Metabolic Targeting of Cancer Cells: Two Molecular Mechanisms Involving Glucose

Metabolism

by

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Date: May 15th, 2008

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pathology in the Graduate School of Duke University 2008
ABSTRACT

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Abstract

Selective therapeutic targeting of tumors requires identification of differences between the homeostatic requirements of cancer and host cells. One such difference is the manner in which cancer cells acquire energy. Cancer cells often grow in an environment of local hypoxia; under these conditions tumor cells depend on glycolysis for energy, but are unable to perform oxidative phosphorylation. Many tumor cells, despite normoxic conditions, continue to perform glycolysis without oxidative phosphorylation. The net result of glycolysis without oxidative phosphorylation is twofold: the need to consume a greater amount of glucose than a non-cancerous host cell, and the burden of increased intracellular lactic acid. The proteins responsible for the transport of lactic acid in and out of cells are known as the monocarboxylate transporters (MCTs). Monocarboxylate Transporter 1 (MCT1) and Monocarboxylate Transporter 4 (MCT4) are the MCTs that play a major role in the transport of lactic acid. Tumor cells depend on MCT1 and MCT4 activity to excrete excess intracellular lactic acid to maintain neutral intracellular pH and homeostasis. Using human neuroblastoma and prostate cancer cell lines this work demonstrates that tumor cells can be selectively targeted tumor under conditions of hypoxia or acidosis in vitro with the drug lonidamine, with a small molecule inhibitor selective for MCT1, or with RNA interference of MCT1. Inhibition of MCT1 activity in neuroblastoma cells under acidic
extracellular conditions results in intracellular acidification and cell death. MCT1 mRNA is expressed in human neuroblastoma and positively correlated with clinical risk profile. Inhibition of MCT1 activity in hypoxic prostate cancer cells results in a reduction of lactate excretion, decreased intracellular pH, inhibition of ATP production, and subsequent cell death. MCT1 expression in sections of human prostate tumors has been demonstrated to validate MCT1 as a target in prostate cancer.

Through the Pasteur and Warburg effects, tumors have an increased demand for glucose. Some cancers store glycogen, but the reasons for this are largely unknown. It is hypothesized that tumor glycogen is used to promote tumor survival during transient hypoxia or low glucose, and that the mechanisms by which glycogen is stored is a potential therapeutic target in cancer. Tumors from human cell lines (WiDr, PC3, FaDu) have been grown in nude mice, sectioned and stained to measure glycogen storage. Using consecutive frozen sections, levels of hypoxia, glucose, lactate, ATP, and CD31, an endothelial cell marker, have been determined. These sections have been employed to elucidate the “architecture” of tumor metabolism in terms of vessel distance. Additionally, PAS-stained EF5 labeled human tumor samples were used to obtain calibrated hypoxia measurements to correlate with PAS. These studies demonstrate a correlation between hypoxia and the formation of glycogen deposits in human tumors and nude mouse xenografts. In cell culture, formation of glycogen deposits after
exposure to hypoxia has been demonstrated, in addition to expression of glycogen synthase in human cancer cell lines.

The development of novel selective cancer chemotherapeutics will require the identification of differences between cancerous cells and normal host cells to exploit as targets. Several differences in metabolism, including the need to excrete excess lactic acid and store glycogen under hypoxic conditions, are such targets. Novel therapeutics exploiting these targets should be effective against cancer cells and minimally toxic to host cells.
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List of Abbreviations

CNCn, α-cyano-4-hydroxy cinnamate

C.SNARF-1AM, carboxyl semi-naphthyl rhodofluor-1 acetoxymethyl ester

FBS, fetal bovine serum

GADPH, glyceraldehyde phosphate dehydrogenase

MCT1/SLC16A1, H+-linked monocarboxylate transporter isoform 1

MCT4/SLC16A3, H+-linked monocarboxylate transporter isoform 4

MCT2/SLC16A2, H+-linked monocarboxylate transporter isoform 2

LON, lonidamine (TH-070; 1-(2,4-dicholorobenzyl)-1H-indazole-3-carboxylic acid)

MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]

VEGF, vascular endothelial growth factor

GLUT1, glucose transporter-1

Myc, myelocytomastosis viral oncogene

MYCN, transcription factor Myc from neuroblastoma

KRAS, Kirsten rat sarcoma viral oncogene

HUVEC, human umbilical vein endothelial cells

BACEC, bovine adrenal capillary endothelial cells

LDH, lactate dehydrogenase
STAUR, staurosporine
MITO, mitoxantrone
IHC, immunohistochemistry
PAS, periodic acid Schiff stain
HIF-1, hypoxia inducible factor-1
HRE, hypoxia responsive element
RNAi, RNA interference
siRNA, short interfering RNA
shRNA, short hairpin RNA
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Matthew 25:13-30

13Therefore keep watch, because you do not know the day or the hour.

14Again, it will be like a man going on a journey, who called his servants and
entrusted his property to them. 15To one he gave five talents of money, to another two
talents, and to another one talent, each according to his ability. Then he went on his
journey. 16The man who had received the five talents went at once and put his money to
work and gained five more. 17So also, the one with the two talents gained two more.
18But the man who had received the one talent went off, dug a hole in the ground and
hid his master’s money.
19 After a long time the master of those servants returned and settled accounts with them. 20 The man who had received the five talents brought the other five. 'Master,' he said, 'you entrusted me with five talents. See, I have gained five more.'

21 His master replied, 'Well done, good and faithful servant! You have been faithful with a few things; I will put you in charge of many things. Come and share your master's happiness!'

22 The man with the two talents also came. 'Master,' he said, 'you entrusted me with two talents; see, I have gained two more.'

23 His master replied, 'Well done, good and faithful servant! You have been faithful with a few things; I will put you in charge of many things. Come and share your master's happiness!'

24 Then the man who had received the one talent came. 'Master,' he said, 'I knew that you are a hard man, harvesting where you have not sown and gathering where you have not scattered seed. 25 So I was afraid and went out and hid your talent in the ground. See, here is what belongs to you.'

26 His master replied, 'You wicked, lazy servant! So you knew that I harvest where I have not sown and gather where I have not scattered seed? 27 Well then, you should have put my money on deposit with the bankers, so that when I returned I would have received it back with interest.'
28 Take the talent from him and give it to the one who has the ten talents. 29 For everyone who has will be given more, and he will have an abundance. Whoever does not have, even what he has will be taken from him. 30 And throw that worthless servant outside, into the darkness, where there will be weeping and gnashing of teeth.’
1. Introduction

1.1 Variations in tumor metabolism, a potential target

A major goal in cancer research is to identify and utilize differences between host and tumor cells, to kill tumor cells, while minimizing damage to the host. The most commonly used difference is the high rate of proliferation of cancer cells when compared with the low rate of proliferation of well differentiated host cells. While these treatments have met with some success, they are toxic to populations of host cells that actively proliferate, and as of yet have been unable to successfully eradicate many cancers. In order to increase the selectivity and effectiveness of chemotherapeutics, new differences between cancerous and normal host cells must be identified and targeted in ways that are non-toxic to the host.

Cancer cells differ in their metabolic activity from host cells, as early as 1930 Otto Warburg and others recognized that tumor cells use more glucose than non-tumor cells [1-3]. This phenomenon of increased glycolysis regardless of oxygenation state is called the Warburg Effect. The Warburg Effect is the phenomenon that cancer cells perform glycolysis without oxidative phosphorylation whether they are in normoxic or hypoxic conditions. The reasons for this are not completely clear, it may be the result of early clonal selection for tumor cells that were capable of surviving repeated intermittent episodes of hypoxia [4]. Alternatively, it may simply be the result of mutations in the
chain of cytochromes necessary to complete oxidative phosphorylation [5]. Whether it is the result of early selective pressures on dysplastic cells or a breakdown in the machinery of the mitochondrial membrane, oxidative phosphorylation is omitted by many cancer cells even in the presence of adequate oxygen.

Prior to Warburg, Louis Pasteur demonstrated that well oxygenated cultures of yeast cells grew faster and produced less of the products of fermentation than poorly oxygenated cultures. These results were later demonstrated in mammalian cells by Hans Krebs and others and called the Pasteur effect [6]. The Pasteur effect, a primal survival mechanism, describes continued glycolysis with the cessation of oxidative phosphorylation in hypoxic cells. The Pasteur effect occurs in both normal and cancerous human cells when exposed to hypoxia. Under resting conditions most normal cells are rarely hypoxic. However, cancer cells more commonly exhibit the Pasteur effect as the high rate of proliferation and poor vascular supply of cancer cells typically results in tumor hypoxia. The combined result of the Warburg and Pasteur effects is a pronounced increase in the consumption of glucose in tumor cells in the absence of oxidative phosphorylation, which has been demonstrated by both radiologic and direct measurements [7-9]. High glucose consumption without oxidative phosphorylation rapidly exhausts the cellular pool of nicotinamide adenine dinucleotide (NAD+) necessary for the redox reactions of glycolysis. To replenish the supply a cell reduces the glycolytic product pyruvate to lactic acid, coupled with the oxidation of NADH to
NAD+. This reaction is catalyzed through the activity of lactate dehydrogenase (LDH), and results in increased production of intracellular lactic acid. A diagram of glycolysis and glucose metabolism is shown in Figure 1.

![Glycolysis Diagram](image)

**Figure 1: Glycolysis and Glucose Metabolism**

Thus, cancer cells must remove excess lactic acid from the intracellular space to the extracellular space as it builds up as a product of fermentation. If a tumor cell is unable to remove excess lactic acid from its cytosol, it will result in a decline in intracellular pH (pHi) below the range in which most cellular enzymes function optimally[10]. Additionally, low pHi activates several cell death pathways including caspases and
endonucleases which result in apoptosis [11-18]. A buildup of intracellular lactate also feedback inhibits the activity of lactate dehydrogenase (LDH) [19]. This causes arrest of anaerobic glycolysis, eliminating the major source of energy for a hypoxic cancer cell [20]. The proteins responsible for lactic acid transport across the cell membrane are the monocarboxylate transporters (MCTs)[21, 22]. The MCT family of proteins consists of passive membrane transporters for a wide range of monocarboxylates. MCT1 and MCT4 are the MCTs that play a major role in the transport of lactic acid[23]. Both MCT1 and MCT4 are symporters for a proton and lactate, and have the capacity for bidirectional transport[21, 22]. Tumor cells depend on MCT activity to excrete excess intracellular lactic acid to maintain neutral intracellular pH, a positive energy balance, and homeostasis. To withstand the continued production of excess lactic acid, the cancer cell must remove it to the extracellular space. This state of aerobic or anaerobic glycolysis represents a difference between cancer cells and most resting normal cells. This metabolic variance can be targeted to kill cancer cells, while leaving normal cells unharmed.

Tumor hypoxia is a broadly studied and well reported phenomenon that has been examined by both direct and fluorescent measurement techniques [9, 24, 25]. Cancer cells likely begin to experience hypoxia relatively early in tumor development as hyperplasia and growth away from blood vessels limits the oxygen supply of many tumor cells [4]. Later in tumor development, a larger tumor will have developed
vasculature of its own. However, tumor vasculature is characteristically poorly developed, leaky, and typically inadequate to supply the oxygen requirement of a rapidly proliferating tumor. [26, 27]. Both of these largely cancer specific effects mean that cancer cells in the body will be consuming more glucose than surrounding host cells and producing a great deal more lactic acid. This results in the unique tumor microenvironment. The buildup of lactate in tumors has been measured and is indicative of a poor prognosis [28]. The characteristically poorly vascularized, hypoxic, and acidotic tumor microenvironment may actually confer some survival advantage to tumor cells that adapt to living there. Firstly, cancer cells residing in a poorly vascularized tumor bed are less accessible to blood borne chemotherapeutics and immune surveillance [29]. Secondly, hypoxic, fermenting tumor cells are more protected from oxidative stress [30, 31], the reactive oxygen species utilized by the cell mediated immune response [32, 33], and the reactive oxygen species that are generated by radiation therapy [34, 35]. Finally, it has been hypothesized that the acidic microenvironment of many tumors plays a role in their ability to invade surrounding tissues by degrading surrounding extracellular matrix, disguising the tumor cells from immunosurveillance, and by causing the environment to become disadvantageous for the growth of host cells [36]. While it is possible to hypothesize and demonstrate ways in which a microenvironment of hypoxia and acidosis and an increased rate of glycolysis and fermentation could confer a benefit to tumor cells it clearly also belies a potential
target. Most normal host cells are well vascularized, well supplied with glucose, and well oxygenated. Therefore most normal host cells engage in very little fermentation. Well oxygenated host cells are also capable of using substrates other than glucose as an energy source. Although many host cells transport lactate across their cell membranes, few, if any, are as dependent on the ability to transport lactate for their survival as are cancer cells.

The precise mechanisms by which glycolysis is constitutively upregulated in cancer cells are not well defined. It is presumed, however, that the glucose transporters and hexokinases are the key molecules in upregulating glycolysis, as they are the rate controlling molecules in normal cells. The selective advantage conferred by upregulating glycolysis is likely insensitive to the mechanism by which it is upregulated; although different cancer cells might acquire different mutations to upregulate glycolysis. A key player in the glycolytic response is the transcription factor hypoxia-inducible factor-1α (HIF-1α). This factor mediates a pleiotropic response to hypoxic stress by inducing several survival genes, including glucose transporters; angiogenic growth factors (such as VEGF); hexokinase II, and hematopoietic factors [4, 37-39]. In some systems, constitutively increased HIF-1α levels are sufficient to result in constitutively high glucose consumption rates [4, 40, 41]. Although, increased HIF-1α activity is strongly associated with glycolytic tumors glycolysis can be constitutively activated by a number of pathways. In some models, upregulation of glycolytic
enzymes can be coordinated in response to oxidation-reduction changes by the Sp1 transcription-factor complex [4, 30]. Glucose Transporter-1 (GLUT1) can be upregulated directly by MYC or indirectly by KRAS [4, 42-44]. Hexokinase II can be transcriptionally activated by mutant p53 or through demethylation of its promoter [4, 45, 46]. The glycolytic phenotype is not a secondary phenomenon. Rather, it is directly selected because it provides a survival advantage in the unreliable conditions a tumor develops in and abundant energy to achieve a growth advantage. Constitutive upregulation of glycolysis occurs through mutations, epigenetic changes such as an alteration of promoter methylation, or a combination of such events [4]. The consequences of constitutive glycolysis are a steady supply of energy for the developing cancer and a substantial increase in the amount of lactic acid produced by the cancer cell.

1.2 Adaptations to and dangers of acidosis

The side effect of continual glycolytic metabolism is the continual production of lactic acid inside the cell. When intracellular lactic acid levels rise, the decline in intracellular pH (pHi) will eventually cause the cell to undergo apoptosis or necrosis [47]. There are many mechanisms by which the cell is sensitized to apoptosis, or apoptosis is induced in response to low pHi. DNaseII, a ubiquitous enzyme in mammalian tissues, has been shown to mediate internucleosomal DNA digestion characteristic of apoptosis following cytosolic acidification [12, 13, 47]. Other pH-
dependent endonucleases have also been identified, these endonucleases have similar functions to DnaseII, and also play a role in apoptosis initiation. Some of these endonucleases are activated by changes in intracellular calcium in addition to pH changes [11, 16, 18, 47].

In addition to increasing endonuclease activity, a decline in pH also increases caspase activity. The two caspases most likely to be sensitive to pH activation are caspase 9 and caspase 3, the cytochrome c activated caspases [17, 48-50]. In addition to exhibiting greater activation and greater sensitivity to activation at acidic pH, procaspase 3 has an intrinsic ‘safety catch’ mechanism provided by an Asp-Asp-Asp tripeptide that is removed upon acidification such that it cannot be activated to caspase 3 under alkaline conditions [17, 47]. After activation caspase 3 and 9 activity will continue at acidic or alkaline pH, it is the activation step that is pH sensitive.

Clearly a decline in pH below normal levels is deleterious to cancer cell survival. The levels of lactic acid produced by rampant glycolysis would cause a major reduction in pH unless the cell was able to acquire mechanisms to eliminate the excess protons. The primary way that cells can combat this increase in lactic acid in the cytoplasm is by increasing the plasma membrane expression or activity of monocarboxylate transporters 1 and 4 (MCT1 and MCT4). Both MCT1 and MCT4 are transporters with high affinity for lactate and a proton. Both MCT1 and MCT4 transport lactate and a proton through the plasma membrane passively according to the concentration gradient of lactate and
protons. Since the pKa of lactic acid is 3.86 it dissociates almost entirely to the lactate anion and a proton at physiological pH. MCT’s are the primary mechanism to transport these charged species across the plasma membrane [21].

