to a 0.72 decrease in pH_i. AACID values were unchanged in contralateral tissue. The combined drug treatment with glucose produced a large acute CEST MRI contrast indicating tumor acidification, which could be used to help localize brain cancer and monitor tumor response to chemotherapy.

4.1 Introduction

Glioblastoma multiforme (GBM) is a highly aggressive cancer and the most common primary brain tumor of the central nervous system (CNS). The current standard of treatment combines surgical resection of the primary tumor followed by concurrent radiation and chemotherapy (6-8,171,184). However, due to the high migratory nature of GBM cancer cells, many escape treatment leading to recurrence in 90% of GBM patients (171). As a result, GBM patients often survive only 12-18 months following diagnosis (7,8,171). In the United States, glioblastomas represent the second leading cause of death among neurological diseases and one of the greatest challenges in the cure of cancer worldwide (6-8,171).

Cancer cells are characterized by a distinctive intracellular/extracellular pH gradient, that is reversed compared to normal cells. Specifically, in cancer cells, the extracellular space is acidic and the intracellular compartment is neutral or slightly basic (24,28-32,185). This reversal of the pH gradient in tumors occurs because (16,186) aerobic glycolysis even in the presence of oxygen, known as the Warburg effect (16), (186) produces a large quantity of acidic metabolites that are exported to the extracellular space. The acidification of the extracellular tumor microenvironment contributes to cancer cell evasion of apoptosis (22,33), drug resistance (34), proliferation (33), and increased metastatic potential (16,22,24,36-40). Aggressive tumors often
increase in size too quickly to develop adequate vasculature. Therefore cancer cells that favor glycolysis tend to have an advantage (16,22,41). High glucose uptake (22,35), which is the basis of cancer detection using the glucose analog $^{18}$F-labeled fluorodeoxyglucose ($^{18}$F FDG) positron emission tomography (PET) (16), and up-regulation of pro-glycolytic enzymes such as hexokinase (16) can lead to further extracellular acidification.

Conversely, purposefully decreasing tumor intracellular pH can have several potential beneficial applications for cancer patients. For example, the manipulation of tumor intracellular pH may predict tumor response to chemotherapy and radiation therapy (8,25,44). Additionally, decreasing intracellular pH in cancer cells may increase the efficacy of some anti-tumor treatments (48,49), and may directly induce cancer cell apoptosis (49). Tumor acidification can be achieved by blocking the regulators that extrude H$^+$ and lactate, which maintain a relatively alkaline tumor pH$_i$. These regulators include carbonic anhydrase inhibitors, anion exchangers, the Cl$^-$/HCO$_3^-$ exchangers, Na$^+$/HCO$_3^-$ co-transporters, Na$^+/H^+$ exchangers, monocarboxylate transporters (MCT), and the vacuolar ATPase and ATP synthase (25,26). We have previously shown that blocking specific regulators of pH can acidify the intracellular tumor environment within two hours of dosing in a U87 GBM mouse model. Specifically, we found the MCT inhibitor lonidamine decreased pH$_i$ by 0.25 at a dose of 50 mg/kg and decreased pH$_i$ by 0.45 at a dose of 100 mg/kg (85), the carbonic anhydrase inhibitor topiramate 120 mg/kg decreased pH$_i$ by 0.17 (86), the MCT and V-ATPases inhibitor dichloroacetate (200 mg/kg) decreased pH$_i$ by 0.16 (88), the Na$^+/H^+$ exchange inhibitor cariporide (6 mg/kg) decreased pH$_i$ by 0.48 (187), and the MCT inhibitor quercetin (200 mg/kg) decreased pH$_i$ by 0.27 (89). However, it is currently unknown whether combining two or more drugs to simultaneously block multiple pH regulatory mechanisms can produce greater tumor acidification.
The purpose of the current study was to determine whether acute glioblastoma intracellular acidification could be enhanced in-vivo in the U87 GBM mouse model using a combined therapy that simultaneously blocked several major pH regulators, and to determine whether cancer cell apoptosis increased following short term acidification treatment. We hypothesize that combining five drugs targeting different pH regulatory pathways would induce greater acidification within tumors than that previously found with only a single drug. We further hypothesized that pre-treatment with glucose to provide additional substrate for aerobic glycolysis in combination with the five drug acidification treatment would further increase intracellular acidification. Finally, we hypothesized that short term acidification treatment would increase tumor apoptosis. In all studies, intracellular pH was monitored in-vivo using amine/amide concentration independent detection (AACID) chemical exchange saturation transfer (CEST) magnetic resonance imaging (MRI).

