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**PROBING THE ANTI-CANCER MECHANISM OF
SELENITE: A METABOLIC APPROACH**

By

Alex Belshoff

B.S., University of Louisville, 2008

A Dissertation

Submitted to the Faculty of the
School of Medicine of the University of Louisville
In Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

Department of Pharmacology and Toxicology
University of Louisville
Louisville, Kentucky
August 2013

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A Dissertation Approved on

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DEDICATION

I would like to dedicate this dissertation to my parents, Mark Belshoff and Lynn Bolton, and my grandparents, Kenneth and Donna Bolton. They have continuously provided me with support for the entirety of my upbringing, and I would not have succeeded in any of my studies or accomplishments, had it not been for their efforts.

ABSTRACT

PROBING THE ANTI-CANCER MECHANISM OF SELENITE: A METABOLIC APPROACH

Alex Belshoff

June 19, 2013

The following dissertation was aimed at characterizing the metabolic disruptions that occur with selenite treatment in human lung cancer. Specifically, the pathways of glycolysis, TCA cycle, nucleotide metabolism, pentose phosphate pathway, hexosamine biosynthetic pathway, and glutaminolysis were examined thoroughly. A stable isotope-resolved metabolomic (SIRM) approach utilizing the tracers $^{13}\text{C}_6$ -glucose and $^{13}\text{C}_5, ^{15}\text{N}_2$ -Gln was employed in order to trace the flow of atoms through the metabolic network. By utilizing this approach and a combination of NMR and MS analyses, the metabolic pathways perturbed by selenite were reconstructed based on measured isotopologues and isotopomers and known biochemical reaction mechanisms. These methods were employed in both cell-based and individualized ex vivo lung cancer tissue models developed from an ongoing NSCLC patient study. In the A549 cell model, glycolysis was upregulated by selenite treatment with a corresponding decrease in flux through the TCA cycle and increase in flux through lactate dehydrogenase. These effects were propagated through nucleotide synthesis by inhibiting the rate of carbon

and nitrogen incorporation into nucleobases. Furthermore, UDP-GlcNAc synthesis was diminished by selenite treatment, particularly in terms of its uracil subunit and acetyl group incorporation. These findings were specific to cancer cells and not observed in non-transformed NHBE cells and were recapitulated in the patient data. The KGA splice variant, GAC, was greatly reduced by selenite treatment, which was consistent with the metabolic data and reduction in glutaminase activity. Again, this effect was not observed in NHBE cells. Proliferation of A549 cells was at least partially restored upon supplementation of selenite-treated cells with Glu, the product of the glutaminase reaction, validating the importance of glutaminase in selenite-mediated cytotoxicity, including the synthesis of glutathione for detoxifying excess ROS. Finally, findings from the tissue slice data suggested that selenite induced a decrease in the synthesis of glycogen. The brain form of glycogen phosphorylase (PYGB) was suppressed using shRNA in order to characterize the metabolic consequences. Glycogen was identified as an important source of fuel for A549 cells, since suppression of PYGB resulted in reduced growth and colony formation accompanied by decreased nucleobase synthesis, TCA cycle activity, and amino acid synthesis.

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CHAPTER 1

1.1 Introduction

It is estimated that 1.66 million people in the US will be diagnosed with some form of cancer and that 580,000 people will die from cancer in 2013 [1]. The current risk for a cancer diagnosis during one's lifetime is approximately 40%, which is a reduction of only 0.9% and 0.3% for men and women, respectively, from 1999-2010 [1]. Furthermore, the reduction in cancer deaths for this time period decreased by only 1.5% [1]. In the US, death from cancer is currently the second highest cause of death behind only cardiovascular disease, but is above chronic obstructive pulmonary disease (COPD), stroke, and accidents [2]. The problem is particularly severe for lung cancer, which causes the greatest number of cancer deaths worldwide with an estimated 228,000 diagnoses and 159,000 deaths in the US alone for 2013 [3]. Currently lung cancer is the 3rd most common form of cancer behind breast and prostate, despite decreases in its prevalence and death rate of 2.6% and 2.2%, respectively, from 2005-2010 [1]. However, these reductions are mostly due to a decrease in the prevalence of smoking with only small improvements in lung cancer diagnosis or management. Obviously, smoking is a huge environmental risk factor of lung cancer that increases with number of cigarettes smoked and the duration of the smoking

history. Cessation remains one of the most effective means of prevention and reduces one's risk of developing lung cancer approximately 3-fold [4]. In a study following men born in the 1920's, cancer risk was reduced to that of a non-smoker if cessation was achieved by age 30 and was reduced by half if it was achieved by the age of 50 [4].

Lung cancer is a particularly severe issue for Kentucky, which leads the nation in incidence and deaths [1]. In addition, Kentucky carries the 2nd highest incidence rate for all cancer diagnoses and deaths [1]. Furthermore, lung cancer related medical expenditures are a huge burden on the healthcare system with an estimated direct cost of 12.1 billion dollars in 2010, corresponding to approximately 10% of total cancer-related expenses [5]. Clearly, lung cancer research is of vital importance not only to improve management, but also to improve diagnostic capabilities.

1.2 Current practices in lung cancer diagnostics and management

The long-term goal of applying SIRM in lung cancer research is both to aid in improved diagnostics and therapeutics for better management of lung cancer. The cure rate for lung cancer remains woefully low despite new management strategies for other types of cancers. These poor outcomes are largely due to late diagnoses where surgery is not an option due to significant metastasis. Only 15% of tumors are localized and operable at the time of diagnosis, which carries the best 5-year survival rate of 53.5% [1]. However, 5-year survival drops

precipitously to 26.1% and 3.9% for regional and distant diagnoses, respectively [1]. Distant metastasis comprises the majority of diagnoses at 57% [1]. Adenocarcinomas of the lung carry the best prognosis with a 5-year survival rate of approximately 60% for localized disease; however small-cell cancer prognosis is poor even with a localized diagnosis (20.1%) [6]. The squamous and large cell subtypes comprise roughly 20% and 5% of lung cancers, respectively, and there is discrepancy over whether the prognosis of these subtype differ significantly from adenocarcinoma [1].

1.2.1 Diagnosis.

As stated previously, much of the problem underlying poor lung cancer survival is late diagnosis. When symptoms present, the tumor has usually distantly metastasized, and screening historically has been largely ineffective. However, a risk-benefit analysis of the ongoing national lung cancer screening trial (NLST) indicated that low-dose computed tomography (LDCT) screening was beneficial for a select group of patients [7]. Therefore, the American Cancer Society recently issued recommendations for annual screening of people aged 55-75 years old who are current smokers or who have quit within the past five years.

Diagnosis also involves a thorough evaluation of tumor stage that incorporates the tumor size, invasion, number of lymph nodes involved, and presence or absence of metastasis. This step is crucial in order to determine

prognosis as well as surgical candidacy since surgery generally yields better outcomes than radiation or chemotherapy.

NSCLC is staged according to the recently revised TNM staging system laid out by Goldstraw et. al [8]. Stage I disease is diagnosed when the primary tumor size is up to 5 cm in diameter with minimal invasion no further than the main bronchus. No regional lymph node involvement or distant metastasis can be present for this stage. Tumors greater than 7 cm in diameter are included in stage II disease that have more extensive invasion and involvement of regional lymph nodes not extending to the mediastinum, but without distant metastasis. Both stage III and IV disease can involve primary tumors of any size with extensive invasion and regional lymph node involvement; however, stage IV includes distant metastasis. Accurate staging involves requires diligent surgical dissection as well as proper histopathological examination and is essential for prognosis and management.

This staging approach has recently been suggested and is in the process of being implemented for SCLC. Historically SCLC was categorized into either limited or extensive disease, which essentially referred to the presence or absence of distant metastasis. The more detailed TNM staging system has been validated for SCLC to improve prognostic value; however, management has remained largely unchanged [8].

1.2.2 Management

Lung cancer treatment is dependent on the stage at the time of diagnosis as well as other general health factors of the patient that govern surgical candidacy. Also, the treatment plan is typically defined for non-small cell lung cancer (NSCLC) or small-cell cancer (SCC). For early stage (I and II) tumors surgical resection, if possible, is the standard of care [9]. Lobectomy is commonly done; however, recent evidence suggests that sublobar (e.g. wedge) resection provides comparable outcomes. The standard of care for early stage inoperable tumors is radiotherapy (RT), but has poorer outcomes (5-year survival 15-48%). However, the use of stereotactic body RT (SBRT) is increasing for these patients, as it yields better outcomes with substantially less toxicity for surrounding tissues.

For advanced stage tumors, surgery is generally not an option, and combined chemoradiotherapy is typically used as it yields better outcomes than chemotherapy or radiotherapy alone. However, research is ongoing to determine whether induction chemotherapy, i.e., shrinking the tumor mass before surgery, may benefit a select patient population. For late stage NSCLC, platinum (Pt) based chemotherapy is generally adopted. Bevacizumab, a VEGF binding drug that inhibits angiogenesis, has also been recently combined with Pt-based therapy to achieve better outcomes. There are also some drugs that are used for tumors containing specific mutations. These include Crizotinib and Erlotinib, which are ALK kinase and tyrosine kinase inhibitors, respectively.