Other proton transporters also play a role in keeping the intracellular pH in the neutral range. The Na⁺-H⁺ exchanger and vacuolar H⁺-ATPases have both been observed to be upregulated in cancers and likely play a role in pH regulation in some cancers [47, 51-54]. Plasma membrane expressed ATP synthase plays a role in the regulation of pH when it is expressed on cancer cells [55]. Regardless of which pH regulatory mechanism(s) a cancer cell is using the end result is that protons generated inside the cancer cell are removed to the outside of the cell preserving the pH while acidifying the extracellular pH (pHe). Cancer cells adapt to extracellular acidification both acutely and chronically. Chronic adaptation results in a significant increase in the expression of pH regulatory proteins, and alteration in the metabolism of cancer cells [56, 57]. Once the tumor cell has adapted to be able to efficiently remove protons from the intracellular space to the extracellular space, the reduction in pHe is an alteration to the local microenvironment that is less harmful to the tumor cell than to competing populations [4]. Acidification of the microenvironment facilitates tumor invasion both through destruction of adjacent normal populations that cannot adapt to extracellular acidification and through acid-induced degradation of the extracellular matrix and promotion of angiogenesis [4].
1.3 H⁺ Linked Monocarboxylate Transporters 1 and 4 and other proton transporters

Central to understanding how tumor cells eliminate lactic acid is an understanding of MCT1 and MCT4. MCT1 and MCT4 are members of the monocarboxylate transporter family of proteins. They are expressed in the plasma membrane where they function to transport charged lactate and a proton across the hydrophobic membrane. The proton and lactate are always co-transported in the same direction. Transport is passive and driven only by the concentration gradient [58, 59]. Structurally both MCT1 and MCT4 have 12 membrane spanning regions arranged in a circular pattern to form a pore with both the NH₂ and COOH-terminal ends of the protein protruding intracellularly [60, 61]. MCT1 and MCT4 are both tightly associated with CD147 or less often gp70 in the plasma membrane; no other MCT’s have been shown to associate with CD147, although MCT2, a pyruvate transporter, requires association with gp70 for its functional expression [62]. CD147 facilitates the membrane expression of MCT1 and MCT4; when these MCT’s are overexpressed experimentally in the absence of CD147 they remain in the cytoplasm. If CD147 is co-expressed with the MCTs, they will be expressed in the plasma membrane along with CD147 [63].

Although both MCT1 and MCT4 are capable of transporting lactate and a proton in either direction based on the concentration gradient, some clues as to their function in vivo may be taken from their expression levels in various normal human tissues, and
from *in vitro* experimental data. Skeletal muscle is the main producer of lactic acid in the body, with erythrocytes and leukocytes being secondary producers to a lesser degree [21]. Skeletal muscle produces the most lactic acid in the transition from rest to very heavy work. This is because the maximal glycolytic work capacity of a muscle exceeds its maximal oxidative work capacity [64]. Additionally, the acceleration of glycolysis as an energy source is more rapid than that of oxidative metabolism as an energy source [23, 59, 65]. In examining muscle fibers, white fast twitch muscle fibers have a low mitochondrial content are mostly glycolytic and have a need to export lactic acid with almost any level of work, red muscle slow twitch fibers have a high mitochondrial content are mostly oxidative and import lactate to convert to pyruvate as a fuel source under most work rates only needing to export lactic acid at the highest work rates.

MCT1 and MCT4 expression has been confirmed in skeletal muscle by western blotting and immunofluorescence microscopy. The level of expression of either transporter can vary considerably from muscle to muscle and from person to person [66-69]. MCT1 expression correlates with a muscle’s overall mitochondrial content. In mammalian species, muscles that are composed of mostly slow twitch oxidative fibers such as the soleus express a high level of MCT1; whereas muscles that are composed of mostly fast twitch glycolytic fibers such as the gastrocnemius express a low level of MCT1 [23, 67, 69-71]. These data suggest that high levels of MCT1 expression in a muscle reflect the need to import large amounts of lactate as an oxidative fuel source. In rats, MCT4 is
present in all muscles but it has much lower expression in highly oxidative muscle such as the soleus [67]. Unfortunately, the situation regarding MCT4 expression in humans is not as clear cut. MCT4 expression level is independent of fiber type and variation from individual to individual is large [71]. This may suggest that human muscle fibers have the need to resort to glycolytic metabolism more so than rat muscle fibers or simply that MCT1 and MCT4 are playing a more mixed role as lactate importer and exporter in human muscle than in rat muscle. Experimental data in vitro have demonstrated that MCT1 is capable of a lower rate of lactate transport than MCT4 [23, 68, 71, 72]. Additionally, MCT1 has a higher affinity for lactate compared to MCT4 (Km~5mM versus Km~20mM) [23, 68, 72]. These data support the hypothesis that MCT1 is specialized for import of lactic acid for use as a substrate in oxidative metabolism and that MCT4 is specialized for the export of lactic acid as a byproduct of glycolytic metabolism. However, there are well documented cases where MCT1 functions for lactic acid export. Mature human erythrocytes metabolize entirely anaerobically as they lack mitochondria; these cells express high levels of MCT1 for lactate efflux and little or no MCT4 [21, 58, 73]. Also, human T lymphocytes increase protein expression of MCT1 upon activation to aid in lactic acid efflux during rapid proliferation, a time during which they predominantly use glycolysis for energy [74]. Based on tissue expression and in vitro experimentation, these data suggest that MCT1 has preferential activity in lactate import and MCT4 has preferential activity in lactate export, however considerable
variation exists between the roles played by MCT1 and MCT4 in individual tissues and species.

While MCT1 and MCT4 are central players in the cellular response to excess lactic acid production, other molecules may also play an important role in removing excess protons. Cell surface ATP synthase, the Na⁺/H⁺ exchanger, the Cl⁻/HCO₃⁻ exchanger, and plasma membrane and vacuolar H⁺-ATPase can also play a role in maintaining intracellular pH in tumor cells [53, 55, 75-77]. Cell surface-associated ATP synthase functions as a proton channel, probably enabling protons to flow down their concentration gradient into caveolae where they can be released into the extracellular space along with ATP generated at the cell surface [76]. The Na⁺/H⁺ exchanger (NHE) and the Cl⁻/HCO₃⁻ (AE) are structurally homologous, electroneutral plasma membrane ion transporters. Both molecules are predicted to have 12 transmembrane domains with NH₂ and COOH-terminal cytoplasmic domains. The NHE has a relatively short NH₂-terminal and long COOH-terminal domain; while the AE has a relatively long NH₂-terminal and short COOH-terminal domain making the molecules rough mirror images of each other [77]. Vacuolar H⁺-ATPase couples ATP hydrolysis with proton transport into a vacuole or out of the plasma membrane depending on where the enzyme is situated. The H⁺-ATPase shares many structural features with ATP synthase and in many ways can be thought of as the synthase running in reverse [78, 79]. These molecules have varied levels of expression in different cancer cell lines, and the extent to
which they are important to a particular cell line usually correlates with their level of expression. An example of when these other proton transporters are absolutely critical is observed when a cell imports lactate to convert to pyruvate for oxidative metabolism. For every molecule of lactate that is imported via MCT1, a proton is imported. A cell is severely limited as to how much lactate is transported into the cell unless it can eliminate excess protons. This is especially important for hepatocytes involved in the Cori cycle, oxidative muscle fibers using lactate as a fuel source, and cancer cells in a well-vascularized portion of a tumor that sometimes use lactate as a fuel source.

1.4 Pharmacologic Targeting of Monocarboxylate Transporters

Four classical categories of MCT inhibitors exist: (1) the substituted aromatic monocarboxylates such as α-cyano-4-hydroxycinnamate (CHC) and phenylpyruvate; (2) inhibitors of anion transport such as the stilbenedisulphonates, including niflumate and 5-nitro-2-(3-phenyl-propylamino)benzoate; (3) the bioflavenoids such as phloretin and quercetin; (4) miscellaneous inhibitors such as thiol reagents including p-chloromercuribenzenesulphonate (p-CMBS) and amino reagents such as pyridoxal phosphate and phenylglyoxal [21]. The indole derivative compound lonidamine, which is an MCT inhibitor, has been utilized as an experimental anti-cancer drug in several studies and clinical trials. Lonidamine has been successful when used on cancer cells known to express MCTs and have a highly glycolytic metabolism, or when intracellular pH was measured as an endpoint [80-84]. More recently, potent and specific inhibitors
of MCT1 have been synthesized [74, 85]. These compounds inhibit MCT1 activity in the nM range [74, 85]. They are approximately 20 fold less potent inhibitors of MCT2 activity, and they exhibit nominal inhibition of MCT4 [74, 85]. These compounds also have significant immunomodulatory activity as they inhibit the high rate of glycolysis and lactic acid efflux associated with the rapid proliferation of lymphocytes [74, 85]. Such compounds should also exhibit excellent inhibitory effects on tumor cell proliferation if utilized in an appropriate model. MCT4 inhibition has not been as extensively studied as MCT1. Most of the classical pharmacologic inhibitors of MCT1 have inhibitory effects on the activity of MCT4, although the potency of a given drug can vary widely [68].

1.5 Glycogen Storage in Cancer

The hypothesis that glycogen is used by cancer cells to promote survival under conditions of hypoxia or glycopenia, was developed in collaboration with Thies Schroeder in the laboratory of Mark W. Dewhirst.

Glycogen production is closely tied to glucose metabolism (Fig 1), as glycogen is the biologic storage form of glucose. Some cancers are known to store glycogen, however, the reasons for this are largely unknown [86-98]. Glycogen storage also occurs in numerous human cancer cell lines [99]. Cancer cells have an increased demand for glucose due to a combination of the Pasteur and Warburg effects [4, 7-9]. Tumor
glycogen may play a role analogous to an energy “savings account” allowing tumor cells to store glucose for future times of need when the cancer cells may be exposed to hypoxia or glycopenia. Glycopenia, is a state of reduced availability of glucose in the extravascular compartment of tissues, as hypoglycemia refers specifically to low blood glucose concentrations.

A physiological precedent for this behavior is observed with muscle tissue exposed to hypoxia. Skeletal and cardiac muscle tissue both store glycogen under basal conditions [100, 101]. However, when muscle tissue is severely depleted of glycogen stores by exposure to hypoxia or a high level of exercise under glycopenic conditions, then allowed to rest, it “supercompensates” for the lost glycogen by depositing glycogen stores of at least double its basal levels [100, 101]. These glycogen deposits confer a survival benefit to myocytes when they are exposed to conditions of hypoxia and glycopenia as a result of ischemia [102-106]. Therefore, continuously well perfused resting muscle only stores a basal amount of glycogen, but muscle that is heavily exercised or has had interruptions in perfusion ‘anticipates’ the need for future stores of glucose by dramatically increasing its glycogen stores. If cancer cells, which are known to endure and survive transient exposures to hypoxia [4, 9, 24, 107, 108], exhibit the same sort of behavior then the mechanisms of glycogen storage in cancers would be a potential target in cancer therapy. The attractiveness of this target is enhanced by the
fact that glycogen storage is not known to be necessary for the survival of any normal, resting tissue. Mice with a knockout in muscle-specific protein phosphatase PP1G/RGL experience an approximate 90% reduction in glycogen deposits compared to control mice with no adverse effects [109]. While glycogen is necessary for sustained activity in exercising or metabolically active glucose deprived tissues, resting tissues can survive without it.

While the potential role of glycogen in cancer cell survival is adequate reason for investigation of the mechanisms of glycogen deposition as a potential target, there is a second reason glycogen could be important in cancer. It has long been known that the most deadly event in the vast majority of cancers is metastasis [110]. Indeed, few cancers are capable of killing their host without the additional burden of widely disseminated metastatic lesions [110]. The process of metastasis itself is a multi-stage event which requires cancer cells to invade surrounding tissues, enter the bloodstream, depart the bloodstream, invade tissues surrounding the distant capillary, and grow in this new environment [111]. While metastasizing cells would clearly be normoxic and well supplied with glucose while in the bloodstream, as the process of extravasation, invasion, and proliferation in distant tissue begins, the metastasizing cells will become hypoxic and glycopenic [24, 112]. During this critical phase of metastasis, well developed stores of glycogen would aid immensely in early proliferation and invasion.
Indeed the first 48-72h, before the process of angiogenesis can provide the cancer cells with a blood supply [24, 112], may be the most critical phase in metastasis. If cancer cells are capable of glycogen supercompensation in the manner that myocytes are, then studies of hypoxic cancer cells in a metastasis model may already be supporting this hypothesis. Studies by Cairns et al [113, 114] have demonstrated that cancer cells acutely treated with hypoxia prior to injection in a murine model of metastasis have an increased capacity to form metastasis. This intriguing finding, coupled with an understanding of glycogen “supercompensation” in muscle tissues, demands an exploration of the potential for glycogen deposition and “supercompensation” in cancer cells and its potential role in cancer pathophysiology and metastasis.

2.1 Introduction

Neuroblastoma is a childhood malignancy postulated to originate from precursor cells of the postganglionic sympathetic nervous system [115]. The disease is remarkable for its clinical heterogeneity, with spontaneous regression commonly seen in infants diagnosed before the age of 12 months, but relentless malignant progression commonly observed in children diagnosed subsequently. This latter category of “high-risk” patients represents 50% of all cases. Moreover, mortality in this subset remains greater than 60% despite aggressive chemoradiotherapy [116, 117]. Clearly, new treatment approaches are needed.

High lactic acid production is a common feature of many solid-tumor cells [118-120], and the transporters responsible for its export are well characterized [121]. Further, it is known that MCT isoforms 1 (MCT1) and 4 (MCT4) both have elevated activity in human melanoma cells in response to low extracellular pH [122]. This suggests that the level and/or affinity of proton export transporters may be increased in cancer cells and that these transporters function to prevent lethal intracellular pH decreases as tumors grow and become more acidic. In a recent microarray screen, we noted differential expression of MCT1/SLC16A1 mRNA, but not of any other SLC16A family members in
relation to tumor phenotype [123]. It was therefore hypothesized that expression of one or more of the MCT proteins would be associated with more malignant subsets of neuroblastoma. It is demonstrated that pharmacologic manipulation of these transport proteins is feasible and that the inhibition of such proteins may define a new class of novel therapeutics for neuroblastoma, and perhaps other neural-crest derived malignancies such as melanoma [122, 124].

2.2 Methods

2.2.1 Cell lines and primary tumors

The neuroblastoma cell lines IMR32, Sk-N-SH, and NGP were obtained from the American Tissue Culture Collection (Manassas, VA). CHP134 cells were isolated from a patient at the Children’s Hospital of Philadelphia. Rho-negative SY5Y neuroblastoma cells were generously provided by Dr. W. Davis Parker, Jr. (U. of Virginia School of Medicine, Charlottesville, VA). The endothelial cell line BACEC was a generous gift of Dr. M. Judah Folkman (Harvard Medical School, Boston, MA). Cells were grown in Dulbecco’s Modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and 26 mM sodium bicarbonate. Media was supplemented with 2 mM glutamine and 1% penicillin/streptomycin. Cells were maintained in a humidified 37°C incubator at 5%
CO₂ for a pH of 7.3, or 15 mM sodium bicarbonate, 17% CO₂ to lower extracellular pH to 6.5. These cell lines were maintained in logarithmic growth at 37°C as monolayers in 75 cm² flasks.

Eighty-three primary neuroblastomas were obtained at diagnosis from children enrolled in Children’s Oncology Group (COG) Biology studies. Patient clinical characteristics and risk group were determined using standard COG algorithms as described [125]. Biopsy samples were snap frozen in liquid nitrogen and a diagnosis of neuroblastoma was confirmed by central pathologic review. MYCN gene copy number status was determined by fluorescence hybridization in situ; DNA index was determined by flow cytometry following cell dissociation [117, 126]. We estimated the percent of tumor cell content in pilot sections before nucleic acid extraction. Only samples with a greater than 70% neuroblastoma cell content were included in this study. We isolated RNA from exponentially growing cell lines or from 50-100 mg of snap-frozen tumor samples using TRIzol reagent (Invitrogen, Carlsbad, CA). All RNA samples were subjected to DNase I (Ambion, Houston, TX) digestion to eliminate contaminating genomic DNA, then purified with QIAGEN RNeasy Mini Kit (Valencia, CA). RNA quality was checked by spectrophotometry, 1% agarose gel, as well as by microfluidics-based electrophoretic analysis (Agilent 2100 Bioanalyzer). The Children’s Hospital of Philadelphia Institutional Review Board approved this study.
2.2.2 Reagents

The fluorochrome Carboxy SNARF-1 acetoxyethyl ester (C.SNARF-1AM) and the detergent Pluronic F127 were obtained from Molecular Probes (Eugene, OR). Matrigel® was purchased from BD Biosciences (Bedford, MA). The MTS kit was obtained from Promega (Madison, WI). All PCR reagents except Tri Reagent were from Invitrogen (Carlsbad, CA). Tri Reagent and all other reagents, including lonidamine and α-cyano-4-hydroxy cinnamate (CNCn), was obtained from Sigma Chemical Co. (St. Louis, MO). Lonidamine was diluted into media from a 16 mM stock solution of DMSO. DMSO and other vehicle controls were negative with respect to effects on intracellular pH and viability. MYCN siRNA was obtained from Ambion (Austin, TX) siRNA ID# 114526 Sense strand 5′ GGAGCAUGUUUGUAUACAtt 3′ Antisense Strand 5′ UGUAUACAAAACAUCCUCCtc 3′, Silencer Negative Control #1 siRNA was also obtained from Ambion. Lipofectamine 2000 was obtained from Invitrogen Corporation (Carlsbad, CA).