4.2 EXPERIMENTAL

4.2.1 Subjects

Nineteen different female Crl:-Nu-Foxn1Nu (NU/NU) (Charles River Laboratories, Canada) adult mice were included in the current study. Mice were group housed in ventilated racks, on a 12h/12h light/dark cycle. All animal procedures were performed according to a protocol that was consistent with the guidelines established by the Canadian Council on Animal Care and was approved by the University of Western Ontario Animal Use Subcommittee.

For the assessment of the acute intracellular tumor acidification produced by the drug combination, six NU/NU mice with U87MG brain tumors were used (Group A1). An additional six NU/NU mice with U87MG brain tumors were used to evaluate the acute intracellular tumor
acidification produced by the drug combination preceded by glucose administration (Group A2). To assess apoptosis following brief continuous treatment (8 days), three groups of mice were studied. The first group consisted of three mice from Group A1 that were treated with the five-drug combination (Group T1). The second group was a control group of three mice with brain tumors that received only vehicle (Group T2). The third group was also a control group of four mice without tumors that were treated with the five-drug combination to assess the impact on normal brain (Group T3).

4.2.2 Selection of Drugs to Block pH Regulation

Intracellular acidification can be achieved by inhibiting different regulators of intracellular pH including vacuolar H\(^+\)-ATPases (188,189), Na\(^+\)/H\(^+\) exchangers (190,191), monocarboxylate transporters (16,22,192), and carbonic anhydrases (193,194). In each case, drugs already used for human use have been shown to inhibit these mechanisms. We designed a combination therapy to simultaneously target these five pH regulatory mechanisms using drugs already approved for human use including quercetin, pantoprazole (PPZ), acetazolamide, dichloroacetate (DCA), and cariporide. Quercetin (Sigma–Aldrich, Canada) is an inhibitor of monocarboxylate transporters (MCTs) (94,114-117) with linear formula C\(_{15}\)H\(_{10}\)O\(_{7}\). It specifically inhibits MCT1 and MCT2 (114). The quercetin dose used was 100 mg/kg every 8 hours (195). Pantoprazole (United States Pharmacopeia, Maryland, USA) is a proton pump inhibitor (PPI) with linear formula C\(_{16}\)H\(_{14}\)F\(_{2}\)N\(_{3}\)NaO\(_{4}\)S\(\cdot\)1.5H\(_{2}\)O that targets the vacuolar H\(^+\)-ATPase and has been shown to decrease intracellular pH and increase apoptotic cell death in cancer cells (142,143). The pantoprazole dose used was 2 mg/kg every 8 hours. Acetazolamide (Sigma–Aldrich, Canada) with linear formula C\(_{4}\)H\(_{6}\)N\(_{4}\)O\(_{3}\)S\(_{2}\) inhibits carbonic anhydrase activity and decreases expression of the aquaporin-1
(AQP1) water channel (128-136). It may also inhibit angiogenesis and endothelial cell proliferation in various cancers (137-140). The acetazolamide dose used was 50 mg/kg every 8 hours. Dichloroacetate (Sigma –Aldrich, Canada) with linear formula Cl2CHCO2Na inhibits pyruvate dehydrogenase kinases (PDKs) and redirects pyruvate back into the mitochondria (160,162), which reverses the Warburg effect by activating pyruvate dehydrogenase (PDH). However, DCA also decreases the expression of monocarboxylate transporters MCTs and V-ATPase (163) in tumor cells, leading to reduced pHi in tumors. DCA has been shown to induce cell death in several different types of cancer (47,154,155,160,161). The DCA dose used was 100 mg/kg every 8 hours. Finally, cariporide (Cayman Chemical Company, Michigan, USA) with linear formula C12H17N3O3S selectively inhibits the Na+/H+ exchange isoform 1 (NHE1) (172) with little effect on other ion transport systems (173) and has been shown to suppress the invasion and migration of cancer cells (175). The cariporide dose used was 2 mg/kg every 8 hours.

### 4.2.3 Drug Delivery

All drugs were obtained in powder form and dissolved in dimethyl sulfoxide (DMSO). To assess the acute effects on pH of the drug combination using CEST MRI (Groups A1 and A2), the drugs were injected together in DMSO only without dilution. Specifically, the single injection contained drug doses equal to that used for eight hours of treatment as described above. To assess whether glucose increased tumor intracellular acidification using CEST MRI, glucose (Sigma –Aldrich, Canada) with linear formula C6H12O6 was dissolved in PBS and administered by i.p. injection (Group A2) at a dose of 5 g/kg in 0.2 ml. Glucose was provided 20 mins before injection of the drug combination. For mice receiving treatment (Groups T1 and T3), the drugs were diluted with phosphate buffered saline (PBS) at a ratio of 1:19 and administered by intraperitoneal (i.p.) injection in a total volume of 1 ml every 8 hours for eight days. Control mice with brain tumors (Group T2) received DMSO+PBS at a ratio of 1:19 in a total volume of 1 ml every eight hours for eight days.
4.2.4 Animal Model of Glioblastoma