Finally, SCLC is typically managed with combined chemoradiotherapy for limited disease (e.g., etoposide/cisplatin with radiotherapy) and chemotherapy alone for extensive disease; however, these cancers carry a particularly grim prognosis despite ongoing research.

1.3 Selenium nutrition and anti-cancer efficacy

1.3.1 Nutrition and toxicity

Selenium is an essential micronutrient and is incorporated into the '21st' amino acid, selenocysteine [10]. Natural foods rich in selenium include Brazil nuts, seafood, and organ meats, among others [11]. Most of these natural food sources contain organic seleno-compounds such as selenomethionine as the predominant selenium species. The composition of inorganic selenium such as selenite and selenate in soil has large regional variation, which is propagated into organic forms such as selenomethionine through plant metabolism [12]. Selenite and selenate as well as organic forms are also available in the form of nutritional supplements. Selenium is utilized primarily in the redox reactions of enzymes like glutathione peroxidase and thioredoxin reductase by functioning similarly to a thiol. Selenium deficiency can lead to a constellation of symptoms including cardiomyopathy, weakened immune response, thyroid abnormalities, and bone deformities [13, 14]. Kashin-Beck disease is a well-characterized selenium deficiency endemic to China that involves cardiovascular disease and bone deformities [15]. However, selenium compounds can also be toxic at high doses (selenosis) and leads to brittle nails, hair, and skin abnormalities in humans due

to chronic overconsumption [16]. Selenosis is typically more of a problem in livestock than in humans.

1.3.2 Selenium compounds possess anti-cancer activity

Selenium has a long history in cancer research as both a chemopreventive and chemotherapeutic agent. A large number of organic and inorganic seleno-species exist, such as methylseleninic acid (MSA) or selenite, which are cytotoxic to cancer cells. It is important to differentiate the various seleno-compounds outside of the umbrella term, selenium, due to a large variation in anti-cancer and chemopreventive efficacy. MSA has been studied extensively in prostate cancer and has been linked to decreased expression of prostate specific antigen [17-19]. Apoptosis has also been observed for prostate cancer cells treated with MSA [20]. Selenite also induces apoptosis in cancer cells including lung adenocarcinoma A549 cells and rodent models [21]. Recently, selenite was shown to cause ROS mediated autophagy in A549 cells by Park et al. [22]. Fan et al. has demonstrated the use of stable isotope-resolved metabolomics (SIRM) combined with transcriptomics for probing metabolic alterations caused by selenite [23]

Despite these findings, however, the mechanism behind selenite toxicity is only partially understood. The prevailing theory of selenite toxicity involves successive reductions of selenite, eventually to some selenide species, generating toxic ROS in a process that causes damages to DNA and other cell machinery leading to death [24, 25].

1.3.2.1 Chemopreventive clinical trials

Selenium supplementation has been the subject of several clinical trials that yielded mixed results. The double-blind clinical trial by Clark et al. showed a statistically significant decrease in prostate cancer incidence with a selenized yeast supplementation [26]. Selenized yeast comprised of a mixture of selenium-containing compounds. Unfortunately, the large scale SELECT trial (selenium and vitamin E cancer prevention trial) was prematurely terminated due to slightly increased cancer incidence with selenium supplement among the participants (reviewed in [27]). These results were disappointing; however, they are difficult to interpret due to the choice of the selenium agent, selenomethionine (SeM). SeM alone has very low cytotoxicity based on our findings and its cytotoxicity is enhanced only when combined with the methionine lyase treatment, which was missing in the SELECT trial [28]. Therefore, further mechanism-based research is necessary in order to determine the optimal choice of selenium-based chemoprevention and/or therapy.

1.4 Cancer is a metabolic disease

1.4.1 The Warburg effect

Historically, altered metabolism in cancer was established in the early 1920's by Warburg [29]. In an elegant experiment, he demonstrated that even under normoxic conditions, the rate of glucose utilized by tumor tissue for lactic fermentation was ten times that used for aerobic respiration. Glycolytic

stimulation, i.e., the “Warburg effect”, has since been established as a hallmark of human tumors. Warburg actually postulated that stimulated glycolysis was the fundamental origin for tumorigenesis; however, the Warburg effect is now considered as one of the many metabolic consequences of accumulated mutations that enable the anabolic and energetic requirements for tumor growth.

1.4.1.1 Clinical manipulations of the Warburg effect

The Warburg effect is so prevalent among cancers that it is routinely used for cancer diagnostics by measuring the uptake of 2-deoxy-2-¹⁸F-D-glucose into suspected tumor tissues using positron emission tomography (PET) scanning. Increased rate of glycolysis has also been studied as a potential target for chemotherapy. 2-deoxyglucose is a hexokinase inhibitor that showed promise pre-clinically; however, the clinical trial yielded disappointing results [30]. Recently, 3-bromopyruvate (3BP) has been investigated in *in vitro* and *in vivo* experiments [31]. 3BP has shown low non-cancer toxicity and high selectivity for the inhibition of glyceraldehyde 3-phosphate dehydrogenase. Furthermore, the potent 6-Phosphofructo-2-kinase (PFKB3) inhibitor, PFK15, has generated very favorable results and is entering phase I clinical trials [32].

1.4.2 Tumors require increased anabolism and favorable energetics for growth

In order to sustain the rapid and uncontrolled growth that defines cancer, tumor cells need to increase their anabolism. By utilizing tracer-based

experimental design, Fan et. al. have shown that non-small cell lung cancer (NSCLC) tumors possess increased rates of glycolysis, Krebs' cycle activity, pyruvate carboxylase (PC) activity, and nucleotide synthesis [33]. Increased flux through these pathways is vital to generating lipids, reducing equivalents, amino acids, and nucleotides necessary for new biomass production. The high rate of nucleotide synthesis that must occur to generate nucleic acids for rapidly growing cancer cells has led to the use of anti-metabolites such as thioguanine, 5-fluorouracil, and methotrexate as chemotherapeutics.

1.4.2.1 Altered metabolism is caused in part by the loss of tumor-suppressor genes and the activation of oncogenes

Changes of metabolic regulation in human cancer have been an important focus of biomedical research and have been shown to be induced by a combination of genetic mutations and altered expression of transcriptional factors. These factors include increased transcription of *MYC*, increased activity of the transcription factor NF- κ B, the protein kinase AKT, and stimulation of the mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase signaling pathways, among many others [34]. For glycolysis in particular, a combination of the loss of the tumor suppressor gene phosphatase and tensin homolog (*PTEN*) and activation of AKT and mTOR signaling cascades increases hypoxia-inducible factor 1 α (HIF-1 α) activity. HIF is stabilized under hypoxic conditions and positively regulates nine out of the 10 enzymes of glycolysis [35]. Also associated with elevated HIF-1 activity is the increase in glucose

transporters (GLUTs) to supply accelerated glycolysis [36]. Interestingly, many tumors also express the fetal M2 form of pyruvate kinase (PKM2). PKM2 binds to tyrosine-phosphorylated proteins, which in turn removes the positive regulation imposed by fructose-1,6-bisphosphate [37]. These events have the effect of diverting glucose for anabolic processes such as nucleotide metabolism by increasing the flux through the pentose phosphate pathway.

Gln is another essential nutrient for rapidly growing tumors as glutaminolysis is an important anapleurotic input for the TCA cycle and regeneration of anabolic precursors. Gln is converted to Glu enzymatically via glutaminase (GLS), which is up-regulated by expression of the proto-oncogene *MYC* [38]. Gln consumption is often increased in tumors and glutaminase (GLS) inhibition has been shown to induce apoptosis in kidney and lung cancer cells, which overexpress *MYC* to drive GLS expression [39].

1.4.2.2 Mutations of specific metabolic enzymes characterize certain familial cancers

Interestingly, several familial cancers are associated with deficiencies of the TCA cycle enzymes fumarate hydratase (FH) or succinate dehydrogenase (SQR). Hereditary leiomyomatosis and renal cell carcinoma (HLRCC) is characterized by a germline mutation and loss of function of the gene expressing FH, followed by a profound rearrangement of TCA cycle metabolism [40]. SQR deficiency, again caused by germline mutation, is associated with both renal cancer and gastrointestinal stromal tumors (GIST) [41].

1.4.3 O-GlcNAc modification of protein targets is increased in cancers

Uridine diphospho-*N*-acetyl glucosamine (UDP-GlcNAc) is a product of the hexosamine biosynthetic pathway (HBP) that is used in the synthesis of glycosaminoglycans, proteoglycans, and glycolipids [42]. UDP-GlcNAc is also the substrate for O-GlcNAc transferase (OGT), which catalyzes the post-translational modification of Ser and Thr residues in certain proteins [43, 44]. The expression of OGT is increased in a variety of cancers including lung that correlates with increased post-translational modification of protein targets [45, 46]. Furthermore, targets are often co-regulated via phosphorylation with either synergistic or competitive effects [47, 48]. These two modes of regulation are dynamic and can even occur on the same residue.