2.2.3 Western Blot Materials

Primary antibodies to MCT1 (C-20) and MycN (H-50) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibody IR dye conjugates donkey anti-
goat IR Dye 800 nm and goat anti-rabbit Alexa Fluro 680 nm were obtained from Rockland (Gilbertsville, PA) and Molecular Probes (Carlsbad, CA) respectively.

2.2.4 Western Blotting Methods

Neuroblastoma cells were solubilized in aqueous buffer containing 2% sodium dodecyl sulfate, 62.5 mM Tris, and 20% glycerol at pH 6.8, scraped from T-25 cell culture flasks, and placed in microfuge tubes. Cells were pulsed for 5 seconds with a probe sonicator, vortexed for 10 s, and centrifuged at 1000xg for 2 min. The protein levels of the samples were measured by BioRad BCA assay. Samples were all standardized to 0.5 mg/mL protein by dilution with gel loading buffer containing 2% sodium dodecyl sulfate, 62.5mM Tris, 20% glycerol, 10% 2-mercaptoethanol, and 0.01% bromophenol blue at pH 6.8. Samples were heated in boiling water for 5 min and then centrifuged at 1000 x g for 5 minutes. 20 μL of each sample was loaded into each well of a 4-15% polyacrylamide Tris HCl Bio Rad Ready Gel for a total of 10 μg of protein per lane. PAGE was performed at 180V constant voltage in Tris/Glycine/SDS running buffer 25 mM/192mM/0.1% pH 8.3 for 1 h. Protein was transferred to Amersham Biosciences Hybond-C Extra® Nitrocellulose 45 μm for 30 min at 15V constant voltage on a BioRad Transblot Semi-dry transfer cell in Tris/Glycine buffer 25 mM/192 mM containing 10% methanol at pH 8.3. Membranes were blocked in Rockland blocking buffer for near infra red fluorescent Western blotting at room temp for 1 h. Primary antibody was
added at a dilution of 1:1000 in blocking buffer and incubated with the blots overnight at 4°C. Membranes were washed five times for five minutes in PBS with 0.05% Tween 20 after which the membranes were placed in blocking buffer with secondary antibody IR dye conjugate at a dilution of 1:10,000. Membranes were again washed five times for five minutes in PBS with 0.05% Tween 20 after which they were rinsed once with PBS. Western blots were read on a Li-cor Odyssey Infrared Imager.

2.2.5 Primary tumor real-time quantitative RT-PCR (Q-RT-PCR)

Q-RT-PCR was used to investigate expression of MCT1/SLC16A1 in 83 primary neuroblastoma samples. One μg of total RNA was used for cDNA synthesis with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Q-RT-PCR was performed using an ABI Prism SDS-7900HT thermal cycler (Applied Biosystems, Foster City, CA) in two separate reactions with differently labeled MCT1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). RNA-specific primers and probes were obtained from ABI “Assay-on Demand” (catalog number Hs00161826m1 for MCT1). Relative expression of target genes was determined by normalization to GAPDH according to the manufacturer’s instructions. All Q-RT-PCR experiments included a no-template control and were performed in triplicate.
2.2.6 RT-PCR on established cell lines

Three neuroblastoma cell lines, IMR32, Sk-N-SH, and NGP, were cultured at pH 6.5. Extracellular pH was adjusted by raising the CO₂ to 17% for 48 h, then cells were harvested and total RNA was extracted by using Tri Reagent according to the manufacturer’s instructions. Subsequently, RT-PCR was performed. In brief, reverse transcription was carried out using the cloned AMV cDNA Synthesis Kit (Invitrogen; Carlsbad, CA) with random hexamers (25°C for 10 min followed by 50°C for 50 min). For the PCR reaction, Platinum Taq polymerase was used. The sequences of specific primers were as follows:

- MCT-1 sense, 5’-TTTCTTTTCGGCTTCCGTTGTTG-3’;
- MCT-1 antisense, 5’-TCAATTTACCCCTTCAGCCCCATGG-3’;
- MCT-4 sense, 5’-TTTTGCTGCTGGGCAACTTCTTCTG-3’;
- MCT-4 antisense, 5’-TCACGTTGTCTCGAAGCATGGTGT-3’;
- MYCN sense, 5’-CACAAGGCCCTCAGTACCTC-3’;
- MYCN antisense, 5’-GATCAGCTCGTGACTGAG-3’;
- β-actin sense, 5’-TGCGTGACATTAAGGAGAAG-3’;
- β-actin antisense, 5’-CTGCATCCTGTCGGCAATG-3’.

The cycling parameters are as follows: for MCT1 and MCT4, 35 PCR cycles of 30 s for denaturing at 94°C, 30 sec for annealing at 62°C, and 45 s for DNA synthesis at 72°C; for MYCN, 40 PCR cycles of 30 sec for denaturing at 95°C, 30 s for annealing at
55°C, and 1 min for DNA synthesis at 72°C; for β-actin, 25 PCR cycles of 15 s for denaturing at 94°C, 30 s for annealing at 55°C, and 1 min for DNA synthesis at 68°C. PCR products then underwent electrophoresis on ethidium bromide-stained 2% agarose gels.

2.2.7 Intracellular pH measurements

2.2.7.1 Cell preparation

Cells were plated on coverslips affixed to microwell dishes at a density of 0.2 × 10^6 cells in 2 ml of medium, 24-48 h prior to experiments. Coverslips were coated prior to plating with a 1:3 dilution of Matrigel® in serum-free medium as previously described [127]. Dye leakage during an experiment of 1 h or less was undetectable under these conditions (data not shown).

2.2.7.2 Dye Loading

Cells were incubated for 15 min with 9 μM C.SNARF-1AM [57] in medium containing 10% FBS in a 37°C incubator under 5% CO₂ as previously described [127, 128]. Following a change of medium, we further incubated the cells were further incubated for 20 min at 37°C, 5% CO₂ to complete hydrolysis of the dye ester to C.SNARF. Each plate was then mounted on the microscope stage and warmed to 37°C under flowing humidified air containing 5% CO₂. In experiments in which extracellular
pH was lowered, this was done by lowering the bicarbonate concentration in the medium while keeping the sodium concentration constant.

2.2.7.3 Fluorescence microscopy and calibration

Intracellular pH values were obtained from intracellular whole emission spectra of the pH-sensitive dye C.SNARF-1 (cleaved from the membrane permeable AM form of the dye). The dye was loaded into cells before mounting them on an inverted fluorescence microscope. The whole-spectrum approach to measurement, calibration technique, and experimental methodology have been described in detail [122].

2.2.7.4 Protocol for extended intracellular pH time courses

The experiments were performed on substrate-adherent cells at 37°C in complete growth medium. Initially, steady-state for intracellular pH were determined of cells in 2 ml of complete growth medium. The initial steady-state intracellular pH at extracellular 7.3 was measured several times on a field of 8-15 cells. We then replaced the medium with medium containing lonidamine and/or having an extracellular pH of 6.5. We then measured the intracellular pH for 30 min, during which it equilibrated. The fluorescence signal used to measure intracellular pH was collected on a single field of cells for 5 s of every 10 s during the course of experiments.
2.2.8 [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) assay

The MTS assay [129] was performed to determine effects of lonidamine on metabolic viability under conditions designed to match the experiments in which intracellular pH measurements were made. Cells were seeded in 96-well culture plates (10,000 cells/well) and after an overnight pre-incubation, cells were exposed to varying concentrations of lonidamine for 48 h. The MTS reagents were then mixed and added to quantitate in a colorimetric assay the number of viable cells present in each treatment group. Color development was monitored with a spectrophotometer at $\lambda=490$ nm. The effect of lonidamine on metabolic viability was expressed as the percentage of cells that were viable relative to the percentage of such cells in untreated controls.

2.2.9 Cell Death Assay

Cell Death Detection ELISA Plus 10x was obtained from Roche Applied Science (Indianapolis, IN). Cell death assays were carried out according to the manufacturer’s suggested methods for measuring both apoptotic cell death and necrotic cell death in adherent cells.
2.2.10 Statistical Analysis

Since distributions for gene-expression data are skewed, non-parametric analyses were used. For the RT-PCR data and clinical correlations, the two-sample Wilcoxon rank-sum test was used to explore possible associations between MCT1 gene expression and age (dichotomized at 1 year of age), stage (4 versus other), risk group (high-risk versus other), Shimada histopathology (unfavorable versus favorable), DNA index (diploid versus other), and MYCN status (amplified versus not amplified). The Kruskal-Wallis test was used to examine gene expression with risk group of three categories. Intracellular pH and MTS data are expressed as means ± SE.Student’s t-test was used to determine the significance between each experimental group. Box-plots were used to describe gene expression by clinical and biological factors.

2.3 Results

2.3.1 MCT1/SLC16A1 mRNA is differentially expressed in neuroblastoma.

Table 1 shows the distribution of clinical and biological co-variates for the 83 neuroblastomas studied. Q-RT-PCR first showed high concordance with Affymetrix microarray information [123].
### Table 1: Neuroblastoma MCT1 Expression

**Summary of Taqman PCR (MCT1/GAPDH ×10)**

<table>
<thead>
<tr>
<th>Variables</th>
<th>N</th>
<th>Median (Min, Max)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age at Diagnosis</strong></td>
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<tr>
<td>&lt;1 year</td>
<td>36</td>
<td>5.89 (3.24, 10.5)</td>
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<td>&gt;1 year</td>
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<td>59</td>
<td>7.10 (2.17, 14.4)</td>
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<tr>
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<td>1, 2, 3</td>
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<td>5.65 (2.02, 10.1)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>7.63 (2.87, 14.4)</td>
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<tr>
<td><strong>Risk Group</strong></td>
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<td>.0016</td>
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<tr>
<td>Low</td>
<td>24</td>
<td>5.30 (2.02, 9.55)</td>
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<tr>
<td>Intermediate</td>
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<td>6.55 (2.17, 10.1)</td>
<td></td>
</tr>
<tr>
<td>High and Ultrahigh</td>
<td>40</td>
<td>7.96 (2.87, 14.4)</td>
<td></td>
</tr>
<tr>
<td><strong>Risk Group</strong></td>
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<td>Low and Intermediate</td>
<td>43</td>
<td>5.87 (2.02, 10.1)</td>
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<tr>
<td>High and Ultrahigh</td>
<td>40</td>
<td>7.96 (2.87, 14.4)</td>
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<td><strong>Shimada</strong></td>
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<tr>
<td>Favorable</td>
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<tr>
<td>Unfavorable</td>
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<td>7.76 (2.87, 14.4)</td>
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<td>Diploid</td>
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<tr>
<td>Hyperploid</td>
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<td><strong>MYCN</strong></td>
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<td>Amplification</td>
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<td>Single Copy</td>
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</table>
High MCT1/SLC16A1 mRNA expression was significantly associated with age > one year at diagnosis, stage 4 disease, unfavorable Shimada histopathology diploid DNA index, and MYCN amplification, and high-risk clinical group by COG criteria as shown in Figure 2.

Figure 2: Correlation of MCT1 mRNA expression with stage classification in neuroblastoma.

The categories that the NB biopsies were divided into are as follows: Human Fetal (H.F.) Brain (normal), Low Risk, Intermediate (Int.) Risk, High Risk, and Ultra High Risk NB Groups. These clinical categories are further defined as follows: Low risk NB were completely resectable, with no metastases. Moderate Risk were not completely resectable (incomplete gross excision), with no metastases. High Risk were those with ipsilateral lymph node involvement. Ultra High Risk were those with widely disseminated disease (commonly to liver, bone marrow, and/or skin, and other organs).
2.3.2 Neuroblastoma cell lines exhibit \textit{MCT1} message.

Analysis of cell lines by RT-PCR at normal and low pH showed that \textit{MYCN} and \textit{MCT1} were expressed in the IMR32 and NGP cell lines, both of which have \textit{MYCN} amplification (Fig. 3). In contrast, Sk-N-SH cells with no genomic amplification of the \textit{MYCN} locus, expressed low amounts of \textit{MCT1} and little \textit{MYCN}. In NGP cells, \textit{MYCN} and \textit{MCT1} were high at both normal and low extracellular pH. In all cases, \textit{MYCN} expression and \textit{MCT1} expression were positively correlated.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Expression of \textit{MCT1}, \textit{MCT4} and \textit{MYCN} mRNA in three neuroblastoma cell lines by RT-PCR.}
\end{figure}

Cells were cultured at pH 7.3 or 6.5 for 48 h, after which the cells were harvested and their total RNA extracted for RT-PCR. Lane 1, IMR32 cells at pH 7.3; lane 2, IMR32 cells at pH 6.5; lane 3, Sk-N-SH cells at pH 7.3; lane 4, Sk-N-SH cells at pH 6.5; lane 5, NGP cells at pH 7.3; lane 6, NGP cells at pH 6.5. The control in all lanes was \textit{β}-actin. This experiment was repeated with similar results.
2.3.3 Neuroblastoma cell lines exhibit MCT1 protein.

Analysis of cell lines by Western blot indicate that MCT1 is expressed in all of the cell lines tested. Figure 4 shows similar expression of this protein in IMR32, NGP, and Sk-N-SH cells.

![Figure 4: MCT1 Protein Expression in Three Neuroblastoma Cell Lines.](image)

Cells were cultured at pH 6.5 or 7.3 for 48 h, after which the cells were harvested and their protein was extracted for Western blot. Lane 1: Molecular weight standards, Lane 2, Sk-N-SH cells at pH 6.5; lane 3, Sk-N-SH cells at pH 7.3; lane 4, NGP cells at pH 6.5; lane 5, NGP cells at pH 7.3; lane 6, IMR32 cells at pH 6.5; lane 7, IMR32 cells at pH 7.3. The western blot with anti-MCT1 is shown in part A. The control in all lanes was β-actin shown in part B. This experiment was repeated with similar results.

2.3.4 Lonidamine dose response during acute acidification of CHP134 cells.

Figure 5 shows the effect of lonidamine, an MCT inhibitor, on the intracellular pH of CHP134 neuroblastoma cells having an extracellular pH of 6.5. Lonidamine immediately exerted a lowering effect on intracellular pH, which reached a minimum level within 15-
On continued exposure to lonidamine, a new steady-state intracellular pH was sustained. In this cell line at the highest dose of lonidamine the intracellular pH decreased to below 6.5.

Figure 5: Intracellular pH dose response to lonidamine with and without acute acidification in CHP134 cells.

Cultured cells were subjected to increasing doses of lonidamine at pH 6.5. Cell viability was measured using the MTS assay. A dose response was observed from 80-320 μM. Intracellular pH was lowered to 6.5 or lower from 160-320 μM. Filled squares: untreated. Open squares: acute acidification to 6.5. Filled diamonds: acute acidification plus DMSO vehicle. Open diamonds: 80 μM lonidamine plus acute acidification. Filled triangles: 160 μM lonidamine plus acute acidification. Open triangles: 240 μM lonidamine plus acute acidification. Small filled squares: 320 μM lonidamine plus acute acidification.

2.3.5 Lonidamine lowered intracellular pH at low extracellular pH.

2.3.5.1 Intracellular acidification in Neuroblastoma Cells
After extracellular acidification, intracellular pH was measured in IMR32, Sk-N-SH, and NGP cells after extracellular acidification combined with various concentrations of lonidamine to demonstrate a dose response (Table 2).