The GBM animal model has been published previously but is provided for completeness (85,88,89,187) GBM brain tumors were induced in 22-27g, NU/NU mice (N=15) using U87MG glioma cells established from a human GBM (ATCC; Rockville, MD, USA) as described previously (165). Briefly, U87MG cells were grown in Dulbecco’s modified Eagles’ medium supplemented with 10% fetal bovine serum (Wisent Inc., St-Jean-Baptiste, QC, Canada) at 37 °C in a humidified incubator with 5% CO2 and passaged twice a week. On the day of injection, U87MG cells were washed and dissociated with versene solution (phosphate-buffered saline (PBS) plus 0.5 mM EDTA), then washed twice with PBS, counted and re-suspended to a final concentration of 1 x 10^5 cells in 2 mL PBS. Before injection, mice were anesthetized by inhalation of 4% isoflurane and maintained using 1.5% isoflurane. The mouse was placed in a stereotactic head frame (Stoelting instruments, Wood Dale, IL, USA). The scalp was swabbed with betadine, and an incision was made in the scalp to expose the bregma. A 1 mm diameter hole was drilled at coordinates measured from the bregma (1 mm anterior and 2 mm lateral). U87MG cells (2 µl) were injected at a rate of 0.5 µL/min, at a position 3 mm deep from the bregma into the right frontal lobe using a Hamilton (Reno, NV, USA) syringe with a 27-gauge needle attached.
4.2.5 Mouse Preparation for *In-vivo* Imaging

Approximately 11±1 days after cancer cell injection, the mice in Groups A1 and A2 were scanned on a 9.4 T small animal MRI system equipped with a 30 mm millipede volume coil (Agilent, Palo Alto, CA, USA). Anesthesia was induced using 4% isoflurane in oxygen and maintained with 1.5%–2.5% isoflurane in oxygen. Each mouse was secured on a custom-built MRI-compatible stage, and the head was secured using a bite bar (86) and surgical tape to limit motion due to respiration. Animal temperature was monitored with a rectal temperature probe, and respiration was monitored with a respiratory sensor pad connected to a pressure transducer that was placed on the thoracic region. Body temperature was maintained at 36.9-37.1 °C throughout imaging by blowing warm air over the animal using a model 1025 small-animal monitoring and gating system (SA Instruments Inc., Stony Brook, NY, USA). Following baseline imaging, the mouse was injected with the drug combination inside the MRI through fine plastic tube ended with a needle to deliver into the peritoneum. Following imaging, three animals from Group A1 were treated with the five drug combination for eight days (Group T1), while all others animals were sacrificed immediately after MR imaging.

4.2.6 *In-vivo* Magnetic Resonance Imaging and pH-weighted imaging

The imaging protocol used in this study has been published previously (85,88,187). Briefly, T$_2$-weighted images were used for tumor detection acquired using a 2-dimensional fast spin echo pulse sequence (FSE) with parameters: TR/TE = 3000/10 ms, ETL = 4, effective TE = 40 ms, FOV = 25.6 x 25.6 mm$^2$, matrix size = 128 x 128, slice thickness= 1 mm. Two slices from the series of T$_2$-weighted images with maximum tumor coverage were selected for CEST imaging. CEST images were acquired using a fast spin-echo (FSE) pulse sequence (TR/TE = 7000/7 ms,
ETL = 32, effective TE = 7 ms, FOV = 25.6 x 25.6 mm², matrix size = 64 x 64, slice thickness = 2 mm) preceded by a continuous wave radiofrequency (RF) pulse with amplitude 1.5-µT and 4s duration. The CEST images were acquired at different saturation frequencies (from 1.2 to 4.5 (Δ=0.1) ppm, from 5.4 to 6.6 (Δ=0.1) ppm, and -1000 and 1000 ppm images were acquired as a reference, total 49 images). A complete series of CEST images were acquired three times before and three times after drug injection to improve the signal-to-noise ratio. For B₀ correction, the water saturation shift referencing (WASSR) technique was used (166). A linearly spaced 37-point WASSR CEST spectrum with saturation frequencies ranging from -0.6 – 0.6 ppm was acquired using the same pulse sequence except preceded by a short RF saturation pulse (100 ms) with low amplitude (0.2µT).