UDP-GlcNAc biosynthesis is complex and incorporates intermediates from glycolysis, the PPP, TCA cycle, glutaminolysis, and nucleotide synthesis. Therefore, O-GlcNAcylation has been described as a nutrient sensor and appears to regulate phosphofructokinase (PFK) by diverting glucose carbon through the PPP under hypoxic conditions [49]. Interestingly, glucose starved cancer cells maintain flux through the HBP and synthesis UDP-GlcNAc by utilizing carbon from glycogen [50]. Other targets of OGT include OGT itself, MYC, p53 and histones, among many others [51-54]. However, the functional significance of the majority of these events is unclear.

Elucidation on the synthesis of UDP-GlcNAc from glucose and Gln is essential to understanding upregulation of O-GlcNAcylation in lung cancer .

Incorporation of glucose carbon into UDP-GlcNAc has been modeled by Moseley et al. and this pathway is one focus of this study[55].

1.5 Rigorous analysis of the metabolic network requires a systems-biochemical approach

In order to ascertain the breadth of metabolic changes that is induced by either cancer transformation or a stressor such as an anti-cancer agent, one must utilize an approach that is able to simultaneously measure the multitude of metabolites that participate in many pathways.

1.5.1 Stable isotope-resolved metabolomics (SIRM)

SIRM is an approach that utilizes a tracer-based experimental design in order to measure the incorporation of stable isotopes into metabolites. By utilizing tracers like $^{13}\text{C}_6$ -glucose or $^{13}\text{C}_5, ^{15}\text{N}_2$ -Gln, one can trace the flow of ^{13}C and ^{15}N through glycolysis, PPP, HBP, TCA cycle, nucleotide biosynthesis, lipid biosynthesis, and glutaminolysis by measuring the different combinations of mass isotopologues or positional isotopomers that are generated for a given metabolite (Figs. 2.6, 2.14, 2.18, 4.15). Isotopologues and positional isotopomers both refer to structures that differ in isotopic composition; however, positional isotopomers refers to the positional location of the isotope, e.g., $^{13}\text{C}_1$ -lactate has three distinct positional isotopomers. One can then begin to reconstruct the metabolic network and assess flux through specific pathways. By understanding which pathways are affected by a given stressor, one can then follow up with

more specific and complimentary analyses such as enzymatic activity assays, immunoblot, or qPCR in order to determine why and how a pathway is altered.

The use of stable isotopes offers several important advantages over radioisotopes, including elimination of biohazard risk and ready detection by both NMR and MS. The incorporation of stable isotopes into a metabolite will not significantly alter its enzymatic transformation with respect to an unlabeled metabolite. Although the kinetic isotope effect on enzyme reaction rate can occur for different isotopes, this effect is largely negligible in ^{13}C or ^{15}N -based SIRM because these isotopes have small relative mass change with respect to their natural abundance atoms. Furthermore, the bond-breaking steps are usually not rate-limiting within a pathway.

Fan et al. have utilized SIRM extensively to characterize metabolic alterations in lung cancer [33, 55-60]. The use of nuclear magnetic resonance (NMR) and mass spectrometry (MS) technologies are essential for SIRM and offer complementary analyses. Nuclei such as ^{13}C and ^{15}N are observable by NMR to provide structural information such as the position(s) of a heavy atom within a given metabolite (isotopomer) based on the spin coupling patterns, covalent linkage, and chemical shift information obtainable from NMR analyses. MS readily differentiates different isotopologues due to their mass differences. Furthermore, with the advent of Fourier transform-ion cyclotron resonance-mass spectrometry (FT-ICR-MS), one can distinguish the tiny mass difference between ^{13}C and ^{15}N (e.g. $^{13}\text{C}_1, ^{15}\text{N}_0$ and $^{13}\text{C}_0, ^{15}\text{N}_1$) labeled species due to ultra-high mass resolution and accuracy.

Finally, one must not overlook the importance of bioinformatics for systems-biochemical approaches. The enormously rich data sets coupled with high-throughput analysis generated from metabolomic investigation make efficient bioinformatics essential to data analysis for generating meaningful and comprehensive conclusions or new hypotheses.

1.6 Rationale for this study

This study aims to test the hypothesis that the anti-cancer mechanism of selenite can be elucidated, at least in part, by a thorough and rigorous metabolic approach. Lung cancer is an important focus of study due to its prevalence and high lethality worldwide, and especially in Kentucky. Metabolic reprogramming elicited by oncogenic transformations is increasingly apparent in various types of cancer. Thus a systematic metabolic approach utilizing SIRM is expected to advance understanding in the diagnosis and management of lung cancer by uncovering key reprogrammed events, which then can be targeted for therapy or help uncover early detection markers. The use of individualized *ex vivo* lung cancer tissues adds a translational element to this investigation and can offer an exciting view of cancer metabolism from actual patients. Selenite is an excellent candidate to probe lung cancer metabolism given its history of anti-cancer activity and potential for chemoprevention. Further research into selenite's mechanism of action is imperative to help clarify the recently failed SELECT trial.

CHAPTER 2-SELENITE INDUCES CANCER-SPECIFIC CHANGES IN METABOLISM

2.1 Introduction

An emerging trademark of human tumors is increased O-linked modification of proteins by *N*-acetylglucosamine (O-GlcNAcylation) [51]. Increased O-GlcNAcylation has been observed and reported in lung, colon, and breast cancers in both pure cell culture and human tumors [45, 46, 61]. The expression of the transfer enzyme responsible for this post-translational modification, O-linked *N*-acetylglucosamine transferase (OGT) is increased in these tissues and thus is likely responsible for the observed increase in GlcNAc modification [45]. However, what has been lacking in these investigations is a detailed study of the biosynthesis of uridine diphospho-*N*-acetylglucosamine (UDP-GlcNAc), the GlcNAc donor and substrate of the transferase, in human lung cancer associated-upregulation of GlcNAc modification.

UDP-GlcNAc is the nucleotide hexosamine that contributes GlcNAc for O-linked glycosylation. The synthesis of UDP-GlcNAc is a complex processes that comprises multiple pathways including the pentose phosphate pathway, hexosamine biosynthetic pathway, glutaminolysis, nucleotide biosynthesis, Krebs' cycle, and glycolysis. The connection of this metabolite to glucose and glutamine metabolism has established UDP-GlcNAc synthesis as a nutrient

sensor [47, 49, 62, 63]. As such, UDP-GlcNAc biosynthesis, including its contributing metabolic pathways, needs thorough SIRM-based analysis to determine the regulatory mechanisms underlying synthesis of this important metabolite.

The utility of SIRM for the analytical dissection of cancer transformed lung metabolism has been well established [33, 56, 58]. Furthermore, UDP-GlcNAc biosynthesis in prostate cancer cells was previously modeled using a non-steady-state algorithm and a SIRM based approach by Moseley et al. [55]. Here, I expanded upon previous work reported by Fan et al. on metabolic alterations induced by the anti-cancer agent selenite [60]. Our goal was to uncover a more detailed mechanistic understanding of selenite-induced anti-cancer activity by determining the time course enrichment of glucose and Gln derived metabolites associated with the UDP-GlcNAc biosynthetic network and comparing these profiles to an immortalized normal human bronchial epithelial (NHBE) cell line.

This approach was then repeated in parallel experiments utilizing NSCLC surgical patient tumor and adjacent benign tissue. These individualized lung cancer models offer a more realistic interpretation of the metabolic changes that occur with exposure to selenite because they contain much of the tumor infrastructure, including stromal cells as well as potentially tumor-suppressive immune cells. Furthermore, these experiments are derived from actual NSCLC patients and are thus representative of the variability of early-stage operable lung cancer. However, the heterogeneous cell population of this model is likely to produce a more mixed set of results than those obtained from traditional

monolayer cell culture. But, this approach also includes a measure of the different metabolic adaptations that are likely to occur that support tumor growth in actual humans. Finally, by comparing the individualized lung cancer models to cell-based experiments one can begin to assess the power and/or limitations of conclusions drawn from cell culture experiments.

2.2 Methods

Cell culture: A549 cells were grown in glucose-free, glutamine-free DMEM medium supplemented with 10% FBS, 0.2% glucose, 4 mM glutamine, and 100 I.U./mL penicillin 100 μ g/mL streptomycin at 37 °C and 5% CO₂. Na₂SeO₃ (Sigma) solutions were prepared immediately prior to use and sterilized using 0.22 μ m syringe filter. Cells were grown to 70% confluence for all experiments. Two hours prior to treatment, medium was aspirated and replaced with half volume of the tracer medium containing either ¹³C₆-glucose (Sigma Isotec, St. Louis, MO) or ¹³C₅, ¹⁵N₂-Gln (Cambridge Isotopes, Andover, MA). The remaining medium was then added with doubled Na₂SeO₃ concentration in order for cells to be conditioned with the tracer medium prior to treatment.