### Table 2: Acidification with and without MCT inhibition in neuroblastoma cell lines

<table>
<thead>
<tr>
<th>Extracellular pH before and after drug addition</th>
<th>NGP cells Intracellular pH</th>
<th>Sk-N-SH cells Intracellular pH</th>
<th>IMR-32 cells Intracellular pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.3→7.3</td>
<td>7.30±0.01</td>
<td>7.41±0.01</td>
<td>7.47±0.01</td>
</tr>
<tr>
<td>7.3→6.5+vehicle</td>
<td>7.07±0.06</td>
<td>7.25±0.003</td>
<td>7.03±0.02</td>
</tr>
<tr>
<td>7.3→6.5+160 μM lonidamine</td>
<td>6.96±0.02</td>
<td>7.20±0.02</td>
<td>6.64±0.01</td>
</tr>
<tr>
<td>7.3→6.5+240 μM lonidamine</td>
<td>6.78±0.03</td>
<td>7.17±0.04</td>
<td>6.54±0.01</td>
</tr>
<tr>
<td>7.3→6.5+320 μM lonidamine</td>
<td>6.75±0.02</td>
<td>7.10±0.02</td>
<td>6.23±0.24</td>
</tr>
<tr>
<td>7.3→6.5+40 mM NaLactate</td>
<td>7.04±0.02</td>
<td>7.22±0.02</td>
<td>6.68±0.06</td>
</tr>
<tr>
<td>7.3→6.5+80 mM NaLactate</td>
<td>6.95±0.02</td>
<td>7.13±0.02</td>
<td>&lt; 6.0</td>
</tr>
<tr>
<td>7.3→6.5+160 mM NaLactate</td>
<td>6.88±0.02</td>
<td>7.08±0.02</td>
<td>&lt; 6.0</td>
</tr>
<tr>
<td>7.3→6.5+1.25 mM CNCn</td>
<td></td>
<td></td>
<td>&lt; 6.0</td>
</tr>
<tr>
<td>7.3→6.5+2.5 mM CNCn</td>
<td></td>
<td></td>
<td>&lt; 6.0</td>
</tr>
<tr>
<td>7.3→6.5+5 mM CNCn</td>
<td></td>
<td></td>
<td>&lt; 6.0</td>
</tr>
</tbody>
</table>

All three cell lines showed concentration-dependent accentuation of the intracellular acidification achieved by lowering extracellular pH alone. However, the MYCN-
amplified cell lines IMR32 and NGP showed more intracellular acidification. Similar results were obtained by incubating cells with increasing concentrations of extracellular sodium lactate at concentrations of 40, 80, and 160 mM (Table 2). Lonidamine had no effect on intracellular pH when the extracellular pH was maintained at pH 7.3 (data not shown). When the experiments were repeated at an extracellular pH of 6.5, the intracellular pH of lonidamine-treated cells decreased significantly relative to the values achieved with extracellular acidification alone. For SKNSH cells, acute acidification to 6.5 caused intracellular pH to decrease from 7.41 ± 0.003 to 7.25 ± 0.003, a decrease of 0.16 unit. When lonidamine was added at 160, 240, or 320 μM, the net decreases in pH when combined with acidification were 0.21, 0.24, and 0.31 pH unit. For NGP cells, acidification alone decreased intracellular pH from 7.30 ± 0.07 to 7.01 ± 0.06, an average decrease of 0.23 unit. When lonidamine was added at 160, 240, or 320 μM, the net decreases in pH were 0.34, 0.52, and 0.55, respectively. For IMR32 cells, the initial intracellular pH of 7.47 ± 0.07 was decreased to 7.03 ± 0.05 by acidification to 6.5 giving an average decrease of 0.44 unit. When lonidamine was added at 160, 240, or 320 μM, the net decreases in pH were 0.83, 0.93, and 1.24 units, respectively.

Comparing the results from these cell lines, we found that Sk-N-SH cells, which expressed negligible MCT1 (Fig. 3), showed the least decrease in intracellular pH after lonidamine treatment. IMR32 and NGP cells, which express higher levels of MCT1 (see Fig. 2), exhibited much greater decreases in intracellular pH under the same conditions,
thus correlating the level of MCT1 expression with the lonidamine response. As shown in Fig. 5, lonidamine had a concentration-dependent cytotoxic effect in all cell lines studied. Compared with treatment at an extracellular pH of 7.3, lowering the extracellular pH to 6.5 induced a significant decrease in metabolic activity with increasing lonidamine concentration.

2.3.5.2 Addition of Exogenous Lactate

The results of using increasing concentrations of extracellular lactate or lonidamine in separate experiments measuring intracellular pH are shown in Table 2. When cells were incubated with 10-160 mM lactate, the effects on intracellular pH were virtually identical to those of lonidamine, as well as to those of α-cyano-4-OH-cinnamic acid (CNCn), an established MCT inhibitor [130] which has been shown to produce this effect in vitro in human melanoma cells [122]. These results support the hypothesis that the effect of lonidamine on intracellular pH is through MCTs, as removing the concentration gradient with exogenous lactate achieves a similar effect.

2.3.5.3 Affect of Lonidamine on Rho-Negative Cells

Figure 6 shows the effects of acute acidification plus lonidamine on neuroblastoma cells lacking mitochondria. In these rho-negative cells, an intracellular pH decline following the acute extracellular pH decrease from 7.3 to 6.5. This decrease was sustained during the time course of treatment. The dose response to lonidamine indicates that these cells, in spite of their lack of mitochondria, are responsive to
lonidamine. Lonidamine exerts effects on mitochondria, but the similarity of activity on rho-negative and mitochondrialy intact cells demonstrate that the intracellular acidification achieved through lonidamine treatment is not dependent on the mitochondria.

![Figure 6: Response of rho-negative Sy5Y cells to lonidamine when coupled with acute acidification.](image)

A steady-state intracellular pH was obtained, followed by acute extracellular acidification from pH 7.3 to pH 6.5 with or without lonidamine in a dose response. Diamonds: Vehicle plus acute acidification. Squares: 120 μM lonidamine with acute acidification. Triangles: 240 μM lonidamine with acute acidification. Circles: 320 μM lonidamine with acute acidification.

2.3.6 Cancer cell line metabolic activity was compromised by acidification and lonidamine administration.

Figure 7, A. IMR32 B. Sk-N-SH C. NGP. Cultured cells were subjected to increasing doses of lonidamine at pH 7.3 and 6.5 for 48 h. Viability was measured using
the MTS assay. A dose response was observed from 160-320 μM. The percentage of IMR32 cells that died during exposure to lonidamine at pH 6.5 was 23.9 ± 6.3%; the percentage of Sk-N-SH cells that died under the same conditions was 38.3 ± 3.0%.

Figure 7A-C: Cell viability as a function of lonidamine concentration.

A. IMR32  B. Sk-N-SH  C. NGP Cultured cells were subjected to increasing doses of lonidamine at pH 7.3 and 6.5 for 48 h. Viability was measured using the MTS assay. A dose response was observed from 160-320 μM. The percentage of IMR32 cells that died during exposure to lonidamine at pH 6.5 was 23.9 ± 6.3%; the percentage of Sk-N-SH cells that died under the same conditions was 38.3 ± 3.0%.
Figure 8 A-C: Cancer cell viability as a function of lactate concentration.

A. IMR32  B. Sk-N-SH  C. NGP Cultured cells were subjected to increasing doses of lactate at pH 7.3 and pH 6.5. Viability was measured using the MTS assay. A dose response was observed from 10-80 mM. In all cell lines, there was a statistically significant difference between results obtained at pH 7.3 and pH 6.5; this was most pronounced in IMR32 cells (Fig. 4A). Data are means ± SE; *, P < 0.01 (n = 8).
2.3.7 Cytosolic acidification and inhibition of metabolic activity were sufficient to cause cell death in cancer cells cultured in an acidic environment.

To determine whether the sudden decline in intracellular pH subsequent to lonidamine treatment was sufficient to cause cell death the Cell Death Detection ELISA was performed. Figure 9 shows that both IMR32 and Sk-N-SH cells undergo apoptotic death in a dose dependent fashion when subjected to lonidamine treatment at pH 6.7. An increase in absorbance at 405 nm corresponds to an increase in the concentration of nucleosomes, complexes of fragments of DNA and histones, in the cytoplasm of cells lysed in the apoptosis assay. IMR32 cells showed a three-fold increase in nucleosome concentration with 160 μM lonidamine treatment at pH 6.7, Sk-N-SH neuroblastoma cells showed a four-fold increase in nucleosome concentration with 160μM lonidamine treatment at pH 6.7. Enrichment in nucleosomes in the cytoplasm of cells is due to the fact that DNA degradation occurs several hours before plasma membrane breakdown occurs during apoptosis. Very little or no increase in cell death resulted from lonidamine treatment at pH 7.3.
Figure 9: Apoptosis assay of two neuroblastoma cell lines exposed to lonidamine and acidification.

A cell death detection assay was performed by ELISA that allows for determination of whether cytoplasmic histone-associated DNA fragmentation occurs after induction of cell death. This assay distinguishes apoptosis from necrosis. Cells were cultured at pH 6.7 or 7.3 and treated with vehicle or lonidamine for 24 h. Solid bars represent results in experiments performed at pH 6.7. Open bars represent experiments performed at pH 7.3. An increase in absorbance at 405 is indicative of increased cell death. Panel A is IMR-32 cells and Panel B is Sk-N-SH.
2.3.8 Lonidamine Treatment and Extracellular Acidification Does Not Result in an Increase in Cell Death in Benign MCT1 Expressing Endothelial Cells.

To demonstrate that cell death from extracellular acidification and MCT inhibition is selective to cancer cells that produce a large amount of lactic acid we performed an identical cell death assay on the benign endothelial cell line, BACEC at the same cell density as the cancerous neuroblastoma cell lines (Fig 10). The fact that BACEC are unaffected is presumably due to their low production of intracellular lactic acid. This means that even under conditions of extracellular acidification, BACEC are not under the same degree of pH stress as malignant neuroblastoma cells.

In order to ensure that BACEC expressed MCT1, and that simple lack of expression of the protein was not responsible for the lack of cell death seen with lonidamine treatment and extracellular acidification, a Western blot was performed. As seen in Figure 11, BACEC express MCT1 protein. BACEC expression of MCT1 is unchanged by after 24h of culture under acidic or neutral conditions (Fig 11).
A cell death detection assay was performed by ELISA that allows for determination of whether cytoplasmic histone-associated DNA fragmentation occurs after induction of cell death. Cells were cultured at pH 6.7 or 7.3 and treated with vehicle or lonidamine for 24 h. Solid bars represent results in experiments performed at pH 6.7. Open bars represent experiments performed at pH 7.3. An increase in absorbance at 405 is indicative of increased cell death.
2.4 Conclusion

These data have demonstrated, using primary biopsies and cell lines, that $MCT1$ activity is highly associated with $MYCN$ amplification in human neuroblastoma. Thus, as is the case with $MYCN$, $MCT1$ expression appears to correlate with high-risk disease features such as the presence of metastatic disease at diagnosis. However, $MYCN$ is not likely to be a good therapeutic target since it is a transcription factor affecting the activity of numerous proteins [131]. This work supports the hypothesis that $MCT1$ may be a more useful therapeutic target. The functional assays of MCT activity show that
inducibility of the gene at low pH or amount of protein by Western blot are not predictive of how much an MCT inhibitor can affect intracellular pH. The findings indicate that inducibility as a function of extracellular pH was variable. The finding that the MCT1 can be constitutively on or off or pH inducible is indeed a surprising finding. This may be indicative of variations in regulation of this gene. It may indirectly be affected by the relative amounts of oxidative aerobic metabolism vs. anaerobic metabolism that varies within a tumor in space and time. It may also be due to the deregulation of normal signal transduction pathways, common to many forms of cancer. Experiments in which knock-down of MYCN was attempted to look at effects on MCT1 were inconclusive, as were similar experiments performed on MYCN by Tang et al [132].

Treating neuroblastoma cells with the MCT inhibitor lonidamine induced a dramatic and immediate decrease in intracellular pH (Fig. 5, Table 2). This correlated with a significant loss of viability (Fig. 7) when extracellular pH decreased to 6.5, which is similar to the acidic microenvironmental pH of most tumors. MCT inhibitors can thus take advantage of the acidic pH tumor microenvironment to confer selectivity.

These studies suggest that the mechanism by which sustained low intracellular pH can bring about cell death is primarily apoptosis (Fig 9). Watching closely as the cell medium was acidified and test compounds were added, we observed not only that the SNARF fluorescence per cell decreased 75% within the first 15 m of treatment, but also that the cells did not detach and float. Dye leakage is an indication of compromised
membrane integrity and viability, and impending karyolysis. No signs of this were seen from acute extracellular acidification without drug nor did extracellular acidification without drug cause any significant increase in apoptosis or necrosis by cell death ELISA (Fig 9).

The declines in intracellular pH to below 6.8 are significant in that intracellular metabolism ceases below 6.8; acidic endonucleases are activated and apoptosis can ensue [133]. Also, all cellular enzymes have pH optima for their activity, such that effects on the myriad of cytosolic and mitochondrial enzymes within the cells are certain to be multiple. Another effect of lowering intracellular pH is the modification of protein folding in membranes, causing alterations in ligand binding to membrane-bound proteins. The plurality of effects caused by intracellular acidification has led to considerable confusion in the literature regarding the primary mechanism of action of lonidamine.

Members of the MCT family of transporters are responsible for the export from cells of lactate, pyruvate, and a variety of other monocarboxylates [130]. They are also referred to as organic anion symporters because they transport monocarboxylates in the same direction as protons. Consequently, we predicted that inhibition of these transporters could alter intracellular pH regulation, resulting in an inability of tumor cells to live in their acidic microenvironment.
To date, 14 MCT isoforms with various substrate specificities are known [121]. MCT1 is ubiquitous, with its highest levels found in skeletal muscle [134]. In most tissues, MCT1 is primarily a lactic acid transporter. The present study demonstrates that although MCT exchangers export lactic acid rather than regulate intracellular pH, inhibition of their activities can dramatically affect intracellular pH.

MCT1 gene expression has been reported in a few tumor cell lines other than neuroblastoma or melanoma [135], but MCT4 levels had not been measured in any tumors before our demonstration of elevated MCT1 and MCT4 activity in human melanoma cells grown at low extracellular pH [122]. The present study provides the first demonstration that the MCT transporters can be critical for maintaining intracellular pH in the viable range in neuroblastoma. Our results indicate that inhibitors of MCT could be useful adjuncts to neuroblastoma therapy, having particularly high activity where extracellular pH is low. Selectivity for the tumor microenvironment is based on the known low pH properties of the tumor. It has recently been reported that siRNA silencing of MCT in malignant glioma cells resulted in a precipitous decline in intracellular pH and cell death [136].

Lonidamine was tested as an anti-cancer drug in the 1980s and 1990s before much information was available regarding its mechanism of action. It was first reported that lonidamine inhibits lactate production in malignant gliomas but not in normal tissue, although the molecular mechanism for the lactate export was unknown [137].
Based on subsequent studies using magnetic resonance spectroscopy, lonidamine was proposed to be an inhibitor of lactic acid efflux that causes intracellular acidification [138]). Increases in intracellular acidification in brain tumors were demonstrated. The present study provides \textit{in vitro} support for these conclusions and extends the observation that inhibition of lactate efflux might be a therapeutically beneficial means of bringing about acidification to treat neuroblastoma.

These studies suggest that lonidamine is an inhibitor of MCT isoforms 1 and 4 [138-145]. Clinical studies testing the anti-tumor activity of lonidamine in adults found that its intravenous administration caused mild muscular and testicular discomfort [146], the tissues in which MCT1, MCT2, and MCT4 isoforms are highly prevalent [62, 130, 147, 148]. When lonidamine was administered orally, however, there were no significant side effects, and an oral administration schedule is currently being studied in benign prostatic hyperplasia.

The present study, with its measurement of the immediate effect on intracellular pH, provides direct evidence of the inhibition of lactic acid export from cells subsequent to lonidamine treatment. Floridi et al. (1981) suggested that a micromolar amount of lonidamine has major effects on aerobic and anaerobic glycolysis and lactic acid production [142], but suggested that the activity was primarily due to targeting hexokinase, the first enzyme in glycolysis (Fig 1). These studies demonstrate that treatment with lonidamine in an acidic environment results in intracellular acidification.
in less than 15 min (Fig 5 and 6, Table 2). This is counter to what would be expected if the primary mechanism of action of lonidamine was through hexokinase inhibition. Hexokinase inhibition and cessation of glycolysis would result in a decrease of lactic acid production and would not cause an immediate decline in intracellular pH. However, this would be the expected outcome if the primary mechanism of lonidamine was MCT inhibition, which is the conclusion of this study. Additionally, a major mitochondrial contribution to the mechanism of lonidamine has been ruled out, since rho-negative (mitochondria-deficient) cells exhibited dose-dependent acidification in response to increasing concentrations of lonidamine in an acidic extracellular environment just as mitochondrially intact cells do.

No ion gradients are involved other than the gradients of lactate anions and the protons themselves for MCT activity. In a tumor with an acidic milieu, a lactate gradient could be used to drive H⁺ transport against its transmembrane gradient, as originally described by Warburg [149]. The combined lactate and proton gradients provide the only requirement for transport into or out of the cell, since each requires the other to have electroneutral movement through the plasma membrane.