4.2.7 CEST Data Processing

All acquired CEST MR data were processed on a pixel-by-pixel basis using custom MATLAB (Mathworks, Natick, MA, USA) code for analysis as previously described (85,88,89,187). Each WASSR spectrum and CEST spectrum was interpolated to achieve 1-Hz resolution. All CEST spectra were smoothed using the ‘smooth’ algorithm from the MATLAB curve fitting toolbox. Each CEST spectrum was then frequency shifted, using the corresponding WASSR spectrum, to account for B₀ variation. B₀ variations were corrected on a pixel-by-pixel basis. The three pre- and three post-injection CEST spectra were summed following B₀ corrections to increase signal to noise ratio. As previously shown (85), the B₁ variation in the CEST slice was less than 5%, so no B₁ correction was applied (85).
4.2.8 Mapping Tissue Intracellular pH

Tissue pH\(_i\) was monitored using amine and amide concentration-independent detection (AACID), which uses the ratio of CEST effects from amide (\(\Delta \omega = 3.5\) ppm) and amine (\(\Delta \omega = 2.75\) ppm) protons to generate pH\(_i\) dependent contrast independent of tissue macromolecule concentration and temperature (60). The CEST contrast originates from exchangeable amine and amide protons that are found in tissue proteins and peptides (14,58,77,159). However the AACID CEST measurement of tissue pH is highly weighted to the intracellular compartment (14) because almost 90% of total protein content exists in the intracellular space (77). AACID values were measured on a pixel-by-pixel basis using the associated B\(_0\)-corrected and smoothed CEST spectra. The AACID value represents the ratio of the CEST effects of amine protons resonating at 2.75 ppm and amide protons at 3.50 ppm, normalized by MT effects measured after saturation at 6.0 ppm and is calculated using equation (1) (60).

\[
AACID = \frac{M_{Z3.5 ppm} \times (M_{Z6.0 ppm} - M_{Z2.75 ppm})}{M_{Z2.75 ppm} \times (M_{Z6.0 ppm} - M_{Z3.5 ppm})}
\]  

(1)

Following drug administration, the change in pH was estimated by Equation (2) obtained using the calibration provided by Equation (8) in McVicar et al. (60).

\[
\Delta pH = -4 \times \Delta AACID
\]

(2)
4.2.9 Immunohistochemistry on mouse brains

On day 18-19 after injection of cancer cells, mice were euthanized and perfused with 4% paraformaldehyde in PBS as described previously (196). After 24-hour incubation in 4% paraformaldehyde at 4 C, brains were cut using a vibratome into 80-µm coronal sections. Randomly chosen sections containing tumor were then immunostained as described previously (197), using an anti-cleaved caspase-3 (CC3) antibody (Cell Signaling, cat#9664, 1:500) and Alexa Fluor 488 secondary anti-rabbit antibody (Thermo Fisher, cat#A-11008), followed by nuclear staining with Hoechst 33342. Stained brain sections were mounted on glass slides using Immu-mount (Thermo Scientific, cat#9990402) and imaged using a FV1000 confocal microscope (Olympus) equipped with a 10x/0.4 or a 20x/0.75 objective. Images were stitched using FluoView software (Olympus) and analyzed using ImageJ Measure and Cell Counter plugins (NIH, Bethesda, MD). At least three sections per animal and at least three animals per condition were analyzed. Apoptosis was quantified using the number of CC3-positive cells per mm$^3$.

4.2.10 Statistical Analysis

Regions of interest (ROIs) containing tumor tissue and contralateral tissue were drawn manually in each mouse brain using the MATLAB (‘roipoly’ function) using the contrast observed in the T2-weighted images as a guide. Average AACID values were calculated before and after injection of the drug combination within each ROI. A paired t-test was used to calculate differences in mean AACID values measured in the tumor and contralateral ROIs before and
after injection of the drug combination and the drug combination plus glucose. The number of CC3 positive cells per mm$^3$ were compared between treatment groups using a Student’s t-test in Prism (GraphPad). In all comparisons, $p<0.05$ was considered statistically significant.