Tissue slices: During NSCLC surgical resection, very thin slices (0.5-1 mm) were prepared from tumor and adjacent benign tissue by the operating surgeon (Dr. M. Bousamra II). Tissue slices were immediately placed in T-25 culture flasks with 8 mL DMEM medium supplemented with 10% FBS, 100 I.U./mL penicillin 100 μ g/mL streptomycin, 0.2% glucose, and 4 mM Gln. Either ¹³C₆-glucose or

$^{13}\text{C}_5^{15}\text{N}_2\text{-Gln}$ was used for tracer experiments. Slice medium was then treated with or without $6.25\ \mu\text{M}\ \text{Na}_2\text{SeO}_3$. Flasks were then placed on a rotator in a cell culture incubator at $37\ ^\circ\text{C}$ and $5\%\ \text{CO}_2$ for 24h. After 24h, medium was collected for downstream analysis, and tissue slices were washed with PBS before flash freezing in LN_2 .

Proliferation assays: Proliferation of A549 cells was measured in 96-well plates according to the protocol by Rasputnig et al. [64]. Briefly, 24 h after treatment with selenite, cell medium was aspirated and replaced with 50% ethanol in H_2O for 30 min. Ethanol was then aspirated and replaced with minimal volume of 0.2% Janus green dye in PBS for 5 min. Dye was aspirated and plates were rinsed in tap H_2O and drained by gravity. Dye was then dissolved in 0.5 N HCl and absorbance was measured at 595 nm. Growth was reported as the fraction of the absorbance for a given concentration of selenite divided by the control absorbance. Increasing concentrations of Na_2SeO_3 were used and the EC_{50} concentration was interpolated from the logarithmic transformation of the dose-response curve. Growth did not reach 0% for the concentrations of Na_2SeO_3 used.

Extraction of metabolites: At the time of harvest, medium was collected before all plates were washed three times with ice cold PBS, and PBS was removed as much as possible by aspiration. The cells' metabolism was then quenched with 1 ml of CH_3CN kept at -20°C before 0.75 ml of nanopure water was added and

cells were harvested by scraping. The process was repeated once before CHCl_3 addition to a final $\text{CH}_3\text{CN}:\text{H}_2\text{O}:\text{CHCl}_3$ ratio of 2:1.5:1 to obtain the polar, non-polar and insoluble proteinaceous interface fractions for downstream analysis [65, 66]. For metabolite extraction from the medium, protein was first precipitated in ice-cold 10% trichloroacetic acid (TCA), and supernatant was lyophilized immediately to remove the acid. Since some metabolites such as glutamine are sensitive to prolonged storage in acidic conditions, metabolite analysis of medium samples by NMR or GC-MS was performed immediately after lyophilization.

NMR experiments: All NMR experiments were conducted on a Varian Inova 14.1 T instrument equipped with 5 mm HCN cold probe. Cell and medium extracts were dissolved in D_2O with 30 and 50 nmol d_6 -DSS, respectively, as internal standard. Shigemi tubes were used for polar extracts to minimize sample volume and thus to concentrate the metabolites. 1D proton spectra were recorded with an acquisition time of 2 seconds after initial presaturation of the H_2O signal with a recycle time of 5 seconds. 1D ^{13}C -filtered HSQC spectra were measured using an acquisition time of 150 ms. 1D proton spectra were processed using a 1 Hz exponential line broadening function and a zero fill of 128 k points. 1D ^{13}C -filtered HSQC spectra were processed with a 6 Hz line-broadening exponential and 0.07 Hz Gaussian functions and zero filled to 16 k points. Both types of spectra were processed manually for baseline and phase corrections. 1D proton spectra were referenced using the methyl signal of D_6 -DSS at 0 ppm, and 1D ^{13}C -filtered

HSQC spectra were referenced to the C3-lactate peak at 1.32 ppm and 1.41 ppm for cell and medium extracts, respectively. Metabolites were identified in reference to an in-house standard database and quantified by calibration with the methyl resonance of the DSS internal standard using the MestReNova NMR processing software (Mestrelab Research, Santiago de Compostela, Spain) [59].

GC-MS analysis: An aliquot of the lyophilized extracts was derivatized in 50% *N*-*tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% *tert*-Butyldimethylchlorosilane (MTBSTFA + 1% TBDMSCI) in CH₃CN before GC-MS analysis. All GC-MS data were acquired on a ThermoFinnigan PolarisQ GC-Ion trap MSn instrument (ThermoScientific, Rockford IL). One μ L of the derivatized sample was separated on a SGE Forte (Victoria, Australia) 5% phenyl capillary column with the following dimensions: 50 m x 0.15 mm; 0.25 μ m film thickness. The GC parameters were as follows: injector and transfer line, 280 °C; oven, 60 °C for 2 min, 60 to 150 °C at 20 °C/min ramp, 150 to 300 °C at 6 °C/min ramp; He carrier gas with flow rate of 1.5 mL/min. Spectra were acquired in segscan mode with ranges of 140-206, 209-280, and 283-650 m/z with an acquisition time of 0.97 s. Spectra were processed using Xcalibur MS software and quantified using a mixture of 63 standards at 50 μ M each except for glutamate at 500 μ M. All samples and standards were spiked with 1 μ M norleucine as internal standard.

Nucleotide processing for FT-ICR-MS (Fourier transform-ion cyclotron resonance-mass spectrometry) analysis: Nucleotides were ion-paired with hexylamine and extracted from polar extracts as described previously [57]. Briefly, dried extract aliquots (e.g. 1/16 of one 10 cm plate) were dissolved in 5 mM hexylamine in H₂O (buffer A) with the pH adjusted to 6.3 with acetic acid. Dissolved nucleotides were adsorbed onto C18 ZipTips (Millipore, Billerica, MA) pre-conditioned with MeOH and buffer A. Bound nucleotides were then washed with buffer A followed by elution with 70% buffer A/30% buffer B (90% methanol and 10% 10mM ammonium acetate adjusted to pH 8.5 with aqueous ammonia). Eluent was then lyophilized and reconstituted in 2:1 MeOH:buffer B for FT-ICR-MS analysis.

FT-ICR-MS analysis: FT-ICR-MS analysis was performed as described previously [57]. Briefly, data were recorded on a hybrid linear ion trap–FT-ICR mass spectrometer (Finnigan LTQ FT, Thermo Electron, Bremen, Germany) using an automated nanospray ion source (TriVersa NanoMate, Advion BioSciences, Ithaca, NY) set with an “A” chip run at 1.5 kV and 0.5 psi head pressure. Measurements were recorded for 15 min, which consisted of 14 min of high resolution FT-ICR scans, followed by 30 s of low resolution ion trap scans (LTQ). FT-ICR-MS scans were recorded from 150-800 Da with a mass resolving power set to 400,000 at 400 m/z. At this resolution and mass accuracy the mass difference between ¹²C₁, ¹⁵N₁ and ¹³C₁, ¹⁴N₁ is resolved and therefore can be independently measured in a double labeling experiment. Each transformed

spectrum consisted of 5 summed transients at 2 s per transient for a cycle time of 10 s. All FT-ICR-MS scans were summed to produce the final spectrum. Spectra were then exported via Xcalibur MS software and peaks were corrected and assigned using an in-house software (PREMISE [56]).

Western Blot: At time of harvest cells were lysed directly on the plate using 62.5 mM Tris, 2% SDS buffer at pH 6.8 before harvest by scraping, and protein concentration was measured via the BCA method (Pierce). All protein extracts were diluted in 4X loading buffer (50% glycerol, 4% SDS, 150 mM Tris pH 6.8, 1 mM DTT, 0.02% bromophenol blue, 10% β -mercaptoethanol) to 1X strength and then boiled for 10 minutes prior to electrophoresis. Approximately 30 μ g protein was loaded for each sample onto a denaturing SDS 10 % acrylamide gel with a 6% stacking gel. Protein extracts were stacked for 30 min at 100 V and then resolved for 1.5 h at 200 V using commercially available Tris/Gly/SDS buffer (National Diagnostics, Atlanta, GA). Proteins were then transferred onto a PVDF membrane (Immobilon-P, Millipore, Bedford, MA) at 25 V for 2 h at room temperature in an Invitrogen transfer apparatus. All primary antibodies were incubated with membrane at 4 °C on a rocker overnight as follows: β -o-linked *N*-acetylglucosamine (Sigma, St. Louis, MO), 1:1000 in PBST with 5% BSA; α -tubulin (epitomics), 1:50,000 in PBST with 5% non-fat powdered milk. Membranes were then washed 3 times with PBST before incubating 1 hour at room temperature with an HRP-conjugated secondary antibody (Pierce, 1:2000 in PBST with 5% non-fat powdered milk). Blots were then incubated in Pierce

supersignal extended duration chemiluminescent reagent before exposure to x-ray film (SCBT) for visualization.