In conclusion, the results presented here indicate that MCT activity in neuroblastoma is high and correlates with high amplification of MYCN. Inhibitors of MCT have great potential when used to achieve selective compromise of neuroblastoma either by directly interfering with the viability of cells or improving the effectiveness of
Chemotherapeutic agents that function best at low extracellular pH, such as alkylating agents and platinum containing compounds, while sparing normal and surrounding cells at normal extracellular pH. Clearly, further exploration is needed into what other tumor and cell types express high levels of MCT1 on their cell surface and more importantly how the inhibition of MCT affects their cellular homeostasis. Additionally, other considerations that pertain in vivo are the level of hypoxia and perfusion, which the present study did not address. This study does indicate that extracellular pH can be a critical parameter of the tumor microenvironment for use in assessing the potential effectiveness of therapeutic anti-tumor agents.
3. Metabolic Targeting of Prostate Cancer Cells via the H+-linked Monocarboxylate Transporter 1 (MCT1/SLC16A1)

3.1 Introduction

In this study, the role of MCT activity in prostate cancer biology is examined. Prostate cancer, the second leading cause of cancer death among men in the United States in 2008 [150], is known to use LDH conversion of pyruvate to lactate to achieve a balanced redox state [151]. Here we report that MCT1 is expressed in human prostate cancer samples and provides an essential mechanism by which hypoxic prostate cancer cells excrete excess lactate. It is shown that inhibition of MCT1 activity in prostate cancer cells under hypoxic conditions, by either a novel small molecule inhibitor with selective activity against MCT1 or depletion of MCT1 by RNAi, results in inhibition of lactate excretion and selective tumor cell death. Further, it is shown that the mechanism of cell death is likely the result of intracellular acidification and cellular starvation brought on by the arrest of anaerobic glycolysis.

3.2 Methods

3.2.1 Cell Culture, Reagents, and Antibodies

1-LN human prostate cancer cells were a gift from Philip Walther, (Division of Urology, Duke University) [152]. PC-3 and LNCaP cells were obtained from American
Type Culture Collection. Human umbilical vein endothelial cells (HUVEC) were obtained from Cambrex (East Rutherford, NJ). 1-LN, PC3, and LNCaP cells were grown in Dulbecco’s Modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 26 mM sodium bicarbonate, 2 mM glutamine, 20 nM human recombinant insulin, and 1% penicillin/streptomycin. HUVEC were grown in Clonetics endothelial cell basal medium with 5% FBS and EGM-MV SingleQuot (Lonza, Walkersville, MD). Cells were maintained in a humidified 37°C incubator at 5% CO₂ pH 7.3 in logarithmic growth in 75 cm² flasks. To achieve hypoxia, a 37 °C, humidified, cell culture incubator at 5% CO₂ was equipped with a “C-Chamber” inner hypoxia chamber (BioSpherix, Redfield, NY). The sealed hypoxia chamber was filled with a 5% CO₂ / 95% N₂ gas blended with 5% CO₂ air inside the incubator to achieve a 2% O₂ and 5% CO₂ mixture inside the hypoxia chamber. Gas mixture and constant O₂ measurements were accomplished using a Proox Model 110 oxygen gas controller (BioSpherix, Redfield, NY). Oxygen measurements were taken continuously throughout each experiment.

AR-C122982, “compound 2” [74, 85], was a gift from AstraZeneca (London, UK). Antibodies to MCT1 (C-20), MCT4 (C-17), and actin (C-2) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibody IR dye conjugates, donkey anti-goat IR Dye 800 nm and goat anti-rabbit Alexa Fluro 680 nm, were from Rockland (Gilbertsville, PA) and Molecular Probes (Carlsbad, CA), respectively. Blocking peptide to MCT1 (C-20) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).
3.2.2 RNA interference

Silencer siRNA for MCT1 (ID# 117128) and Silencer Negative Control #1 siRNA were from Ambion. Cells were plated at low density in 96-well plates and allowed to achieve 90% confluence over 72 hours before the experiment was performed. Lipofectamine 2000 (Invitrogen; Carlsbad, CA) was used to transfect cells according to the manufacturer’s recommendations, with 0.25 μL/well plus siRNA in 100 μl Opti-MEM (Invitrogen). Cells were returned to the incubator for 4h, after which 100 μL of DMEM with 10% FBS and no antibiotics was added. After overnight incubation, the transfection reagent was removed and replaced with full media. Peak knockdown of MCT1 was observed by western blot between 48-72h after transfection.

3.2.3 Cell Lysis and Western Blot

Cells were lysed in buffer containing 2% SDS, 62.5 mM Tris, and 20% glycerol at pH 6.8 and placed in microfuge tubes. Cells were pulsed for 5 s with a probe sonicator, vortexed for 10 s, and centrifuged at 1000 x g for 2 min. Protein concentrations were measured by BioRad BCA assay. Samples diluted to 0.5 mg/mL by dilution with gel loading buffer (2% SDS, 62.5 mM Tris pH 6.8, 20% glycerol, 10% 2-mercaptoethanol, and 0.01% bromophenol blue). Samples were heated to 100 °C for 5 min and then centrifuged at 1000 x g for 5 minutes. Twenty μL aliquots of each sample were processed by SDS-PAGE and subsequent immunoblot as described [82]. Antibodies to
MCT1 and MCT4 were used at 1:1000, and IR dye-conjugated secondary antibodies were used at 1:10,000. Immunoreactive protein was quantified using the Odyssey Infrared Imager (Li-Cor; Lincoln, NE).

3.2.4 Intracellular pH Measurements

Intracellular pH was measured essentially as described [55]. Briefly, cells were plated on coverslips affixed to microwell dishes at a density of $2 \times 10^6$ cells in 2 ml of medium and allowed to achieve confluence over about 72 h. Preliminary experiments (not shown) demonstrated that dye leakage during an experiment of 1 h or less was undetectable under these conditions. Cells were incubated for 15 min with 9 μM Carboxy SNARF-1 acetoxyethyl ester (C.SNARF-1AM) (Invitrogen, Carlsbad, CA) in medium containing 10% FBS in a 37°C incubator under 5% CO$_2$. If cells had been treated under hypoxic conditions, then dye loading and incubation was carried out under hypoxic conditions. Following a change of medium, cells were further incubated for 20 min at 37°C and 5% CO$_2$ to complete hydrolysis of the dye ester to C.SNARF. Each plate was then mounted on the microscope stage and warmed to 37°C under flowing humidified air containing 5% CO$_2$. Intracellular pH values were obtained from intracellular whole emission spectra of the pH-sensitive dye C.SNARF. Measurements were taken for 2 minutes as pH$\text{i}$ takes approximately 30 s to equilibrate after transfer through room air to 5% CO$_2$ heated stage.
3.2.5 Cell Death, Lactate, and Intracellular ATP Assay

Cell survival was measured using the Cell Death Detection ELISA Plus 10x (Roche Applied Science, Indianapolis, IN). Cells were plated at low density and allowed to achieve 95% confluence over 72 h before the experiment was performed. Cells were treated with AR-C122982 or siRNA for MCT-1 and then incubated under hypoxia or normoxia for 24 h. Cells were then lysed and assayed for intracellular nucleosome fragments according to the manufacturer’s recommendations. Data is reported as nucleosome enrichment (treatment/vehicle alone).

Extracellular lactate in cell culture supernatants was measured using the Enzychrome Lactate Assay (BioAssay Systems, Hayward, CA). Cells were plated at low density and allowed to achieve 95% confluence over 72 h before the experiment was performed. Cells were treated with AR-C122982 or siRNA for MCT-1 and then incubated under hypoxia or normoxia for 24 h. Supernatants were collected and centrifuged at 200 x g for 10 min in V-bottom 96-well plates. Supernatants were diluted 1:10 with H2O prior to assay. Lactate was measured according to the instructions of the manufacturer.

Intracellular ATP was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI). Cells were plated at low density and allowed to achieve 95% confluence over about 72 h before the experiment was performed. Cells were treated with AR-C122982 or siRNA for MCT-1 and then incubated under hypoxia
or normoxia for 24 h. Supernatants were removed and cells were lysed and assayed for intracellular ATP according to the manufacturer’s instructions.

3.2.6 Immunohistochemistry

Tissue microarrays were purchased from US Biomax (cat# PR951) (Ijamsville, MD). Immunohistochemistry was performed essentially as described [153], except heat-induced antigen retrieval was carried out in 10 mM Tris-HCl, 1 mM EDTA, pH 9.0 for 20 min. To reduce background staining after antigen retrieval, sections were incubated with non-immune horse serum for 30 min at room temperature. Sections were then incubated with anti-MCT1 antibody diluted to 10 μg/mL in PBS overnight at 21 °C. Detection of bound anti-MCT1 antibody and counterstaining was accomplished as described [153], except that biotinylated horse anti-goat antisera (Vector Laboratories, Burlingame, CA) diluted 1:250 in PBS was used.

3.3 Results

3.3.1 Prostate cancer cells produce lactate in both normoxic and hypoxic conditions and express MCT1.

To assess the relative contribution of Warburg and Pasteur effects to glucose utilization in prostate cancer cells, lactate release from 1-LN prostate cancer cells was measured under normoxic and hypoxic conditions. Under normoxic conditions 1-LN
cells produced an average of 18.1 mM lactate over 24 h measured in 200μL of media (Fig 12). The relatively large amount of lactate produced by these cells under normoxia shows that these cancer cells indeed exhibit the Warburg effect. For comparison, lactate production of the non-neoplastic HUVEC was assayed at the same density under normoxic conditions (Fig 12). HUVEC produce significantly less lactate, 1.2 mM, under normoxic conditions demonstrating the lack of the Warburg effect in these benign cells. Under hypoxic conditions 1-LN prostate cancer cells nearly doubled their lactate excretion, producing an average of 28.5 mM lactate over 24 h (Fig 12). These results demonstrate that 1-LN prostate cancer cells also exhibit a robust Pasteur effect. HUVEC also increased their production of lactate under conditions of hypoxia to 1.7 mM demonstrating a modest Pasteur effect (Fig 12), though the final lactate concentration was more than 16 fold less than in 1-LN prostate cancer cells under similar conditions.
Figure 12: 1-LN and HUVEC Lactate Production.

Extracellular lactate production was measured in 1-LN prostate cancer cells after 24h of culture under normoxic or hypoxic conditions. For comparison extracellular lactate production was also compared in HUVEC, benign primary endothelial cells, cultured under identical conditions with equivalent cell density.

In order to survive, prostate cancer cells must excrete the abundant lactate that they produce, especially under conditions of hypoxia. Because this process may provide a metabolic target for therapeutic intervention, we investigated the mechanism by which the cancer cells excrete this potentially toxic metabolite. The proteins involved in lactate transport are the monocarboxylate transporters (MCT) [21-23, 82]. MCT1 has been reported to be ubiquitously expressed in human tissues [21]. To assess MCT1
expression in prostate cancer cells, Western blot analysis of the 1-LN prostate cancer cell line, its parent line PC-3, and the LNCaP prostate cancer cell line was used (Fig. 13). As shown in Figure 13, all three cell lines express MCT1.

**Figure 13: MCT1 Expression by Western Blot in Prostate Cancer Cell Lines.**

Western blot was performed for MCT1 in lysates of 1-LN, PC3, and LNCaP prostate cancer cell lines. As indicated, actin was detected as a loading control.

To confirm that MCT1 is expressed in human prostate cancers as well as immortilized cell lines, immunohistochemistry was performed on prostate cancer specimens arranged in a tissue microarray. Samples in this array, which contained 34 cores of prostate cancer and 9 of normal prostate, were scored according the intensity of
MCT1-specific staining: no staining = 0; mild or focal staining = 1; moderate staining = 2; and strong staining = 3. All the prostate cancers tested were positive for MCT1-specific staining; staining intensity ranged from 1-3, with a mean score of 1.9. A representative photomicrograph showing strong staining is in Figure 14A. Benign prostate epithelial cells also expressed MCT1, with staining intensity ranging from mild to moderate (Fig 14B). The mean intensity score of staining was slightly lower at 1.3 (p<0.05). The mild increase in expression of MCT1 in malignant prostate cancer when compared to benign may reflect an increased demand on the monocarboxylate transporters to remove intracellular lactate in the tumors. The MCT1 staining was completely blocked by incubation of the anti-sera with the peptide to which it is directed in consecutive sections (Fig 14C-D).
Figure 14A-D: Prostate Cancer Immunohistochemistry.

Immunohistochemistry was performed on benign and cancerous prostate samples in a tissue microarray. A, a sample of prostate cancer stained for MCT1 exhibiting 3+ staining. B, a sample of benign prostatic tissue stained for MCT1 exhibiting 1+ staining. C and D, consecutive sections of a sample of prostate cancer stained for MCT1 (C), and peptide control pretreated with blocking peptide for MCT1 and then stained (D).
3.3.2 Monocarboxylate Transporter 1 is a critical regulator of lactate excretion in prostate cancer cells under hypoxic conditions.

To assess the contribution of MCT1 to lactate excretion, a small molecule, AR-C122982 (compound 2 [74, 85]), was used that has been shown to have selective inhibitory activity against MCT1 lactate transport (Ki = 0.1nM for MCT1). Lactate excretion by 1-LN cells was measured under both normoxic and hypoxic conditions in the presence and absence of the drug. As shown in Figure 15, AR-C122982 caused a dose dependent inhibition of lactate release under hypoxic conditions but had no significant effect on cells cultured under normoxic conditions.
Figure 15: 1-LN Lactate Excretion with MCT1 inhibitor.

Extracellular lactate production was measured in 1-LN prostate cancer cells cultured under hypoxic or normoxic conditions with vehicle treatment, 0.05 μM, 0.1 μM, 1 μM, or 2 μM AR-C122982 for 24h.

To confirm these results, RNA interference was used to knockdown expression of MCT1. Western blot analysis showed that 50 nM siRNA could achieve an approximate 61% depletion of MCT1 in 1-LN cells, while having no effect on expression of MCT4 (Fig 16-17).
siRNA knockdown of MCT1 was performed followed by western blot for MCT1, MCT4, and actin. Approximately, 61% decline in MCT1 protein was observed, with no decline in MCT4 or actin expression.
Figure 17: siRNA Knockdown of MCT1.

A histographical representation of the intensity of bands for MCT1 after siRNA knockdown.

Similar to the effect of AR-C122982, siRNA-dependent knockdown of MCT1 resulted in statistically significant inhibition of lactate release under hypoxic conditions, p<0.05 (Fig 18). Knockdown of MCT1 under normoxic conditions resulted in a small inhibition of lactate excretion. These results indicate that, under hypoxic conditions, MCT1 is a major regulator of lactate excretion in hypoxic 1-LN prostate cancer cells.
Figure 18: Lactate Excretion after siRNA Knockdown of MCT1.

Extracellular lactate production was measured in 1-LN prostate cancer cells cultured under hypoxic or normoxic conditions for 24h after siRNA knockdown of MCT1.
3.3.3 Inhibition of MCT1 activity results in apoptotic death of 1-LN prostate cancer cells grown under hypoxic conditions, but does not cause cell death of benign endothelial cells.

Since MCT1 inhibition significantly impeded 1-LN lactate excretion (Fig. 15), it was desired to understand the deleterious effects that any resultant intracellular lactate accumulation may have on the cells. It has been reported that lactic acid accumulation can contribute to cell death induced by drugs with MCT inhibitory activity [154]. To measure the effects of MCT1 inhibition on cell survival, prostate cancer cell death was assessed under hypoxic or normoxic conditions. 1-LN prostate cancer cells were cultured under hypoxic or normoxic conditions for 24h in the presence of increasing concentrations of the MCT1 inhibitor AR-C122982. Apoptotic cell death was measured by quantitative detection of intracellular DNA/histone (nucleosome) fragments [155]. As shown in Figure 19, treatment with AR-C122982 resulted in significant, dose-dependent nucleosome fragment enrichment (ratio of drug treatment/vehicle) in 1-LN cells cultured under hypoxic conditions. However, increasing doses of AR-C122982 did not result in any significant enrichment in nucleosomes in cells cultured under normoxic conditions. Cell death was also measured in response to agents that are known to induce apoptosis in a manner independent of oxygenation, staurosporine and mitoxantrone. As expected, treatment with either of these agents resulted in similar 1-
LN apoptosis, whether the cells were cultured under hypoxic or normoxic conditions (Fig 19).

![Bar graph showing cell death in hypoxic 1-LN prostate cancer cells treated with MCT1 inhibitor.](image)

**Figure 19: Cell Death in Hypoxic 1-LN prostate cancer cells treated with MCT1 inhibitor.**

Cell death assay of 1-LN cells treated with 5μM staurosporine (Stauro), 2μM mitoxantrone (Mito), vehicle, 0.05 μM, 0.1 μM, 1 μM, or 2 μM AR-C122982 for 24h under hypoxia or normoxia. Nucleosome enrichment factor = nucleosomes in drug treated cells/nucleosomes in vehicle treated cells.

Inhibition of MCT1 activity does not result in cell death in normal cells producing a small amount of lactic acid. As seen in Figure 20, HUVEC treated with AR-C122982 under normoxic or hypoxic conditions do not show an increase in cell death. However, staurosporine treated HUVEC do show an increase in cell death under normoxic or hypoxic conditions (Fig 20).
Figure 20: HUVEC Apoptosis Assay

Cell death assay of HUVEC treated with 5 μM staurosporine (Stauro), vehicle, 0.05 μM, 0.1 μM, 1 μM, or 2 μM AR-C122982 for 24h under hypoxia or normoxia. Nucleosome enrichment factor = nucleosomes in drug treated cells/nucleosomes in vehicle treated cells.