### 4.3 Results

AACID CEST maps were acquired in all animals 11 ±1 days after U87MG cancer cell implantation. Anatomical FSE MR images were successfully used to identify regions of interest in the tumor and on the contralateral side (Figures 4.1c and 4.2c). AACID CEST maps showed the expected lower AACID value in the tumor region compared to the surrounding brain tissue (Figures 4.1a and 4.2a) indicating a relatively basic intracellular tumor pH. Also, as expected, a large increase in tumor AACID value was observed within the tumor following the injection of the drug combination (Figure 4.1b) indicating rapid intracellular acidification within 2 hours of injection. As expected, the difference between the post and pre images in a single animal showed a greater effect in the tumor ROI compared to the contralateral ROI (Figure 4.1d). Specifically, two hours after injection of the drug combination, there was an average increase in the AACID value of 0.10±0.03 (N=6, $p<0.05$) in the tumor region (Figure 4.3a), but no change in AACID value within the contralateral tissue (Figure 4.3a). When providing glucose prior to the combined drug injection an even larger increase in AACID value was visible (Figure 4.2b) suggesting even greater acidification was achieved. When pre-treating with glucose, there was a significant increase in the AACID value of 0.18±0.03 (N=6, $p<0.0001$) two hours after the combination drug injection in the tumor region (Figure 4.3b). However, there was also a small change in the AACID value within the contralateral tissue (Figure 4.3b). The difference between the post and pre images in a single animal (Figure 4.2d) showed a greater effect in the tumor ROI compared to the contralateral ROI as expected. The measured changes in the average AACID value within
the tumor after the combination drug treatment corresponded to a 0.4 pH drop, while pretreatment with glucose produced a 0.72 pH drop, estimated using Equation 2.

All animals in Groups T1 and T3 completed eight days of drug treatment. Representative immunostained brain sections showing the results of cleaved (active) caspase 3 (CC3) immunostaining as a marker of apoptotic cell death are provided in Figure 4.4. Control mice without tumors showed almost no evidence of CC3 staining (Figure 4.4A). However, within tumors, CC3 staining was increased in the control group (Figure 4.4B, 4.4D) and after treatment (Figure 4.4C, 4.4D) in the treatment group. In the mice treated with the five drug combination there was a significant increase in CC3 levels (p=0.0023) (Figures 4.4C, 4.4D).
Figure 4.1 Mouse brain with GBM tumor 11 ±1 days after implantation: a) baseline AACID map prior to drug injection, b) the AACID map two hours post drug injection, c) coronal fast spin-echo anatomical image showing the ROIs in the tumor (dashed white line) and on the contralateral side (solid white line), and d) AACID difference maps for the same brain post–pre drug injection.

Figure 4.2 Mouse brain with GBM tumor 11 ±1 days after implantation: a) baseline AACID map prior to drug and glucose injections, b) the AACID map two hours post drug and glucose injections, c) coronal fast spin-echo anatomical image showing the ROIs in the tumor (dashed white line) and on the contralateral side (solid white line), and d) AACID difference maps for the same brain post–pre drug and glucose injections.
Figure 4.3 Average AACID value in tumor and contralateral ROIs: a) pre and post intraperitoneal five drug combination injection (N=6). b) pre and post five drug combination + glucose intraperitoneal injection (N=6). Error bars represent the standard error of the mean. The asterisks indicated \( p < 0.05 \) in repeated measures t-test.
Figure 4.4 Cleaved Caspase 3 (CC3) Increases in Tumor after Treatment. (A-C) Representative images of coronal brain sections of (A) non-tumor control mice, (B) mice with U87 tumors, non-treated receiving vehicle only, and (C) mice with tumors, treated for eight days with the five drug combination. Blue corresponds to nuclear stain (Hoechst), red corresponds to CC3 (apoptotic marker). Scale bar: 0.4 mm. (D) Quantification of apoptotic cells in (A-C). ** indicate p<0.01.
4.4 Discussion

This study demonstrates intracellular acidification in brain tumors two hours after injection of a single dose of five drugs designed to block different pH regulatory mechanisms. Tumor acidification was further enhanced by pretreatment with glucose. The magnitude of these changes is larger than that reported previously for single drug treatment. In contrast, there was no change in brain tissue pH detected on the side contralateral to the tumor after combined drug injection, but there was a small decrease in pH on the contralateral side when glucose was given prior to the combined drug injection. Eight days of treatment with the five drug combination significantly increased the number of cells positive for cleaved caspase 3 within the tumor.