GAIMS analysis: GAIMS deconvolution was performed by Dr. Hunter Moseley as described previously [55]. FT-ICR-MS measurements from the selenite time course of A549 were used to identify UDP-GlcNAc isotopologue intensities. Each isotopologue intensity represents combinations of $^{13}\text{C}_6$ -glucose, $^{13}\text{C}_5$ -ribose, $^{13}\text{C}_2$ -acetyl, and/or $^{13}\text{C}_1$, $^{13}\text{C}_2$, and $^{13}\text{C}_3$ -uracil groups in the UDP-GlcNAc molecule. Solutions to the equations generated from these combinations are then solved via the algorithm.

Statistical analysis: All metabolite amounts were normalized to total protein measured via BCA method. All error bars represent the standard deviation of an average of experiments (specific number of experiments detailed in appropriate figure legends). For statistical significance, the two-tailed Student paired t-test was used to calculate p values. Significance was assumed for p values less than 0.05. For experiments involving multiple measurements (i.e., measurement of metabolites from NMR and MS analysis) the false discovery rate method described by Benjamini and Hochberg was applied [67]. Briefly, naïve *p*-values for all measured metabolites were calculated. These values were then ranked in order of decreasing significance and assigned a rank number. A q_i value was then calculated according to the equation $q_i = kp_i/i$ where i =rank of the *p* value among the *k* tests. The false discovery rate (FDR) was then defined for every q_i

value such that $FDR_i = \min(q_i, \dots, q_k)$. Tests with p values < 0.05 and an FDR value < 0.05 were then accepted as significant. The following analytes were measured and included for statistical analysis via FDR: citrate, fumarate, malate, Asp, lactate, glucose, Ser, Ala, UDP-GlcNAc, UTP, ATP, and lactate.

2.3 Results and discussion

2.3.1 Selenite induces dose-dependent cell death in human lung cancer models

2.3.1.1 Cell culture

In order to determine an appropriate treatment concentration of Na_2SeO_3 for subsequent tracer experiments, the dose-response curve was first established in A549 cells, and changes in cell morphology were measured using phase contrast microscopy as shown in figure 2.1. The EC_{50} calculated from the curve in figure 2.1A was $6.25 \mu\text{M}$ for 24 h of treatment. Prominent morphological features of A549 cells treated with $6.25 \mu\text{M}$ Na_2SeO_3 are illustrated in figure 2.1B. Features specific to the treated cells included the widespread appearance of vacuolar inclusions within the cytoplasm by 6 hours. By 24 hours, these vacuoles had diminished in number but were still present. These vacuoles have been reported as autophagosomes by Park et al. [22]. Cell detachment was also visually more prominent in treated cells than in controls. Also, treated cells were noticeably less confluent.

Figure 2.1. Selenite effect on A549 proliferation and morphology

A) A549 cells were treated for 24 h with increasing concentrations of Na_2SeO_3 , and growth was measured via Janus green assay. Growth was plotted as the percentage absorbance of selenite/CTL. B) A549 cells were treated with or without 6.25 μM Na_2SeO_3 , and images were recorded on a phase contrast microscope. Arrows indicate vacuolar inclusions (expanded in inset).

2.3.1.2 Human tissue slices

Thin (0.5-1 mm) slices of tumor and adjacent benign lung tissue from NSLSC surgical patients were also used as an individualized model for human lung cancer in parallel with the following cell experiments. These tissue slices are analogous to those described by Warburg [29], but are placed in medium rather than on wet blotting paper, thereby giving much greater control over nutrient supply and removal of waste products. Such slices represent better models than cultured cells as they contain much of the *in situ* microenvironment that is lost with cell cultures. In order first to establish that these tissue slices were metabolically viable 24 hours *ex vivo*, we incubated both cancerous and adjacent benign lung tissue with $^{13}\text{C}_6$ -glucose tracer, as described. As indicated by the appearance of ^{13}C -1'-AXP satellite peaks at 6.28 and 5.98 ppm in figure 2.2, both the cancerous and benign lung tissue slices had synthesized AXP from $^{13}\text{C}_6$ -glucose at the time of harvest. We then treated the slices with 6.25 μM Na_2SeO_3 for 24 h as in the cell experiments and afterwards prepared sections for histological hematoxylin and eosin (H&E) staining to measure any morphological changes. Surprisingly, we found that selenite induced widespread necrosis in the tumor tissue that was not observed in the adjacent benign tissue (Fig. 2.3). I thus chose to use 6.25 μM Na_2SeO_3 as the treatment concentration for all subsequent tracer experiments based on the dose response of A549 and its efficacy in inducing cancer cell death in the tissue slices.

Figure 2.2. Metabolic viability of benign and tumor tissue slices from NSCLC surgical patients

Tissue slices were prepared from resected tumor and adjacent benign lung tissue of NSCLC patients and incubated for 24 hours with $^{13}\text{C}_6$ -glucose tracer. ^{13}C -1'-AXP satellite peaks (6.28 and 5.98 ppm) flanking the central 1'-AXP peak at 6.14 ppm indicate incorporation of $^{13}\text{C}_6$ -glucose into the ribose subunit of AXP and active AXP synthesis.

Figure 2.3. Selenite induces necrosis in human lung tissue slices

Representative hematoxylin and eosin stained histology images from selenite treated tissue slices. Tissue slices prepared from resected tumor and adjacent benign lung tissue of NSCLC patients were incubated for 24 h with or without 6.25 μM Na_2SeO_3 and prepared for histological staining.

2.3.2 Selenite effects on polar metabolites

A549 cells were grown in medium containing $^{13}\text{C}_6$ -glucose tracer and treated with selenite as described above. Polar metabolites were then extracted and measured via ^{13}C -filtered 1D HSQC NMR after harvesting the cells to assess selenite-induced perturbations (Fig. 2.4). These spectra indicate protons directly attached to ^{13}C atoms and thus represent *de novo* biosynthesis from labeled tracers. Many metabolic pathways were affected by selenite treatment as indicated by differences in the amounts of newly synthesized intermediates. As shown by the peak at 1.32 ppm, ^{13}C -3-lactate increased in selenite-treated A549 cells but not in NHBE cells, suggestive of an increase in glycolytic flux or increased diversion of pyruvate into lactate at the expense of other metabolites. There were also numerous polar metabolites that decreased in the treated cells especially ^{13}C -3-Ala, ^{13}C -4-Glu, ^{13}C -4-Glu-glutathione, ^{13}C -2,5-citrate, and ^{13}C -3-Asp at 1.46, 2.34, 2.54, 2.66, and 2.8 ppm, respectively, indicating decreased amino acid synthesis and TCA cycle activity from glucose. In order to determine if these selenite-induced changes were cancer cell specific, I then treated immortalized NHBE cells with 6.25 μM Na_2SeO_3 and measured the polar metabolites as shown in figure 2.4. Interestingly, the majority of the differences observed in A549 were absent or minimal in NHBE suggesting cancer cell specificity.

I then examined the polar metabolite profile of selenite-treated tumor and benign tissue slices as shown in figure 2.4. Many of the metabolic alterations noted in A549 were recapitulated in the cancer tissue slices including decreased

Figure 2.4. Selenite effect in $^{13}\text{C}_6$ -glucose labeled polar metabolites

Specified cell lines or tissues were treated with 6.25 μM Na₂SeO₃ for 24 h with $^{13}\text{C}_6$ -glucose tracer. Polar metabolites were extracted and measured via ^{13}C -filtered 1D HSQC NMR. Treated and control spectra were superimposed and normalized to total mg protein in order to visualize differences.

^{13}C -3-Ala, ^{13}C -4-Glu, ^{13}C -4-Glu-glutathione, ^{13}C -2,5-citrate, and ^{13}C -3-Asp along with increased ^{13}C -3-lactate. In contrast, with the exception of lactate, other metabolites in the benign tissue showed much smaller effects with selenite treatment, analogous to the comparison between A549 and NHBE cells, again suggesting cancer specificity.

We then repeated the same experiments using $^{13}\text{C}_5,^{15}\text{N}_2$ -Gln in order to determine the effect of selenite on metabolites derived from the anapleurotic precursor Gln (Fig. 2.5). Again, selenite induced a striking depletion of Krebs' cycle intermediates ^{13}C -2,5-citrate and ^{13}C -3-Asp in selenite-treated A549 cells and tumor tissue. Interestingly, ^{13}C -4-Glu decreased while ^{13}C -4-Gln increased, in both treated A549 cells and treated cancer tissue. However, there were substantially fewer differences between the selenite and control groups in the NHBE and benign tissue counterparts. Notably, ^{13}C -4-Glu-glutathione was also reduced by selenite treatment. A more detailed investigation of selenite effects on glutaminolysis is explored in chapter 3.