To confirm that AR-C122982 was exerting its effects through MCT1, we measured cell death under hypoxia or normoxia after siRNA knockdown of MCT1. As shown in Figure 21, knockdown of MCT1 resulted in a greater than 1.6 fold increase in apoptotic cell death under hypoxic conditions over control RNA treatment under these conditions. The stimulation of apoptosis caused by knockdown of MCT1 under hypoxia
was significantly greater than the small change in cell death resulting from MCT1 knockdown in normoxia (p<0.05). The difference between hypoxic and normoxic cell death with siRNA knockdown is less dramatic than the difference between cell death in hypoxic and normoxic cells treated with AR-C122982. This is presumably due to the incomplete knockdown of MCT1 activity, and the overall toxicity associated with administration of siRNA with a lipid based transfection reagent.

**Figure 21: Cell death with siRNA knockdown of MCT1.**

Cell death assay of 1-LN cells treated with 50nM siRNA for MCT1 or control RNA under hypoxic or normoxic conditions for 24h. Nucleosome enrichment factor = nucleosomes in drug treated cells/nucleosomes in vehicle treated cells.
3.3.4 Inhibition of MCT1 activity causes intracellular acidification of 1-LN prostate cancer cells grown under hypoxic conditions.

Intracellular lactic acid accumulation lowers pHi [154], pHi in the acidic range is known to result in activation of endonuclease II, an initiator of apoptosis [11-14]. Intracellular acidification to 6.3-6.8 optimally accelerates the rate of formation of the caspase activation complex; indeed, an intracellular acidification of ~0.4 pH units has been associated with apoptosis from a wide range of stimuli [47, 50]. To investigate the effects of MCT1 inhibition on pHi, we measured the intracellular pH of 1-LN prostate cancer cells treated with AR-C122982 under hypoxic or normoxic conditions. Intracellular pH measurements of 1-LN cells were taken using the fluorescent dye SNARF [55] under hypoxic or normoxic conditions in the presence or absence of the MCT1 inhibitor AR-C122982 (Fig 22). As shown in Figure 22, the pHi of 1-LN cells under normoxic conditions was ~7.5, regardless of the presence of AR-C122982 (Purple and Green lines). Thus, under normoxic conditions not enough lactic acid is retained due to MCT1 inhibition to cause a significant change in intracellular pH, likely because other pHi regulatory proteins are actively regulating pHi. Hypoxia alone was capable of causing a small decrease in pHi to 7.15 (Fig 22, Blue line) corresponding to an increase in lactic acid production, but hypoxic 1-LN cells treated with AR-C122982 demonstrated intracellular acidification to a pHi of ~6.7 (Fig 22, Red line). This significant drop in pHi
is caused by the combined effects of hypoxia increasing lactic acid production and MCT1 inhibition eliminating a major cellular mechanism of proton extrusion.

**Figure 22: Intracellular pH with MCT1 Inhibition.**

Intracellular pH was measured in 1-LN prostate cancer cells treated with 2 μM AR-C122982 or vehicle under hypoxic or normoxic conditions for 24hrs. Intracellular pH measurements were taken for 2 minutes on a heated microscope stage under a 5% CO₂ atmosphere.

To confirm that the effects of AR-C122982 were mediated by inhibition of MCT1, we also measured pH in 1-LN prostate cancer cells cultured under hypoxic or normoxic conditions after siRNA-directed knockdown of MCT1 (Fig 23). As expected, cells
treated with non-targeting siRNA under normoxic conditions had a pHi~7.4 (Blue line), and hypoxia in these control cells resulted in a small decrease in pHi to 7.19 (Red line). In contrast to treatment with AR-C122982, however, siRNA-directed knockdown of MCT1 resulted in a small decrease in pHi under normoxic conditions to 7.19 (Green line). In any event, siRNA-directed knockdown of MCT1 under hypoxic conditions resulted in acidification to pHi of 6.7, similar to drug treatment (Purple line). These results support the hypothesis that AR-C122982 is exerting its effects on pHi through inhibition of MCT1 activity.
Figure 23: Intracellular pH with siRNA knockdown of MCT1.

Intracellular pH was measured in 1-LN prostate cancer cells treated with 50nM siRNA for MCT1 or 50nM control RNA under hypoxic or normoxic conditions for 24hrs. Intracellular pH measurements were taken for 2 minutes on a heated microscope stage under a 5% CO₂ atmosphere.

The experiments in Figures 22 and 23 illustrate that the combination of hypoxia and inhibition of MCT1 activity is sufficient to reduce the pHi into the acidic range. Acidic pHi near this level is sufficient to activate pH dependent endonucleases and caspases [11-18, 50] and induce or accelerate apoptosis. Therefore, the low pHi in hypoxic cancer cells treated with low MCT1 activity is most likely a contributing factor to the cell death observed under these conditions.
3.3.5 Inhibition of MCT1 activity blocks ATP production in 1-LN prostate cancer cells grown under hypoxic conditions.

In addition to causing a decrease in pHi, accumulation of intracellular lactate can also diminish the cell’s ability to utilize glucose as an energy source to produce ATP, principally by inhibition of LDH [19]. In cells that depend on anaerobic glycolysis, such as hypoxic tumor cells, the end result would be a form of cellular starvation and ultimately cell death. To investigate whether the MCT1 inhibition under hypoxic conditions results in this form of starvation, we measured ATP production in 1-LN cells cultured in the presence or absence of AR-C122982 under hypoxic or normoxic conditions. In agreement with the absence of effects in normoxic cells on lactate excretion (Fig. 15), cell death (Fig. 19), and pHi (Fig. 22), AR-C122982 did not significantly affect intracellular ATP production in 1-LN cells under normoxic conditions (Fig. 24). However, 1-LN cells treated with AR-C122982 under hypoxic conditions showed a dose dependent reduction in intracellular ATP (Fig. 24). A dramatic reduction of intracellular ATP in hypoxic cells was observed throughout the range of drug concentrations used. Staurosporine treatment, which non-specifically kills cells regardless of the oxygenation state, resulted in a dramatic reduction in intracellular ATP under both hypoxic or normoxic conditions.
Intracellular ATP was measured in 1-LN cells treated with AR-C122982 under hypoxic or normoxic conditions. 1-LN prostate cancer cells were treated with 5μM staurosporine, vehicle, 0.05 μM, 0.1 μM, 1 μM, or 2 μM AR-C122982 for 24h under hypoxia or normoxia and then assayed for intracellular ATP.
Collectively, the data in Figures 22, 23, and 24 indicate that, under hypoxic conditions, MCT1 inhibition can result in both intracellular acidification and also cellular starvation. Both of these mechanisms can potentially contribute the apoptotic cell death that results from MCT1 inhibition under hypoxic conditions (Fig. 19).

3.4 Conclusion

The metabolism of cancer cells and normal host cells differs in that cancer cells use more glucose than normal host cells. One effect of this difference is that tumor cells will generate more lactate than host cells. Since cells must have a mechanism to excrete this potentially toxic metabolite, lactate transport could be an effective metabolic target for therapeutic intervention in human cancers. In this study, inhibition of a major lactate transporter, MCT1, in prostate cancer cells by either small molecule inhibitor (AR-C122982) or RNAi results in inhibition of lactate release and apoptosis under hypoxic conditions. Further, evidence is provided that MCT1 inhibition under hypoxic conditions results in intracellular acidification and inhibition of ATP production. It is hypothesized that these two effects collaborate to induce apoptotic cell death, resulting from activation of pH dependent caspases and endonucleases and cellular starvation brought on by the cessation of glycolysis in hypoxic cells unable to rid themselves of excess lactate.
The fact that significant tumor cell death is observed only under conditions of hypoxia has relevance to human tumor biology and the potential specificity of lactate transport as a therapeutic target. Under hypoxic conditions, most eukaryotic cells have the ability to continue to use glucose as an energy source, regenerating the cellular pool of NAD+ through the reduction of pyruvate to lactic acid (Pasteur Effect). Though this pathway can be utilized by both tumor and host cells, the phenomenon is more common in cancer cells, because under basal conditions most tumors experience at least some hypoxia, while host cells are rarely hypoxic [9, 24, 107, 108]. Cancer cells likely begin to experience hypoxia relatively early in tumor development as hyperplasia and growth away from blood vessels limits the oxygen supply of many tumor cells [4]. Later in tumor development, a larger tumor will have developed vasculature of its own. However, tumor vasculature is characteristically poorly developed, leaky, and typically inadequate to supply the oxygen requirement of a rapidly proliferating tumor [26, 27]. Both of these largely cancer specific effects mean that cancer cells in the body will be consuming more glucose than surrounding host cells and producing a great deal more lactic acid.

These observations suggest that lactate transport would be most critical in hypoxic cells. Indeed, MCT1 inhibition in prostate cancer cells under normoxic conditions is largely nontoxic. This finding supports the hypothesis that inhibition of MCT activity can be used to selectively target tumor cells because they are more likely to
experience hypoxia. Additionally, Figures 12 and 20 suggest that cells not actively producing lactic acid, i.e., most normal resting host cells, are not adversely affected by inhibition of MCT activity. While many host cells transport lactate across their cell membranes, few, if any are as dependent on the ability to transport lactate for their survival as cancer cells are.

The ability to target hypoxic tumor cells specifically is germane to cancer therapy because, in general, hypoxic cancer cells are most resistant to standard chemotherapeutic or radiation therapy [156-159]. In fact, the buildup of lactate as the result of hypoxia in the extracellular space of tumors is indicative of a poor prognosis in some tumors [28, 160]. The reasons for this resistance are complex and incompletely understood, but in part lie in the fact that hypoxia creates a unique tumor microenvironment that may actually confer some survival advantage to tumor cells that adapt to living there. One contributing factor is that cancer cells residing in a poorly vascularized tumor bed are less accessible to blood borne chemotherapeutics and immune surveillance [29]. Secondly, hypoxic, fermenting tumor cells are more protected from oxidative stress [30], the reactive oxygen species utilized by the cell mediated immune response [32, 33], and the reactive oxygen species that are generated by radiation therapy [34, 35]. Finally, it has been hypothesized that the acidic microenvironment of many tumors plays a role in their ability to invade surrounding tissues by degrading surrounding extracellular matrix, disguising the tumor cells from
immunosurveillance, and by causing the environment to become less suitable for the
growth of host cells [36].

Another novel result of this study is the finding that MCT1 plays a major role in lactate export in hypoxic prostate cancer cells. Under conditions of normoxia, MCT1 inhibition, either by drug or siRNA, had no significant effect on lactate excretion. This suggests that another lactate transporter remains highly active under normoxia, likely MCT4. Either MCT4 is the dominant transporter under normoxic conditions, or it can readily compensate for loss of MCT1 activity under normoxia. However, under hypoxic conditions, inhibition of MCT1 completely blocks the excess lactate excretion that otherwise accompanies hypoxia in these cells (Fig. 15 and 18). These results imply that MCT1 plays an essential role in excess lactate excretion under hypoxic conditions, and that MCT4 is unable to compensate under these conditions. This result is a little surprising, since MCT1 is reportedly not capable of as high a rate of lactate transport as MCT4 [23, 68, 71, 72]. MCT1 has a higher affinity for lactate compared to MCT4 (Km~5mM versus Km~20mM) [23, 68, 72], though it is unclear whether this property plays a role in their observed behavior under hypoxia.

These data also suggest that the mechanism by which MCT1 inhibition causes cell death under hypoxic conditions is twofold: cellular starvation due to an inhibition of anaerobic glycolysis, evidenced by the decline in intracellular ATP (Fig. 24), and a decline in intracellular pH from the retention of lactic acid (Fig. 22). While a significant
decline in intracellular ATP is seen with the increasing dose of AR-C122982 (Fig. 24), it is important to note that the sharpest decline occurs at the lowest dose of 0.05 μM. This suggests that at this low dose of AR-C122982, the increase in intracellular lactate is sufficient to inhibit the activity of LDH. This observation is supported by the fact that a significant increase in cell death is observed at that concentration of drug in hypoxic prostate cancer cells (Fig. 19). Under these conditions a cell dependent on anaerobic glycolysis will quickly exhaust its cytosolic pool of NAD\(^+\). As the redox balance of the cells shifts to the side of overly reduced, anaerobic glycolysis will cease. It is known that cancer cells have several mechanisms by which to maintain a balanced redox state under hypoxic conditions [151], however our data demonstrate that in 1-LN prostate cancer cells, inhibition of LDH activity through a buildup of intracellular lactate is sufficient to inhibit the energy generating capacity of the cell.

These data support the notion that a decline in pH\(_i\) is also part of the mechanism by which MCT1 inhibition results in cell death. Treatment with 2μM AR-C122982 under hypoxic conditions results in a decline in pH\(_i\) of 0.8 units (Fig. 22). Lowering of pH\(_i\) to this degree has been associated with initiation and acceleration of apoptosis [11-18, 47, 50]. Additionally, since doses of AR-C122982 above 0.5 μM under hypoxic conditions do not result in a substantial additional decline in intracellular ATP (Fig. 24), but increasing doses of AR-C122982 do result in a substantial increase in cell death (Fig. 19), it is plausible that this increase in cell death is due to intracellular acidification.
In this study siRNA against MCT1 was employed to verify the specificity of AR-C122982 in targeting MCT1. Using siRNA knockdown of MCT1 it was possible to demonstrate inhibition of lactate release (Fig 18), lowering of pHi (Fig 23), and cell death (Fig 21) in prostate cancer cells under hypoxic conditions. While siRNA is useful to validate the specificity of AR-C122982, the potential toxicity of the lipid transfection reagents and the RNA can complicate interpretation of the data. For example, in Figure 18, the peak levels of lactate measured in the supernatant of the control RNA group are lower than those measured in Figure 15 for the vehicle treatment group. It is concluded that this is due to the toxicity associated with transfection of control RNA. Additionally, the difference in nucleosome enrichment factor in the hypoxic cells versus normoxic cells is less in siRNA-dependent MCT1 knockdown experiment (Fig 21) than the AR-C122982-treated cells (Fig 19). It is again believed that this is due to the toxicity associated with siRNA transfection, as control RNA transfection resulted in greater background cell death than treatment with vehicle. Finally, siRNA was used to knockdown MCT1 and intracellular pH was measured under normoxic and hypoxic conditions (Fig 23). This gave a very similar result to what was seen in Figure 22 with AR-C122982 treatment and pHi measurement. It is believed that this is due to the fact that cells can only be labeled with C. SNARF-AM if they have intact esterase activity and intact plasma membranes. Therefore, cells experiencing toxicity due to siRNA transfection would be excluded from detection.
While the future of cancer therapeutics targeting MCT activity is promising, many questions remain. It is unknown whether some normal tissues in the body may be adversely affected by an inhibition of MCT activity. Lonidamine, a compound with known MCT inhibitory activity [80, 81, 154] has been used in clinical trials in humans for cancer and for benign prostatic hyperplasia [83, 161], it is generally well tolerated and minimal adverse effects have been reported, mostly limited to muscle pain. It should be noted that lonidamine is known to have effects other than MCT inhibition [162], it is therefore more difficult to clearly decipher its molecular mechanism of action. *In vivo* studies with specific pharmacologic and molecular targeting of MCT activity are needed to validate MCT’s as a target in cancer.

Additionally, the respective contributions of MCT1 and MCT4 in lactic acid transport in tumors must be determined. The relative expression of MCT1 and MCT4 in glycolytic vs. oxidative muscle fibers [23, 163-165] has led to the belief that in many tissues, such as glycolytic muscle fibers, MCT4 is specialized for the export of lactic acid. In other tissues, such as oxidative muscle fibers, MCT1 is believed to be specialized for the import of lactate as an oxidative fuel. Our data suggests that in prostate cancer cells MCT1 plays a critical role in lactate export under hypoxic conditions. Moreover, we have shown that inhibition of MCT1 activity in 1-LN prostate cancer cells under hypoxic conditions results in inhibition of lactic acid release, intracellular acidification, inhibition of ATP production, and cell death. These data suggest that hypoxic cancer cells, *id est*,
those most resistant to standard radio and chemotherapy, would be specifically targeted by therapy aimed at MCT activity. Since normal resting host cells are rarely hypoxic, they should be largely spared by a cancer therapeutic targeting MCT activity. This novel finding presents a new avenue for the pursuit of selective cancer therapy.
4. Glycogen Storage in Cancer as a Response to Hypoxia

4.1 Introduction

These studies were performed as a collaborative effort resulting in the manuscript, “Spatial correlation of glycopenia and glycogen accumulation to hypoxia in human head and neck cancer.” Table 3 and Figures 25-28 and text are reprinted from this manuscript with the permission of the authors. I would like to thank the authors for their contribution, and especially acknowledge Thies Schroeder for his help with my thesis. My collaboration contributed to the development of the hypothesis that glycogen deposits are a survival factor for tumor cells, I also performed all the PAS stains in the study.