The drug combination used in the current study was designed to block multiple pH regulatory mechanisms to enhance the acidification produced by any single compound. Quercetin is a natural compound, and a plant product that has been used as a chemotherapeutic agent to treat many cancer types (116,117,198). Quercetin is an MCT inhibitor (94,114-117) that specifically inhibits MCT1 and MCT2 (114). Pantoprazole (PPZ) has also been shown to be an anti-cancer agent (141) due to its ability to induce cancer cell death by inhibiting the V-ATPase causing reversal of H+ homeostasis. Acetazolamide induces intracellular acidification by inhibiting carbonic anhydrase activity and decreasing aquaporin-1 (AQP1) water channel protein expression (128-136). Dichloroacetate (DCA) is a small molecule and has low toxicity compared to other anti-cancer agents (155). DCA has been shown to induce cell death in several different types of cancer (160,161). DCA inhibits pyruvate dehydrogenase kinases (PDKs) and redirects pyruvate back into the mitochondria (160,162), which reverses the Warburg effect by activating pyruvate dehydrogenase (PDH). However, DCA also decreases the expression of monocarboxylate transporters and V-ATPase (163) in cancer cells reducing pH_i in tumors.
Cariporide selectively inhibits the sodium proton (Na\(^+\)/H\(^+\)) exchange isoform 1 (NHE1) (172) with little effect on other ion transport systems (173). In malignant tumors NHE1 plays a significant role in maintaining acidic pH\(_e\), alkaline pH\(_i\), and regulating cell volume (110). It is activated by growth factors and cellular proliferation processes (174). Cariporide effectively inhibits NHE1 and has been shown to suppress the invasion and migration of cancer cells (175). Cariporide also decreased intracellular pH and down-regulates vascular endothelial growth factor (VEGF) secretion in K562 leukemia cells (182). Therefore, using these specific drugs blocks several known pH regulatory mechanisms: Na\(^+\)/H\(^+\) exchange, monocarboxylate transporters (MCTs), carbonic anhydrase and aquaporin-1 (AQP1), pyruvate dehydrogenase kinases (PDKs) and V-ATPase.

In previous studies using the same methodology to evaluate the magnitude of tumor acidification, we found lonidamine decreased pH\(_i\) by 0.25 at a dose of 50 mg/kg and decreased pH\(_i\) by 0.45 at a dose of 100 mg/kg (85) while topiramate decreased pH\(_i\) by 0.17 (86). More recently we also showed that 200 mg/kg of dichloroacetate decreased pH\(_i\) by 0.16 (88), and that 6 mg/kg of cariporide decreased pH\(_i\) by 0.48 (187). At the dose studied, the combination of drugs used in the current study decreased pH\(_i\) by 0.4 alone and by 0.72 in combination with glucose. Although the five drug combination produced a pH\(_i\) change similar to that shown previously with 100 mg/kg of lonidamine and 6 mg/kg of cariporide, the combination used in the current study used mostly drugs considered safe in humans and used a much lower dose of cariporide (one third of that used previously) and DCA (half of that used previously). The dose was lowered in the current study to reduce potential side effects and interactions.

In an effort to further enhance intracellular acidification, glucose was injected 20 minutes prior to drug injection. Cancer cells readily take up and metabolize glucose more so than normal
cells, which forms the basis of cancer detection using fluorodeoxyglucose (\(^{18}\text{F}-\text{FDG}\)) positron emission tomography (PET). The end products of glucose metabolism are protons and lactate, which are normally removed from the cell by the pH regulatory mechanism targeted in this study. Blocking these mechanisms 20 minutes after glucose injection produced a much larger acute intracellular acidification effect. The application of several drugs together, in combination with glucose achieved greater tumor intracellular acidification than any previous single drug studied.

To study the long-term effect of intracellular acidification on cancer cell viability in-vivo, the five-drug combination was given to mice three times per day for eight days. Within the tumor, there was a significant increase in the number of cells staining positive for cleaved caspase 3, suggesting an increase in the number of cells undergoing apoptosis. There were very few cells undergoing apoptosis in normal brain, after being given the same drug treatment. These in-vivo results are consistent with previous studies of cancer cell lines, which have shown that cancer cell acidification can induce apoptosis in a variety of cancer models (149, 199-203). The results from the current study provide further evidence that tumor intracellular acidification may provide a benefit for cancer control and treatment.

The current study has several limitations that should be considered. First, the number of animals used was small. However, as in previous studies by our group, the effect sizes are large, and the ROI based pH measurements have low variability. Also, the test-retest design reduces inter-subject variation. Therefore, the animal numbers used were sufficient to determine whether the drug combination could produce a measurable pH effect. Second, we did not optimize the combination dose in the current study, only one dose of drugs was examined. Future studies should determine whether higher doses of these drugs could produce a greater effect, or if a
similar effect would be produced with lower doses, decreasing the risk of potential side effects. It should also be established whether the effect is repeatable after multiple exposures and whether the treatment would be more effective if started earlier on in the development of the tumor. The timing and dose of glucose supplementation to increase cellular metabolism prior to drug injection should be studied to optimize intracellular acidification, and consequently cancer cell death. Future studies should also examine whether chronic intracellular acidification could enhance the efficacy of existing chemotherapies.