2.3.3 Selenite decreases TCA cycle activity

In order to more fully explore selenite-induced TCA cycle disturbances, I then measured the time course of enrichment of $^{13}\text{C}_6$ -glucose-derived Krebs' cycle isotopologues. By understanding defined reaction mechanisms, one can trace the flow of ^{13}C atoms through central metabolic pathways, as illustrated in figure 2.6, when a time course design is employed. $^{13}\text{C}_6$ -glucose generates $^{13}\text{C}_2$ -acetyl-CoA as it is metabolized through glycolysis and pyruvate dehydrogenase. When $^{13}\text{C}_2$ -acetyl-CoA condenses with unlabeled oxaloacetate, $^{13}\text{C}_2$ -citrate is

Figure 2.5. Selenite effect in $^{13}\text{C}_5, ^{15}\text{N}_2\text{-Gln}$ labeled polar metabolites

Specified cell lines or tissues were treated with 6.25 μM Na_2SeO_3 for 24 h with $^{13}\text{C}_5, ^{15}\text{N}_2\text{-Gln}$ tracer. Polar metabolites were extracted and measured via ^{13}C -filtered 1D HSQC NMR. Treated and control spectra were superimposed and normalized to total mg protein in order to visualize differences.

Figure 2.6. ^{13}C flow through glycolysis, PDH, and the TCA cycle

Filled circles represent ^{13}C atoms. $^{13}\text{C}_6$ -glucose is transformed into $^{13}\text{C}_2$ -acetyl-CoA, and then doubly labeled Krebs' cycle intermediates as it is metabolized through glycolysis, PDH, and the TCA cycle.

generated. As shown in figure 2.7A, the depletion of $^{12}\text{C}_6$ -citrate was slower in selenite treated A549 cells than in controls and had plateaued by 10 hours of treatment. Conversely, $^{13}\text{C}_2$ -citrate increased more rapidly and to a higher level in controls relative to selenite. This implies that glucose is providing less carbon to the Krebs' cycle in the presence of selenite, consistent with the observed diversion to lactate shown in figure 2.4. When these same isotopologues were measured in immortalized NHBE cells that were treated with selenite for 24 hours under the same conditions as A549, the differences between control and selenite were not significant (Figs. 2.7C and D). Finally, we measured $^{13}\text{C}_0$ and $^{13}\text{C}_2$ -citrate in tumor and adjacent benign tissue slices from four separate NSCLC surgical patients. These tissue slices were cultured in $^{13}\text{C}_6$ -glucose, treated with or without selenite, and processed similarly to the A549 and NHBE cells. Analogous to the cell culture models, we found that $^{13}\text{C}_2$ -citrate was significantly reduced in the cancer tissue compared to controls (Fig. 2.7E and F). $^{13}\text{C}_0$ -citrate was not increased to compensate for the selenite-induced reduction in $^{13}\text{C}_2$ -citrate due to an increase in $^{13}\text{C}_1$ -citrate. However, no significant differences were seen between treatments in the benign tissue.

$^{13}\text{C}_2$ -citrate is then transformed into $^{13}\text{C}_2$ - α -ketoglutarate, $^{13}\text{C}_2$ -succinate, and $^{13}\text{C}_2$ -fumarate as it is metabolized through the TCA cycle. When $^{13}\text{C}_0$ -fumarate and $^{13}\text{C}_2$ -fumarate were measured as above in A549 cells, we observed the same isotopologue patterns as in citrate (Figs. 2.8A and B). Fumarate enrichment was unaltered by selenite treatment in the treated NHBE group (Figs. 2.8C and D). When tissue slices were examined, fumarate enrichment was

Figure 2.7. Effect of selenite on $^{13}\text{C}_6$ -glucose enrichment of citrate in A549, NHBE, and human tissue slices

A and B) A549 cells were pre-incubated for 2 hours with $^{13}\text{C}_6$ -glucose followed by treatment with or without 6.25 μM Na_2SeO_3 . Harvest hour denotes time after treatment. C and D) NHBE cells were cultured in $^{13}\text{C}_6$ -glucose supplemented medium and treated with or without 6.25 μM Na_2SeO_3 for 24 hours. E and F) Patient tissue slices were cultured in $^{13}\text{C}_6$ -glucose supplemented medium and treated with or without 6.25 μM Na_2SeO_3 for 24 hours. Polar metabolites from all experiments were extracted and citrate was quantified via GC-MS and normalized to total protein. * $p < 0.005$; $n = 4$.

Figure 2.8. Effect of selenite on $^{13}\text{C}_6$ -glucose enrichment of fumarate in A549, NHBE, and human tissue slices

A and B) A549 cells were pre-incubated for 2 hours with $^{13}\text{C}_6$ -glucose followed by treatment with or without 6.25 μM Na_2SeO_3 . Harvest hour denotes time after treatment. C and D) NHBE cells were cultured in $^{13}\text{C}_6$ -glucose supplemented medium and treated with or without 6.25 μM Na_2SeO_3 for 24 hours. E and F) Patient tissue slices were cultured in $^{13}\text{C}_6$ -glucose supplemented medium and treated with or without 6.25 μM Na_2SeO_3 for 24 hours. Polar metabolites from all experiments were extracted and fumarate was quantified via GC-MS and normalized to total protein. * $p < 0.01$; $n = 4$.

significantly decreased by selenite treatment specifically in the tumor tissue (Figs. 2.8E and F).

$^{13}\text{C}_2$ -fumarate is then converted via fumarase to $^{13}\text{C}_2$ -malate, which is also measurable via GC-MS. The isotopologue distribution of malate reiterated the same pattern described of citrate and fumarate as illustrated in figure 2.9. Finally, $^{13}\text{C}_2$ -malate is converted to $^{13}\text{C}_2$ -oxaloacetate via malate dehydrogenase. One metabolic fate of $^{13}\text{C}_2$ -oxaloacetate is transamination to $^{13}\text{C}_2$ -aspartate. I measured the aspartate isotopologue distribution as another analogue of TCA cycle activity, which reflected the same changes described for citrate, fumarate, and malate (Fig. 2.10). Taken together, selenite induced a profound decrease in the doubly labeled Krebs' cycle intermediates citrate, fumarate, malate, and aspartate in A549 lung cancer cells. These changes were specific to A549 relative to immortalized NHBE cells. Interestingly, $^{13}\text{C}_2$ -malate appeared to be slightly increased with selenite treatment in NHBE. These findings were then recapitulated in the individualized lung cancer tissue slice model indicating a cancer specific decrease in the flux through the TCA cycle caused by selenite.

2.3.4 Selenite stimulates glycolysis in A549

One immediately obvious feature from the selenite-treated A549 spectra in figure 2.4 was increased ^{13}C -3-lactate. $^{13}\text{C}_3$ -pyruvate generated via glycolysis of $^{13}\text{C}_6$ -glucose can in turn generate $^{13}\text{C}_3$ -lactate through lactate dehydrogenase. This process restores oxidizing equivalents for substrate level phosphorylation by replenishing NAD^+ and is a hallmark of tumor metabolism [29]. However, intracellular lactate, which is illustrated in figure 2.4 at 1.32 ppm, represents only

Figure 2.9. Effect of selenite on $^{13}\text{C}_6$ -glucose enrichment of malate in A549, NHBE, and human tissue slices

A and B) A549 cells were pre-incubated for 2 hours with $^{13}\text{C}_6$ -glucose followed by treatment with or without 6.25 μM Na_2SeO_3 . Harvest hour denotes time after treatment. C and D) NHBE cells were cultured in $^{13}\text{C}_6$ -glucose supplemented medium and treated with or without 6.25 μM Na_2SeO_3 for 24 hours. E and F) Patient tissue slices were cultured in $^{13}\text{C}_6$ -glucose supplemented medium and treated with or without 6.25 μM Na_2SeO_3 for 24 hours. Polar metabolites from all experiments were extracted and malate was quantified via GC-MS and normalized to total protein.

Figure 2.10. Effect of selenite on $^{13}\text{C}_6\text{-glucose}$ enrichment of Asp in A549, NHBE, and human tissue slices

A and B) A549 cells were pre-incubated for 2 hours with $^{13}\text{C}_6\text{-glucose}$ followed by treatment with or without $6.25\ \mu\text{M}\ \text{Na}_2\text{SeO}_3$. Harvest hour denotes time after treatment. C and D) NHBE cells were cultured in $^{13}\text{C}_6\text{-glucose}$ supplemented medium and treated with or without $6.25\ \mu\text{M}\ \text{Na}_2\text{SeO}_3$ for 24 hours. E and F) Patient tissue slices were cultured in $^{13}\text{C}_6\text{-glucose}$ supplemented medium and treated with or without $6.25\ \mu\text{M}\ \text{Na}_2\text{SeO}_3$ for 24 hours. Polar metabolites from all experiments were extracted and malate was quantified via GC-MS and normalized to total protein. * $p < 0.05$; $n = 4$.

a small fraction of the total lactate produced as most of the lactate generated from anaerobic fermentation is exported from the cell. Therefore, I also measured extracellular lactate production by quantifying the time course lactate isotopologue distribution in the media of $^{13}\text{C}_6$ -glucose labeled A549 as shown in figure 2.11. The medium measurements reiterated the cellular measurements and showed a substantial increase in $^{13}\text{C}_3$ -lactate production in selenite-treated media suggestive of increased utilization of glucose for lactate at the expense of acetyl-CoA (Fig. 2.11B). In order to determine whether or not increased lactate synthesis is indicative of an increase in flux through glycolysis, one must consider the rate of glucose consumption as shown in figure 2.12. Linear regression analysis of lactate production and glucose consumption indicated ~4 and ~2-fold increases, respectively, in selenite-treated cells (Table 2.1). These values are consistent with a selenite-induced increase in glycolytic flux along with reallocation of glucose carbon for lactate synthesis at the expense of TCA cycle intermediates as further illustrated in figure 2.13.