Solid tumors are characterized by a situation of metabolic substrate depletion, ultimately due to increased intervascular distances that follow uncontrolled cell growth. The most prominent example of diffusion limited substrate availability in solid tumors is diffusion limited hypoxia, which poses a serious therapeutic problem, since it causes resistance towards radio- and chemotherapy. On a microenvironmental level, the severity of hypoxia increases with intervascular distance, peaking in regions adjacent to tumor necrosis [166]. It is also commonly accepted now that such tumors are chronically starved of glucose, a status that can be termed glycopenia. Considering the continued interest in the field of anti-angiogenesis (to “starve the tumor by cutting off of its
vascular supply”), it is surprising that only recently the investigation of the biological causes and consequences of energy starvation, in particular glycopenia, has re-emerged as a point of research interest. Several reports have shown that glucose starvation triggers HIF-1-independent upregulation of the potent angiogenic growth factor VEGF, mediated via the metabolic sensor AMP kinase [167, 168]. AMPK also induces expression of the glucose transporter GLUT-1 and the glycolytic accelerator PFK2, which may lead to more efficient uptake and utilization of glucose [169]. As a consequence, overactivation of AMPK in glucose-starved areas of the tumor, for example by the use of the antidiabetic drug metformin, may lead to less necrotic, better vascularized, and faster growing tumors [170]. It also seems that hypoxia itself may play a role in inducing tolerance of glucose-starved cells to further glucose deprivation [171]. However, while there is a general notion that solid tumors are both “glucose starved” and “hypoxic”, few data exist that show where in the tumor, and why, glucose starvation occurs.

The original aim of this study was to characterize basic gradients of glucose and lactate, in comparison with intervascular distances and hypoxia on a quantitative histological level in human head and neck squamous cell carcinomas. Because these parameters are functionally interrelated, there was hope that the level of hypoxia could be described through the levels of these metabolites, providing a tool to measure hypoxia in tumors. This comparison was done in a very unique set of snap frozen samples from human head and neck cancer patients who had been administered the
hypoxia marker, EF5, prior to biopsy. Glucose and lactate were quantified and mapped using bioluminescence histology, vascular distance was quantified using CD31 immunostain and distance mapping, and EF5 was quantified in the tumor areas using quantitative fluorescence immunohistochemistry and grayscale analysis. Images from adjacent slices were coregistered and compared using pixel to pixel correlation, with Spearman rank correlation coefficient as a statistical endpoint. Further, the intra-tumoral accumulation pattern of glycogen, measured by Periodic Acid Schiff reaction (PAS), with the other parameters mentioned above were compared, using pixel to pixel correlation. It was found that the glucose concentration is – on a microscopic level – inversely correlated with hypoxia and increasing intervascular distances. There is no association of glucose and (tumor) hypoxia, however, when compared across the samples. Our results show that histologically viable human cancer can be virtually free of glucose, whereas in other cases, normal blood levels are frequently reached. Lactate in most cases increases with intervascular distance, and with hypoxia. Again, no correlation could be found between the overall level of lactate and the severity of hypoxia. It was also demonstrated for the first time that glycogen accumulation occurs in regions of high diffusion distances, rather than close to blood vessels, and is spatially correlated to hypoxia. These results indicate that hypoxic, perinecrotic areas of the tumor are particularly sensitive to systemic changes in glucose levels. These results also suggest that hypoxia is involved in inducing glycogen accumulation in cancer cells.
Accordingly, glycogen accumulation may be a mechanism that protects hypoxic cells from starvation.

In order to develop a model by which glycogen deposition could be studied in tumor biology, human cancer cell lines WiDr, PC-3, and FaDu were xenografted into nude mice. These tumors were then frozen sectioned and stained for glycogen, glucose, perfusion, and hypoxia. These results mirrored those seen in human tumors.

To further evaluate the role of hypoxia in triggering the deposition of glycogen, PC-3 cancer cells were cultured under normoxic or hypoxic conditions for 48h. Cells cultured under hypoxic conditions showed more rapid and extensive glycogen deposition peaking at about 24h. At 48h, glycogen deposits began to fade, presumably as glycogen was consumed as a fermentable substrate. Additionally, glycogen synthase expression was demonstrated in four cancer cell lines by Western blot.

Future studies will use RNAi to knock down glycogen synthase, and attempt to demonstrate reduction of glycogen deposits in knock down cultures. Then cell death and viability assays will be used to determine if glycogen deposits enhance the survival of cancer cells in hypoxia or glucose depleted environments. Finally, in the future an orthotopic xenograft model will be used to determine whether glycogen deposits enhance primary tumor development, and a metastasis model with be used to determine whether glycogen deposits enhance the development of metastases.
4.2 Methods

4.2.1 Human subjects, drug administration, and sample acquisition

For the EF5 study, nine patients presented with squamous cell carcinoma of the hypopharynx at Duke University Medical Center in the period between 2002 and 2004 in an IRB approved protocol. Written informed consent was obtained from all patients that entered the study. EF5 ([2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide], obtained through NIH, Bethesda, MD, was infused via an i.v. catheter at a rate of 350 ml/h, over a period of 2 h, as described in more detail elsewhere [166]. Biopsies were snap-frozen over the vapor phase of liquid nitrogen within seconds after removal from the tumor.

Thirty eight additional samples of head and neck tumors and normal mucosa were acquired during 1995 and 1997 at Duke Medical Center, Radiation Oncology Dept in a separate IRB approved protocol, following informed consent. Lactate values from these samples have been published elsewhere [28]. Additional tumor samples and normal oral mucosa were obtained through the Oral and Maxillofacial Surgery Department at the University of Mainz. Normal mucosa was obtained during routine dental extractions. Written informed consent was obtained for all patient material used here.
4.2.2 Cryoslicing and immunohistochemical detection of CD31 and EF5

Slides for immunostaining were routinely cut at 10 μm thickness, whereas those used for bioluminescence imaging were cut at 20 μm. Before staining, slides were fixed for 20 min in acetone at room temperature, and allowed to dry for 1 h. CD31 was detected using a mouse monoclonal anti human PECAM antibody (DAKO, 1:100, 1 h at room temperature), and a Cy2 labeled secondary rabbit anti mouse antibody (Jackson Immunoresearch, West Grove, PA, 1:200, 30 min at room temperature). EF5 was detected using a mouse monoclonal antibody obtained from Dr. Cameron Koch, (University of Pennsylvania) that was directly labeled with Cy3 (1:2 dilution in primary antibody blocking buffer, Biomeda, incubated for 1 h at room temperature). Stained slides were temporarily stored at 4°C in 1% formalin until imaging was performed. Reference tissue was obtained in parallel to the actual biopsy, temporarily stored in Excell medium (Invitrogen, Carlsbad, CA), and the incubated with the EF5 analogue EF3, as outlined in the original EF5 protocol (Evans 2000).

Histopathological classification was done by a certified pathologist, and for every sample, the tumor areas were distinguished into invasive and/or noninvasive cancer.

4.2.3 Identification of tumor, stroma, and necrosis

All tissue types were detected and marked on the basis of the H&E stained sections. Tumor necrosis was identified as the occurrence of bright eosinophilic staining
in combination with small, dark (pycnotic) nuclei. In cases when histologically necrotic tissue areas showed EF5 uptake, this was taken into consideration to determine the necrotic boundaries.

### 4.2.4 Fluorescence imaging of the slides

For fluorescence imaging, slides were mounted with 60 μl of PBS, and then covered with a microscope coverglass, equipped with spacers. Slides were imaged using a Marzhauser 100 x 100 scanning stage (Marzhauser, Wetzlar, Germany), mounted on a Zeiss Axiophot II microscope, and powered by a Ludl Mac 5000 controller (Ludl Electronic Products Ltd., Hamthorne, NY). The microscope was further equipped with a computer controlled shutter (Vincent Associates, Rochester, NY). Imaging was done using a 12 bit CCD microscope camera (Retiga 1300R, Qimaging, Surrey BC, Canada). Metamorph (Molecular Devices, Sunnyvale, CA) software was used to control the camera, stage, and shutter. Image stitching was done in Metamorph as well. After imaging, the slides were re-stained for hematoxylin and eosin for general histology, and imaged using a color microscope camera (Carl Zeiss Corp., Thornwood, NY).

### 4.2.5 Bioluminescence imaging of glucose and lactate

Bioluminescence microscopy was done using a method described in detail elsewhere [172]. We used an Andor 16 bit camera with photon counting capacities for imaging (Andor, South Windsor, CT), and a custom built black box with microscope.
4.2.6 Quantification of glycogen using PAS staining

10 μm thick sections were first fixed (100mL dH2O, 150mL 37% w/w Formaldehyde, 750mL absolute EtOH) for 10 min, washed, and incubated with periodic acid solution (0.5% w/v, 50mg Periodic Acid (Sigma P7875) dissolved in 10 mL deionized water). After washing, slides were incubated in Schiff reagent (10g Basic Fuchsin (Fisher A-803), 18g Sodium Metabisulfite (Sigma S-1516), 1L distilled water, 10mL concentrated hydrochloric acid) for 5 min. After additional washing, slides were dehydrated and permanently mounted. For amylase control, slides were incubated with amylase (0.1% w/v, 0.2 g amylase (Sigma A3176) in 200 mL deionized water) at 37°C for 30 min, before adding the periodic acid. We did not perform a counterstain, in order to maintain the ability to perform grayscale analysis.

4.2.7 Image alignment, thresholding and quantification strategy

Side by side comparison was done in slides that were no more than 10 μm apart from each other. All tissue free areas were cancelled out from the analysis, by appropriate masking. For CD31 analyses, images were thresholded using structural criteria: the threshold was set such that artifacts were removed that were clearly not vascular structures. The thresholded images were then transformed into vessel distance maps, using Image J. Other images were then aligned to the distance maps, using Photoshop. Image correlation was done using an ImageJ plugin (Image Correlator).
Resulting data were either processed for plotting, by calculating median y values for each x, or pooled for statistical analysis.

### 4.2.8 Statistical endpoints

In order to identify spatial interrelation of two parameters within a sample, we have aligned the histographical maps (=images) of these parameters, gathered from two adjacent tissue sections. After appropriate and identical masking of aligned images, pairs of adjacent pixels of identical position were compared in terms of their grayscale values. Pairwise combinations of values were plotted and the resulting matrix was analyzed for interrelationship of parameters, using the Spearman rank correlation coefficient \((r_s)\). This method of analysis is appropriate if no linear relationship can be assumed between the two compared variables, the \(r_s\) range between zero and 1, whereas values close to 1 indicate strong and direct functional relationship of the two variables, and values close to zero indicate a weak interdependence. A p-value greater than 0.05 indicates that predictions based on the results of the analysis cannot be made with confidence. We have used Image J for the generation of pixel-to-pixel pairwise comparison matrices (plugin “Image Correlator”), and Matlab for statistics.

### 4.2.9 Data range

Several of the methods of data acquisition used in this study require a consistent way of determination of evaluation range, in order to exclude artifacts: for example the
failure to detect single microvascular structures by CD31 immunostaining, a quite realistic scenario, may lead to artfactually high intervascular distances in this area. In addition, a microvessel may exert its physiological influence on the microenvironment that is visualized in a histological slide, but passed by in a different layer without being detected. Even careful histological examination cannot always exclude aberrant values arising from such inherent problems. A justifiable way of removing such values is consistent truncation of the data range on one or both ends of the data range. When two variables were compared, only the independent variable required truncation, no truncation was necessary for the dependent variable

1. CD31 immunostaining, intervascular distances: Data ranges from microvessel distance maps have been truncated to include only the lower 95% of the data, in order to exclude artfactually high intervascular distances. No truncation was necessary in the low data range (small intervascular distances), because false positive labelings can be detected and excluded on an individual basis.

2. EF5-immunostaining: Data ranges from EF5 immunolabeling were truncated to include the lower 99% of the values, to exclude general staining artifacts.

3. Bioluminescence microscopy: In the high data range, artifacts arise from unspecific punctual glowing of the enzymes, or from air bubbles. While air bubbles can be largely removed, small artifactual peaks need to be cleared
systematically. Thus we have included only the lower 95% of the data range in our evaluation

4. PAS stain: No truncation was necessary, because PAS was never an independent variable

4.2.10 Pimonidazole and Hoechst Staining

Human cancer cell lines were xenografted into nude mice and grown to a diameter of 1 cm max. Animals were anesthetized with 7.5 μl/ g body weight Nembutal. Pimonidazole was injected at 8 μl/g body weight from a stock solution of 10 mg/mL. Animals were placed on a heating pad to incubate pimonidazole for 3 h. Five min before sacrificing the animal 100 μL of 20 mg/mL Hoechst 33342 was injected into the tail vein. Tumors from the anesthetized animals were snap frozen immediately after separation from the blood supply. Snap freezing was carried out in the vapor phase of liquid nitrogen for 10min. Samples were stored at -80°C until staining or further processing.
4.3 Results

4.3.1 Tissue levels of glucose, lactate, and hypoxia in stroma, tumor and necrosis, and in invasive and noninvasive cancer

Table 3 lists average values, and the range between the minimum (1 percentile) and peak (99 percentile) of the glucose, lactate, and hypoxia values found in the nine EF5 infused samples.

Table 3: Glucose, Lactate, and Hypoxia Values in Nine Head and Neck Tumors

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glucose (μmol/g)</th>
<th>Lactate (μmol/g)</th>
<th>Hypoxia (%</th>
<th>glucose insulin</th>
<th>glucose ischemia</th>
<th>glucose sarcoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stroma</td>
<td>0.0 - 4.6</td>
<td>0.0 - 19.1</td>
<td>0.0 - 9.5</td>
<td>0.0 - 0.5</td>
<td>0.0 - 0.4</td>
<td>0.0 - 0.2</td>
</tr>
<tr>
<td>Tumor</td>
<td>0.0 - 4.6</td>
<td>0.0 - 19.1</td>
<td>0.0 - 9.5</td>
<td>0.0 - 0.5</td>
<td>0.0 - 0.4</td>
<td>0.0 - 0.2</td>
</tr>
</tbody>
</table>

Average glucose values ranged from 0.0 to 4.6 μmol/g (~mM) for individual samples. Individual pixel values ranged from 0.0 and 9.5 μmol/g, respectively.

Average lactate concentrations ranged from 4.8 to 19.1 μmol/g; individual pixels
ranged from 1.6 to 29.9 μmol/g. Hypoxia averages ranged from 0.4 to 4.7 percent of maximum binding; individual pixels ranged from zero to 18.2 %. Glucose values were highest in stroma, and then decreased towards the tumor. Wilcoxon matched pair analyses across all samples revealed that these differences were significant \((p<0.05)\). Glucose values tended to decrease further from viable tumor to necrosis, however, this difference was not significant. Lactate followed the opposite trend, having its lowest concentration in the stroma, and increasing in tumor \((\text{Wilcoxon matched pairs, } p<0.001)\). Average concentration of lactate on the edge of necrosis tended to be higher than in the tumor, but this difference was not significant. The Mann Whitney U-test revealed no significant differences in hypoxia, glucose, or lactate concentrations between noninvasive and invasive cancer.

### 4.3.2 Metabolite concentration and hypoxia in relation to distance from vasculature

Figure 25 shows images of two tumors assayed for hypoxia, glucose, lactate, and glycogen along with H+E staining and CD31 staining. This figure gives a “bird’s eye view” of the architecture of tumor metabolism.
Figure 25: Tumor Metabolism Overview in Three Head and Neck Tumors

Coregistered example images from histological slices stained/processed for H&E, superimposed CD31 (green) and EF5 (orange), percentage of EF5 binding, glucose, lactate, and glycogen by PAS stain. Grey areas represent regions of the slide that were excluded from evaluation: In case of EF5, all non-tumor regions were greyed out. Grey areas in glucose and lactate images were either tissue free areas or artifacts, such as air bubbles. In the PAS images, grey areas represent areas with either no tumor, or, as in the lower panel, areas that could not be evaluated, because it was impossible to draw a mask. H&E and CD31 were obtained from the same slice. EF5, glucose and lactate are color encoded, with blue representing the lowest concentration, and red/white the highest. The sample in the first row consists almost entirely of invasive cancer. The second row shows a sample with some nonmalignant tissue on the top part of the sample (N) and extensive invasive cancer below (I). Note that tumor vessels and occurrence of hypoxia (EF5/CD31) consistently display an inverse pattern, as has been shown before (Evans 2001). The arrow points towards a patch of invasive cancer with a prominent necrosis at the edge of the section (nec, arrow). Note that the region is largely avascular, and perinecrotic cells show strong EF5 signal. In such diffusion-limited areas, particularly close to necrosis, glucose levels drop to a minimum, whereas lactate often increases (arrows).