The use of CEST MRI contrast to detect changes in intracellular pH has many potential clinical applications in cancer detection and treatment evaluation (8,14,157). The results of the current study further demonstrate that acute CEST MRI contrast changes after administration of several drugs in combination with glucose could help localize brain cancer by rapidly and selectively inducing a shift in intracellular pH. The current study demonstrated that the magnitude of intracellular acidification (AACID) of the tumor after combined drug injection was larger when providing glucose as a substrate.
Chapter 5

5 Conclusions and Future Directions

5.1 Summary of Findings

The overall goal of the work presented in this thesis was to maximize intracellular tumor acidification following drug treatment. In the first study, one hour after DCA intravenous injection (200 mg/kg) there was a significant increase in tumor AACID level by 0.04±0.01 corresponding to a 0.16 decrease in pH_i, and no change in AACID in contralateral tissue. In the second study presented, two hours after cariporide intraperitoneal injection there was a significant 0.12±0.03 increase in tumor AACID value corresponding to a 0.48 decrease in pH_i, and no change in AACID value in contralateral tissue. Finally, in the last study presented, two hours after combination drug intraperitoneal injection there was a significant 0.10±0.03 increase in tumor AACID corresponding to a 0.40 decrease in pH_i. After injecting the drug combination with glucose the AACID value decreased by 0.18±0.03 corresponding to a 0.72 decrease in pH_i. AACID values were unchanged in contralateral tissue. The results of the current study further demonstrate that acute CEST MRI contrast changes after administration of several drugs in combination with glucose could help localize brain cancer by rapidly and selectively inducing a shift in intracellular pH. We designed a combination therapy to simultaneously target these five pH regulatory mechanisms using drugs already approved for human use including quercetin, pantoprazole (PPZ), acetazolamide, dichloroacetate (DCA), and cariporide. The current study demonstrated that the magnitude of intracellular acidification (AACID) of the tumor after combined drug injection was larger when providing glucose as a substrate.
5.2 Limitations
There are several limitations to the current work that should be considered.
1. The number of animals used was small. However, as in previous studies by our group, the effect sizes are large, and the ROI based pH measurements have low variability. Also, the test-retest design reduces inter-subject variation. Therefore, the animal numbers used were sufficient to determine whether the drugs could produce a measurable pH effect.

2. Cariporide and quercetin at the doses studied cannot be dissolved in distilled water or PBS. Therefore, these drugs were dissolved in DMSO. DMSO alone caused a small increase in AACID value suggesting a decrease in intracellular pH. This could be considered an advantage for studies aimed at lowering intracellular pH, as long as the concentration of DMSO is sufficiently small to limit any toxic effects.

3. We did not optimize the combination drug doses in the current study, only one dose of drugs was examined. Future studies should determine whether higher doses of these drugs could produce a greater effect, or if a similar effect would be produced with lower doses, decreasing the risk of potential side effects. It should also be established whether the effect is repeatable after multiple exposures and whether the treatment would be more effective is started earlier on in the development of the tumor. The timing and dose of glucose supplementation to increase cellular metabolism prior to drug injection should be studied to optimize intracellular acidification, and consequently cancer cell death. We only studied one animal model in cancer, also, we do not have a calibration of AACID measurement within tumors to make absolute pH measurements. Future studies should also examine whether chronic intracellular acidification could enhance the efficacy of existing chemotherapies.
5.3 Conclusion

The use of CEST MRI contrast to detect changes in intracellular pH has many potential clinical applications in cancer detection and treatment evaluation (8,14,157). The results of the current study further demonstrate that acute CEST MRI contrast changes (86,89,187,204) after administration of several drugs in combination with glucose could help localize brain cancer by rapidly and selectively inducing a shift in intracellular pH. The current study demonstrated that the magnitude of intracellular acidification (AACID) of the tumor after combined drug injection was larger when providing glucose as a substrate could help localize brain cancer by rapidly and selectively inducing a shift in intracellular pH. Since the drugs are approved for use in humans, future studies in people with glioblastoma should be performed to determine whether pharmacologic modulation of tumor pH would aid in cancer localization.

5.4 Future directions

This thesis studied the effect of the MCT and V-ATPases inhibitor dichloroacetate, Na+/H+ exchange inhibitor cariporide, and a combination therapy to simultaneously target five pH regulatory mechanisms using mostly drugs approved for human use including quercetin, pantoprazole (PPZ), acetazolamide, and dichloroacetate (DCA).

We did not optimize the combination dose in the current study, only one dose of drugs was examined. Future studies should determine whether higher doses of these drugs could produce a greater effect, or if a similar effect would be produced with lower doses, decreasing the risk of potential side effects. It should also be established whether the effect is repeatable after multiple exposures.
Also, future work should evaluate the effect of these drugs on tumor pH\textsubscript{i} and determine which combination of drugs cause the greatest decrease in pH\textsubscript{i} with therapeutic dosages. Also, to design experiments to observe the effects of these drug combinations on tumor growth and optimize the dose for maximum growth delay and enhancement of the efficacy of chemotherapy drugs.