Selenite-induced stimulation of lactic fermentation is likely compensating for reduced TCA cycle activity evidenced by the attenuated $^{13}\text{C}_2$ intermediates discussed above. Interestingly, selenite-treated A549 media also accumulated $^{13}\text{C}_2$ -lactate. A variety of labeling patterns of lactate can be generated through two rounds of the TCA cycle and malic enzyme catalyzed conversion of malate to pyruvate followed by lactate production via lactate dehydrogenase as depicted in figure 2.14. If pyruvate is generated from doubly labeled malate via malic enzyme, then the isotopologue distribution should be $^{13}\text{C}_2$: $^{13}\text{C}_1$ 1:1. However,

$^{13}\text{C}_2$ -lactate

$^{13}\text{C}_3$ -lactate

Figure 2.11. Effect of selenite on lactate production in A549

A549 cells were pre-incubated for 2 hours with $^{13}\text{C}_6$ -glucose followed by treatment with or without 6.25 μM Na_2SeO_3 . Harvest hour denotes time after treatment. $^{13}\text{C}_2$ (A) and $^{13}\text{C}_3$ (B)-lactate isotopologues were quantified via GC-MS from medium aliquots taken from the onset of treatment (0 h) and at cell harvest. 0 h values were subtracted from harvest hour values and the resulting quantity was normalized to total protein to calculate the amount of lactate produced.

Figure 2.12. Effect of selenite on glucose consumption in A549

$^{13}\text{C}_6$ -glucose was quantified from 1D proton NMR spectra generated from medium aliquots taken at the onset of treatment (0h) and at cell harvest. Cells were pre-incubated with $^{13}\text{C}_6$ -glucose tracer for 2 hours before treatment with 6.25 μM Na_2SeO_3 . 0h values were subtracted from harvest hour values and the resulting quantity was normalized to total protein.

6.25 μM SeO_3^{2-}	$^{13}\text{C}_3$ -lactate production		$^{13}\text{C}_6$ -glucose consumption	
	-	+	-	+
R^2	.60	.87	.90	.98
slope	108.8	359.7	-2087	-4168
SD	44.16	69.02	349.5	302.9

Table 2.1 Linear regression analysis of lactate production and glucose consumption in selenite-treated A549 cells

Linear regression was applied to data from figures 2.11 and 2.12, and the above variables were calculated. Slopes of selenite-treated cells were significantly different from non-treated controls with p values <0.0005 for both $^{13}\text{C}_3$ -lactate production and $^{13}\text{C}_6$ -glucose consumption.

Figure 2.13. Effect of selenite glucose carbon allocation to lactate

$^{13}\text{C}_6$ -glucose and $^{13}\text{C}_3$ -lactate were quantified from 1D proton NMR spectra generated from medium aliquots taken at the onset of treatment (0h) and at cell harvest. Cells were pre-incubated with $^{13}\text{C}_6$ -glucose tracer for 2 hours before treatment with 6.25 μM Na_2SeO_3 . 0h values were subtracted from harvest hour values for glucose, and lactate was divided by glucose consumed to determine the fraction of glucose converted to lactate. Values were then normalized to mg protein.

Figure 2.14. ^{13}C flow through glycolysis, TCA cycle, malic enzyme, and lactate dehydrogenase

As $^{13}\text{C}_6$ -glucose is metabolized through glycolysis and the TCA cycle eventually symmetric $^{13}\text{C}_2$ -fumarate is generated, which generates two distinct $^{13}\text{C}_2$ isotopomers of malate. If $^{13}\text{C}_2$ -malate is decarboxylated via malic enzyme to pyruvate, $^{13}\text{C}_2$ and $^{13}\text{C}_1$ -pyruvate should be created in equal amounts. If doubly labeled malate continues through another round of the TCA cycle with doubly labeled acetyl-CoA, then $^{13}\text{C}_2$ and $^{13}\text{C}_3$ pyruvate will be generated a ratio of 3:1. Generation of lactate from labeled pyruvate will create the isotopomer distribution displayed above. Blue filled circles represent ^{13}C atoms. Functional groups have been removed from all structures to simplify ^{13}C flow.

very little $^{13}\text{C}_1$ was measured in the media. If doubly labeled malate undergoes another round of TCA cycle conversions with doubly labeled acetyl-CoA, then a predominance of $^{13}\text{C}_2$ -lactate will eventually be generated. Therefore, when the possibilities of lactate generation from glycolysis and malic enzyme (ME) are taken into consideration, a predominance of $^{13}\text{C}_2$ and $^{13}\text{C}_3$ -lactate will be formed. However, it is puzzling that $^{13}\text{C}_2$ -lactate would accumulate in selenite-treated cells, since TCA cycle activity is reduced and lactate production from Gln appears to be negligible. However, if malic enzyme activity is increased upon selenite treatment, doubly labeled Krebs' cycle intermediates could be siphoned off for lactic fermentation ultimately reducing the amounts of measurable $^{13}\text{C}_2$ intermediates. Notably, ME was indeed increased by selenite treatment at the mRNA level as reported by Fan et al. [60].

As another measure of glucose utilization, we measured the serine isotopologue distribution in selenite-treated A549 and NHBE cells. $^{13}\text{C}_6$ -glucose is eventually converted through glycolysis to $^{13}\text{C}_3$ -3-phosphoglycerate, which is a biosynthetic precursor for serine. $^{13}\text{C}_3$ -Ser enrichment in treated A549 cells was increased relative to controls; however, no differences were noted in treated NHBE cells, which is again consistent with an increase allocation of glucose carbon to lactate (Fig 2.15). These patterns indicate selenite induces a cancer-specific increase in glycolysis, which is likely a compensatory mechanism for decreased flux through the TCA cycle.

Finally, I measured both the cellular and extracellular alanine isotopologue distributions as another glycolytic analogue. Alanine can be synthesized from

Figure 2.15. Effect of selenite on $^{13}\text{C}_6$ -glucose derived enrichment of serine in A549 and NHBE

A and B) A549 cells were pre-incubated for 2 hours with $^{13}\text{C}_6$ -glucose and treated with or without 6.25 μM Na_2SeO_3 . C and D) NHBE cells were cultured in $^{13}\text{C}_6$ -glucose supplemented medium and treated with or without 6.25 μM Na_2SeO_3 for 24 hours. Polar metabolites from all experiments were extracted and Ser was quantified via GC-MS and normalized to total protein.

pyruvate through alanine transaminase, and an isotopologue distribution similar to lactate is possible as shown in figure 2.14. Examination of the medium alanine production indicated increased production of $^{13}\text{C}_3\text{-Ala}$ in controls and increased production of $^{13}\text{C}_2\text{-Ala}$ in the treated A549 medium (Figs. 2.16A and B). Quantification of cellular Ala reiterated these findings (Figs. 2.16C and D). Selenite-increased $^{13}\text{C}_2\text{-Ala}$ may arise from increased $^{13}\text{C}_2\text{-lactate}$ as discussed previously. But in this scenario, $^{13}\text{C}_3\text{-Ala}$ was actually decreased in the selenite group relative to controls. The data are puzzling; however, one possible solution for this distribution would be a combination of increased malic enzyme and lactate dehydrogenase activity. Increased flux through malic enzyme could generate $^{13}\text{C}_2$ and $^{13}\text{C}_3\text{-pyruvate}$ in selenite treated cells. If lactate dehydrogenase activity were increased in order to replenish NAD^+ , then most of the pyruvate would be siphoned off for lactate synthesis. Some of the doubly and triply labeled pyruvate could generate correspondingly labeled alanine, which is illustrated by the measured increase in $^{13}\text{C}_2\text{-Ala}$. However, since the controls are generating predominantly $^{13}\text{C}_3\text{-Ala}$, the overall $^{13}\text{C}_3\text{-Ala}$ enrichment is actually greater than with selenite treatment. Taken together these data strongly indicate that selenite increases glycolytic activity.