Glycogen as well shows increases towards necrosis. Hypoxic areas usually contain larger deposits of glycogen (upper panel, EF5 and PAS). Note also that nonmalignant tumor can occasionally become severely hypoxic (lower panel).
Cross correlation analysis of quantitative maps of lactate, glucose, and EF5 binding with vascular distance maps revealed that, as described before, hypoxia increased with vascular distance [166]. To identify possible interrelations between glucose concentration and distance to nearest vessel, we performed image correlation between vascular distance maps and aligned glucose bioluminescence images in two observation sites within a sample. All samples, except those with extremely low glucose levels (#1, #2), showed a consistent significant inverse relation between glucose concentration and distance to nearest vessel (all p<0.0001, Figure 26). Lactate concentrations generally increased with increasing intervascular distance (Figure 26B).
Figure 26: Hypoxia, Glucose, and Lactate in Relation to Vessel Distance

Comparison of metabolite concentrations and vascular density between images acquired from parallel histological slices. Vascular distance maps have been obtained by intensity controlled imaging, thresholding, and transformation of the resulting binary CD31 images, so pixel grayscale values would reflect the proximity to the nearest vessel. These distance maps then underwent pixel-to-pixel correlation to the respective quantitative lactate, glucose, or EF5 image. The resulting matrix contained the number of actual events for every potential combination of x/y grayscale values, and were used to test for the existence of concurrent or anticurrent gradients. From two sites analyzed per biopsy, one is shown as an example. The data range of 0-95% of vascular density grades was considered for evaluation. A: The degree of hypoxia increases with increasing intervascular distance, as demonstrated before (Evans 2001). B: Glucose concentration decreases with increasing vascular distance, or remains constant, if overall low. C: Lactate concentrations increased with vascular density, or remained constant.
4.3.3 Metabolite concentrations and EF5 binding in the tumor

Glucose and hypoxia were inversely correlated in all except two cases: in one case glucose was close to zero (#2, Fig 27), and in one case there were not enough data points to perform correlation analyses (#7, Fig 27).
Pixel to pixel correlation analysis was carried out to identify a correlation between the level of hypoxia and the concentration of glucose in the tumor. Every graph outlines the results of the analysis of two sites of the same biopsy. Except in two cases when glucose levels were very low, and therefore no further decline with increasing hypoxia could be detected (#2,#4), glucose concentrations were always inversely correlated to hypoxia (Spearman, p<0.001 in all cases).
4.3.4 Patterns of glycogen accumulation in head and neck cancer

To analyze the patterns of glycogen accumulation in tumors on a histological basis, it was necessary to cancel out all stromal areas including basement membranes, because the PAS reaction shows strong reactivity with the proteoglycan-rich extracellular components. Additionally, proteoglycan staining by PAS can be distinguished from glycogen because the latter is sensitive to amylase digestion while the former is not. Using cross correlation analysis techniques as outlined above we investigated whether there was a relationship between glycogen accumulation and vascular distance or hypoxia. We also examined whether levels of glycogen expression were affected by tumor grade. Across all samples, there was a strong and consistent increase of glycogen accumulation with distance from the nearest vessel (Figure 28). Glycogen also increased with increasing EF5 binding intensity (Figure 28B). In some cases, glycogen declined again, after an initial increase with vascular distance or hypoxia (Figure 28B+D, sample #8). Glycogen accumulation was significantly stronger in noninvasive than in invasive tumors (Figure 28C).
Figure 28: Glycogen Deposition in Relation to Hypoxia and Vessel Distance

A: PAS signal shows an increase with distance from blood vessels in all samples (Statistical results in Table 2). B: The same can be seen when comparing PAS to EF5 binding. In some cases (#8, #9), PAS exhibits an initial increase with mild hypoxia, and a decline at higher levels of hypoxia (also 5D, sample #8, arrows). C: Noninvasive tumor areas (dysplasia, carcinoma in situ) display significantly higher levels of glycogen than invasive cancer (Mann Whitney U-test, p<0.05). D: Coregistration of H&E/CD31 images (double-stained slides), PAS, and PAS/EF5 (both determined from parallel slides). Samples #7 and #8 contain both, invasive (I) and noninvasive (N) tumor, whereas #9 has only invasive cancer. In sample #7, an example of spatial association between glycogen accumulation and EF5 in invasive cancer can be seen (arrow). Sample #8 shows strong EF5 signal in a dysplastic area of the tumor. Glycogen increases initially, then decreases again (arrow). The typical perinecrotic increase of EF5, and corresponding glycogen signal, can be observed in sample #9 (arrows).
4.3.5 Perihypoxic Glycogen Deposition in Human Tumor Xenografts

Glycogen deposition was examined in a human tumor xenograft model, as a step towards an experimental model targeting glycogen deposition. As seen in Figure 29, hypoxic regions of the tumor stain with the yellow pimonidazole, while the well perfused regions stain with the blue Hoechst dye. Using a bioluminescent technique the presence of glucose can be detected (Fig 29). As is the case in human head and neck cancers (Fig 27) hypoxia and glucose localization are inversely related, and hypoxia is inversely related to the perfusion marker. Diffusion limited hypoxia is demonstrated in the yellow pimonidazole staining regions (Fig 29). Glycogen deposition is seen as pink staining in the PAS method (Fig 29). Glycogen deposits on the periphery of the hypoxic fraction of tumor cells (Fig 29); this was also observed in human head and neck cancers (Fig 28). This supports the conclusion that hypoxia is somehow triggering the deposition of glycogen in cells that are becoming hypoxic. However, in cells towards the middle of the hypoxic fraction, glycogen deposits are less apparent (Fig 29). This suggests that glycogen may be used up as a source of fermentable fuel in these cells.
Figure 29: Glycogen Deposition in Perihypoxic Regions on Human Tumor Xenograft

Pink staining in the panel labeled PAS is glycogen deposition. Yellow staining in the panel labeled Pimo/Hoechst is Pimonidazole (Hypoxia) staining. Blue staining in the Pim/Hoechst panel is Hoechst (Perfusion) staining. Glucose concentration is indicated by the luminescence intensity in the glucose panel.
4.3.6 Glycogen Deposition by Hypoxic Cells In Vitro

To establish a link between hypoxia and glycogen deposition in a more controlled environment, PC3 cells were cultured under normoxic or hypoxic conditions in vitro and then PAS stained to evaluate any glycogen deposits. As seen in Figure 30, PC3 cells cultured under hypoxic conditions quickly formed glycogen deposits while PC3 cells under normoxia only exhibited nominal staining for glycogen. Glycogen deposition peaked at 24h in hypoxic PC3 cells. By 48h, glycogen deposits began to fade, presumably as they are used up as a fuel source by hypoxic cells. This study demonstrates that glycogen deposits are not only associated with hypoxia, but that hypoxia plays a role in the initiation of glycogen deposits in cancer cells. Understanding if glycogen deposits play a role in helping cancer cells survive hypoxia will be the next critical step in determining whether or not this is a therapeutic target in cancer.
**Figure 30: In Vitro Hypoxic Induction and Consumption of Glycogen Deposits**

PAS stain of PC-3 cells treated cultured under hypoxia or normoxia for 6, 24, or 48h. 0h is a PAS stain of cells at the beginning of the experiment.

<table>
<thead>
<tr>
<th>Time</th>
<th>Hypoxia</th>
<th>Normoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>6h</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>24h</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>48h</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>
4.3.7 Glycogen Synthase Expression in Human Cancer Cell Lines

To further evaluate glycogen deposition in vitro four human cell lines; WiDr, FaDu, PC-3, and 1-LN were probed for expression of the liver isoform of glycogen synthase. All four cell lines were positive. This protein will be the initial target for RNAi knockdown.

![Figure 31: Glycogen Synthase Expression in Four Human Cell Lines](image)

4.4 Conclusion

It has been reported previously that epithelial tumors accumulate glycogen, and evidence has been presented that some cancers even increase their glycogen content, compared with their tissue of origin [98]. Experiments in heart muscle cells show that hypoxia increases their glycogen content [101], whereas glucose starvation of these cells could even lead to a supercompensation of the baseline levels of glycogen [100]. A potential mechanism may be the activation of PKB/Akt by hypoxia, leading to phosphorylation and thus inactivation of glycogen synthase kinase 3 (GSK-3), which in
turn activates glycogen synthase [173, 174]. This data however, is the first primary evidence that glycogen accumulation occurs in human tumors in association with hypoxia and high intervascular distances. The strong expression in noninvasive, mildly hypoxic tumor areas may argue that hypoxia is not the only stimulus to induce glycogen formation in epithelium and tumors, but differentiation associated processes play a role as well. However, in particular the perinecrotic increase in glycogen (Figure 28D) is intriguing, since this may represent an emergency pathway, and thus a potentially targetable mechanism, which saves cells from starvation. Glycogen could be synthesized from various sources here, such as glucose and other hexoses, and, under expense of ATP, lactate. The discovery that glycogen accumulates in conjunction with hypoxia, and shows an increase towards poorly vascularized and perinecrotic areas, may point towards a new target for anticancer therapy.

5. Conclusion

5.1 Monocarboxylate Transporter Inhibition as a Potential Target in Cancer Therapy

5.1.1 Review

In conclusion, monocarboxylate transporter inhibition can selectively kill highly glycolytic cancer cells under conditions of acidosis or hypoxia (Fig 9 and 19). The
demonstration that cancer cells can be killed by inhibition of MCT activity is proof of principle that metabolic targeting of cancer cells is a viable avenue to develop novel selective chemo-therapeutics. This is a practical application of observations made over 70 years ago by Otto Warburg, and others, who noted that cancer cells differed in their metabolism from normal cells.

In these studies, two modalities were employed to model the microenvironment of the tumor bed: hypoxia and extracellular acidification. While these modalities are quite distinct in their effects on cells exposed to them, they have an important factor in common; both hypoxia and extracellular acidification put the cell under increased pH stress. Cancer cells under hypoxic conditions produce an increased amount of lactic acid secondary to the Pasteur effect. This increase in intracellular lactic acid means an increase in the amount of protons that the cell must export to maintain cytosolic homeostasis. MCT activity is critical to the export of excess protons and lactate in hypoxic cells. Cancer cells under conditions of extracellular acidification must contend with a tide of excess protons leaking in from the extracellular compartment in addition to the copious acid they already produce as a result of the Warburg effect. Under these conditions removal of intracellular lactic acid through MCT activity is critical for the cells survival of acute extracellular acidification. Using both these modalities selective cancer cell death was observed such that cells exposed to hypoxia or extracellular acidification and MCT inhibition, exhibited cells death; while cells at normoxia or
neutral extracellular pH did not exhibit cell death with MCT inhibition (Fig 9 and 19). Importantly, no cell death was seen with hypoxia treatment or extracellular acidification alone (Fig 9 and 19), as cancer cells are quite adept at surviving these insults.

In this study, MCT activity was targeted in cancer cells by three means: lonidamine, a pharmacologic MCT inhibitor; AR-C122982, a pharmacologic MCT1 specific inhibitor; and siRNA knockdown of MCT1. All three modalities were capable of producing intracellular acidification (Table 2, Fig 5, Fig 22, Fig 23), and cell death in cancer cells under pH stress (Fig 9 and 19). Additionally, all three modalities were nontoxic to cancer cells that were not under pH stress. Another fact that contributes to the understanding of how MCT inhibition causes cell death is that neither lonidamine or AR-C122982 were able to cause an increase in cell death in benign endothelial cell lines (Fig 10 and 20). This is presumably because these cells do not produce a high level of lactic acid. Indeed, even if external pH stress is applied via hypoxia or extracellular acidification, BACEC and HUVEC are not burdened with the same high level of intracellular lactic acid as the cancer cells are. They are therefore less sensitive to MCT inhibition.

5.1.2 Future Study

The next step in the development of MCT inhibitors as a potential therapeutic must involve in vivo studies. To truly demonstrate that MCT inhibitors selectively target cancer cells, they must be applied in an animal model. Specifically, an orthotopic
xenograft model should be performed utilizing human cell lines with clearly defined MCT expression and a highly glycolytic phenotype. These tumors should be allowed to implant and grow to a size where some fraction of the cells is hypoxic. Then MCT inhibition should be applied via a small molecule inhibitor or inducible shRNA knockdown. In such a model, cancer cell death and tumor shrinkage should be observed without deleterious effect to the host. It will likely be a requirement to specifically inhibit some, but not all of the MCT’s to prevent damage to the host. Another factor that should be explored is how the tumor’s glycolytic phenotype impacts the efficacy of MCT inhibition as a potential therapy. It is hypothesized that tumors with a less glycolytic phenotype will not be targeted as effectively by MCT inhibition.

5.2 Glycogen Deposition as a Survival Mechanism and Potential Therapeutic Target in Cancer Cells

5.2.1 Review

While it had been known for some time that cancer cells sometimes store glycogen, the reasons for it remained unknown. These studies have demonstrated for the first time that cancer cells store glycogen as a response to hypoxia (Fig 25, 28, 29, and 30). This has been demonstrated by co-localization of hypoxia and glycogen deposits in both human tumors and human cell line xenografted tumors (Fig 25, 28, and 29). It has also been demonstrated by in vitro studies incubating cultured cell lines under hypoxia or normoxia (Fig 30). A precedent has been set for this by the behavior of cardiac and
skeletal muscle tissue. Cardiac and skeletal muscle, maintain basal stores of glycogen under normal conditions [100, 101]. However, when exposed to hypoxia and exercise these deposits can be depleted [100, 101]. When this occurs, the tissue “supercompensates” by more than doubling its deposits of glycogen [100, 101].

Additionally, muscle glycogen deposits have been shown to be protective from ischemic and hypoxic injury, demonstrating the utility of glycogen under substrate deprived hypoxic conditions [100, 102, 104, 105]. It is therefore hypothesized that glycogen is playing a similar role in tumor biology. Such that, cancer cells increase their stores of glycogen when exposed to hypoxia and that this allows them to survive the intermittent substrate and oxygen deprivation that are typical of the tumor microenvironment [24]. Glycogen stores are not necessary for the survival of normal resting tissues as PP1G/RGL knock-out mice unable to form glycogen deposits are phenotypically normal [109]. For this reason, glycogen synthesis is an attractive target as a selective metabolic cancer therapeutic.

There is an additional reason why glycogen deposition must be explored as a potential target in cancer, its potential role in metastasis. It is known that cells exposed to acute hypoxia prior to being injected into animals in a metastasis model display a greatly increased rate of metastasis [112-114, 175]. However, the reasons for this are not understood. We have demonstrated that cancer cells form glycogen deposits as a response to acute hypoxia (Fig 30). It is also known that during the process of
metastasis, cancer cells must invade into distant tissues without a readily available blood supply, where they will quickly become hypoxic. If the formation of glycogen deposits plays a role in the increased rate of metastasis observed in these models then targeting glycogen synthesis may be a critically important step in preventing cancer metastasis.

5.2.2 Future Study

Several studies remain to be performed to explore the hypothesis that glycogen deposition is a factor in cancer cell survival and metastasis. (1) These include a cell death assay of cancer cells under hypoxic and or glucose deprived conditions in the presence and absence of glycogen deposits. (2) The development of a siRNA knockdown of glycogen synthase or another protein critical to glycogen deposition to clearly show glycogen’s role as a survival factor. (3) An orthotopic xenograft model of cancer in which the ability to make glycogen deposits is inhibited after tumor formation by an inducible knockdown, to validate glycogen synthesis as a survival factor for cancer cells in vivo. (4) A metastasis model comparing cells exposed to acute hypoxia with and without a glycogen synthase knockdown, to determine the role of glycogen deposition in the process of metastasis. (5) Finally, it would be desirable to know the mechanism(s) by which hypoxia induces glycogen formation, including the presence or absence of hypoxia responsive elements in the promoter of glycogen synthase or related regulatory and signaling protein(s), and the role if any for HIF-1.
Bibliography


Biography

Quintin Jose Quinones was born February 20, 1979 in Boynton Beach, Florida to Drs. Jose and April Quinones. Quintin spent his youth learning to love the outdoors and developing a thirst for adventure through boating, snorkeling, S.C.U.B.A. diving, hunting, fishing, aviation, and motorcycling, mostly in South Florida. Quintin graduated from Lake Worth Christian High School in 1997. Quintin’s college education consisted of one year at Calvin College, and three years at Florida Atlantic University where he graduated with a Bachelor of Arts in Chemistry, Cum Laude. Quintin matriculated to the Medical Scientist Training Program to pursue his graduate training in 2001. He expects to graduate with combined M.D. and Ph.D. degrees in May of 2009, and pursue residency training in Anesthesiology with the goal of combining research with clinical practice in his career. In his personal life, Quintin married Maria Johanna Koornneef on March 23, 1998. They are happily married and currently have four children: Jose, Daniel, Julia, and Lucas; they are thinking of trying for one more.
Publications


* indicates authors contributed equally