Also, it is interesting to design experiments to measure pH\textsubscript{e} along with pH\textsubscript{i} (185) to monitor the effect on extracellular-intracellular pH, as it is the pH gradient across the plasma membrane that impacts the efficacy of chemotherapy drugs. This simultaneous measurement of pH gradient may help to design the most effective treatment regime. Most recently, contributed to a published study of longitudinal measurement of the intracellular/extracellular pH gradient in a rat glioma model using MRI. pH\textsubscript{i} was mapped using CEST MRI, whereas regional pH\textsubscript{e} was determined using hyperpolarized \textsuperscript{13}\text{C} bicarbonate magnetic resonance spectroscopic imaging at 3 Tesla (185).

The results from this study indicate the potential value of carbonic anhydrase inhibitors in tumor acidification and hopefully will stimulate future experiments to expand the role of such drugs in cancer treatment.
5.5 References


Appendix A

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Appendix B

AUP Number: 2015-005

PI Name: Bartha, Robert

AUP Title: Development of Novel Targeted MRI Contrast Agents

Approval Date: 04/29/2015

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Development of Novel Targeted MRI Contrast Agents" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, is subject to annual Protocol Renewal.

1. This AUP number must be indicated when ordering animals for this project.

2. Animals for other projects may not be ordered under this AUP number.

3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.
Mohammed Albatany

EDUCATION

I have postdoctoral offer for one year will start on Feb 2019
Western University, Robarts Research Institute- The Centre for Functional and Metabolic Mapping (CFMM)- Molecular Imaging of Cancer.

Doctor of Philosophy 2014-Anticipate completion in Jan 2019
Department of Medical Biophysics, Robarts Research Institute, University of Western Ontario, London, Canada
Specialization: Molecular imaging of cancer; enhance respond of tumors to the treatments; improve CEST MRI contrast using new approaches.

Master of Science September 1994-Feb 1997
Department of Physics
Specialization: electromagnetic theory; radiation field measurement

Bachelor of Science in Physics September 1990-June 1994
Department of physics
Specialization: physics

TEACHING EXPERIENCE

Instructor for medical physics Fall 2009-Winter 2013
Department of Medical Science, Faculty of Medicine
I taught medical physics for the students at the university level.

Instructor for physics Fall 2006-Winter 2008
Higher Institute of Computer Science
I taught the general physics for the students at the university level.

Instructor for medical physics Winter 2003-Winter 2006
Aldalil Institute
I taught medical physics for the students at the university level.
Instructor for medical physics Fall 1997-Winter 2003
Alnajah Institute
I taught medical physics for the students at the university level.

RESEARCH EXPERIENCE

Cancer Imaging:
• Molecular imaging and early detection of cancer using MRI
• Enhance the respond of cancer to the treatment.
• Monitor respond of tumors to the treatments by using MRI.
• Imaging the absolute intracellular pH of brain tumors for humans at 7T

Improve MRI measurement:
• We improved intracellular pH measurement using MRI by 39%.
  I am very interested in Improve MRI measurement to improve the detection of diseases. For example, my previous work was using the 2 ppm amine resonance increased the AACID based pH range by 39% compared to the 2.75 ppm resonance and led to reduced measurement variability across the brain suggesting that using the 2 ppm amine resonance could improve AACID based pH measurement in-vivo. This work was presented at ISMRM 2017 USA.

MRI experiments:
• Running 9.4T MRI scanner during the experiments
• Prepare the animals inside the MR
• Animals anesthesia
• Animals injections and treatments
• Cancer drugs and chemotherapy injections
• Animals perfusion surgery, extract the brains, slices the brains for the histology
• Treat the animals with drugs injections for cancer treatment.
• Analyses all of MRI experiment data from (7T and 9.4T scanners)

LANGUAGES

Arabic - native level of competence
English - advanced level of competence


**Presentations**


5. (2017). In-vivo Detection of Acute Intracellular Acidification in Glioblastoma Multiforme Following a Single Dose of Cariporide and Quercetin. 25th Annual Meeting of the international society of Magnetic Resonance Imaging (ISMRM), Honolulu, HI, USA.

6. (2017). Improved Measurement Precision for AACID CEST MRI of Brain pH using the 2 ppm Amine Resonance. 25th Annual Meeting of the international society of Magnetic Resonance Imaging (ISMRM), Honolulu, HI, USA.


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Awards
PhD Scholarship $30,000 per year 2013-2018
Department of Medical Biophysics, Western University $12,000 per year 2017-2018

MEMBERSHIPS

Western’s Society of Graduate Students (SOGS) May 2014- Present
International Society for Magnetic Resonance in Medicine(ISMRM) 2015-Present