2.3.5 Selenite decreases nucleotide synthesis

In an ongoing effort to characterize selenite-induced metabolic perturbations, I then examined nucleotide synthesis. Analysis of the 1D proton spectra from a set of unlabeled selenite experiments indicated that several of the nucleotide hexoses decreased by ~50% relative to controls after 24 hours,

Figure 2.16. Effect of selenite on $^{13}\text{C}_6$ -glucose derived enrichment of Ala in A549

A and B) A549 cells were pre-incubated for 2 hours with $^{13}\text{C}_6$ -glucose followed by treatment with or without 6.25 μM Na_2SeO_3 . Harvest hour denotes time after treatment. $^{13}\text{C}_2$ (A) and $^{13}\text{C}_3$ (B)-Ala isotopologues were quantified via GC-MS from medium aliquots taken from the onset of treatment (0 h) and at cell harvest. 0 h values were subtracted from harvest hour values and the resulting quantity was normalized to total protein to calculate the amount of Ala produced. C and D) Intracellular Ala measurements from the same experiment. Polar metabolites were extracted and Ala was quantified via GC-MS and normalized to total protein.

including UDP-GlcNAc (Fig. 2.17A). Selenite-induced reduction of these same nucleotide hexoses was substantially less when the experiment was repeated in NHBE cells (Fig. 2.17A). Quantitation yielded a ~2-fold decrease in selenite-treated A549 cells and a somewhat smaller decrease in NHBE cells (Fig. 2.17B and C). Interestingly, UDP-GlcNAc was more substantially affected by selenite treatment than UDP-GalNAc in both A549 and NHBE, suggesting some specificity for UDP-GlcNAc synthesis. I then measured the time course of enrichment of UDP-GlcNAc, UTP, and ATP in selenite-treated A549 cells using both $^{13}\text{C}_6$ -glucose and $^{13}\text{C}_5$, $^{15}\text{N}_2$ -Gln tracers. UDP-GlcNAc is both analytically and biologically interesting as its synthesis involves contributions from glycolysis, pentose phosphate pathway, hexosamine biosynthetic pathway, Krebs' cycle, nucleotide biosynthesis, and glutaminolysis (Figs. 2.18A and B). By analyzing the time course enrichment of UDP-GlcNAc from labeled precursors, one can make conclusions about the flux through the pathways that contribute towards its synthesis. When we measured UDP-GlcNAc isotopologues from selenite-treated A549 cells with $^{13}\text{C}_6$ -glucose tracer, I found that the time course of enrichment was generally attenuated with respect to controls (Fig. 2.19). $^{13}\text{C}_5$, $^{13}\text{C}_6$, and $^{13}\text{C}_{13}$ -UDP-GlcNAc enrichment was delayed in selenite-treated cells as shown in figures 2.19B, C, and D. Furthermore, the enrichment of more fully labeled isotopologues such as $^{13}\text{C}_{15}$ -UDP-GlcNAc was immeasurable in selenite treated A549 cells (Fig. 2.19E).

Presumably, $^{13}\text{C}_5$ and $^{13}\text{C}_6$ -UDP-GlcNAc represent isotopologues having fully labeled ribose and glucose moieties, respectively, due to their rapid accumulation

Figure 2.17. Effect of selenite on nucleotide hexosamines

A) A549 and NHBE cells were treated with or without 6.25 μM Na_2SeO_3 for 24h. Polar metabolites were extracted, and 1D proton spectra were recorded. Spectra were superimposed and normalized to total protein to assess differences. UDP-GlcNAc peaks at 5.5 ppm were quantified in A549 (B) and NHBE (C) using Mnova spectral analysis software and normalized to total protein. * $p < 0.01$; $n = 3$

B

Figure 2.18. Contributions from glucose and Gln in UDP-GlcNAc biosynthesis

A) Heavy carbon from $^{13}\text{C}_6\text{-glucose}$ is incorporated into UDP-GlcNAc at several points during its synthesis. $^{13}\text{C}_2$ or $^{13}\text{C}_3\text{-Asp}$ generated from the flow of ^{13}C through glycolysis, PDH, Krebs' cycle, and transamination of labeled OAA is incorporated into the uracil ring. $^{13}\text{C}_5\text{-ribose}$ of the nucleoside diphosphate is generated from glycolysis the pentose phosphate pathway. The $^{13}\text{C}_6\text{-glucose}$ moiety of UDP-GlcNAc is generated during the first few steps of the HBP. Finally, the $^{13}\text{C}_2\text{-acetyl}$ group is generated from glycolysis and PDH. B) ^{13}C and ^{15}N from $^{13}\text{C}_5, ^{15}\text{N}_2\text{-Gln}$ are incorporated into UDP-GlcNAc at several points during its synthesis. CPS II can generate labeled $^{15}\text{N}_3$ uracil from $^{13}\text{C}_5, ^{15}\text{N}_2\text{-Gln}$ substrate. Uracil $^{15}\text{N}_1$ is generated from transamination of either fully labeled or unlabeled OAA with $^{13}\text{C}_5, ^{15}\text{N}_2\text{-Gln}$. $^{13}\text{C}_4\text{-6}$ of uracil can be generated through glutaminolysis of $^{13}\text{C}_5, ^{15}\text{N}_2\text{-Gln}$ and subsequent transformation of fully labeled Glu and $\alpha\text{-KG}$ through the TCA cycle to $^{13}\text{C}_4\text{-OAA}$, which can then be transaminated from $^{13}\text{C}_5, ^{15}\text{N}_2\text{-Gln}$ to form fully labeled Asp. $^{13}\text{C}_4, ^{15}\text{N}_1\text{-Asp}$ is then incorporated into the uracil ring during pyrimidine synthesis. The amino group of glucosamine can be generated from the GFAT reaction of the HBP using $^{13}\text{C}_5, ^{15}\text{N}_2\text{-Gln}$ as substrate. Finally, the acetyl group of *N*-acetylglucosamine can be generated from $^{13}\text{C}_4\text{-malate}$ followed by malic enzyme mediated conversion to ^{13}C -pyruvate and $^{13}\text{C}_2\text{-acetyl-CoA}$ via PDH.

Figure 2.19. Effect of selenite on UDP-GlcNAc enrichment from glucose

FT-MS quantified $^{13}\text{C}_0$ (A), $^{13}\text{C}_5$ (B), $^{13}\text{C}_6$ (C), $^{13}\text{C}_{13}$ (D), and $^{13}\text{C}_{15}$ (E) UDP-GlcNAc isotopologues. A549 cells were pre-incubated with $^{13}\text{C}_6$ -glucose for 2 hours and then treated with or without 6.25 μM Na_2SeO_3 . Cells were harvested at 0, 1.5, 3, 6, 10, and 24 hours post treatment. Nucleotides were extracted and UDP-GlcNAc was measured via FT-MS. Plots represent the intensity of the designated isotopologue divided by the total intensity as a percentage.

and depletion as more fully labeled species are generated. $^{13}\text{C}_{13}$ - and $^{13}\text{C}_{15}$ -UDP-GlcNAc likely represent ribose plus glucose labeling and combinations of labeling in the acetyl group and uracil ring. Taken together these enrichment patterns indicate delayed enrichment of ribose and glucose with preferential blockage of the uracil and/or acetyl subunits.

In order to more fully explore these enrichment patterns, the FT-MS data were deconvoluted using the *genetic algorithm for isotopologues in metabolic systems* or “GAIMS” developed by Dr. Hunter Moseley. This non-steady-state modeling algorithm deconvolutes the UDP-GlcNAc isotopologue data into the time course enrichment of its subunits: glucose, ribose, uracil, and the acetyl group. When the algorithm was applied, the results indicated that the acetyl group and uracil enrichment were preferentially delayed by selenite treatment as illustrated in figures 2.20C, D, and E. However, ribose and glucose enrichment were less substantially affected by selenite treatment, despite the increased uptake rate of glucose (Figs. 2.20A and B). These findings are again consistent with an increase in glycolytic flux and reallocation of glucose carbon into lactate at the expense of pyrimidine synthesis as well as ribose synthesis via the PPP. Interestingly, the flux of glucose carbon through the HBP appears to be mostly intact with selenite treatment and preferentially blocked downstream of glucosamine synthesis as suggested by the negligible effect on $^{13}\text{C}_6$ -UDP-GlcNAc accumulation.

When we examined the enrichment patterns in NHBE cells incubated with $^{13}\text{C}_6$ -glucose tracer we found that selenite had no effect on UDP-GlcNAc

Figure 2.20. GAIMS deconvolution of UDP-GlcNAc isotopologue distribution in A549

FT-MS data from figure 2.18 were deconvoluted as described. Individual plots represent the time course enrichment of the $^{13}\text{C}_6$ -glucose (A), $^{13}\text{C}_5$ -ribose (B), $^{13}\text{C}_2$ -acetyl (C), and $^{13}\text{C}_{1-3}$ -uracil (D-F) subunits of UDP-GlcNAc. Data are represented as the fraction of selenite treated subunit over the total. Attention should be given to the y-axes as the scales vary widely among subunits.

enrichment (Figs. 2.21A-D). We then measured the UDP-GlcNAc isotopologue distribution generated from tumor and adjacent benign tissue slices treated with selenite and incubated with $^{13}\text{C}_6$ -glucose tracer for 24 hours. Enrichment of the acetyl group and/or uracil ring appeared to be preferentially blocked in both the tumor and benign tissue as evidenced by decreased amounts of $^{13}\text{C}_{13}$ -UDP-GlcNAc.

I then measured the UDP-GlcNAc isotopologue distribution generated when A549 cells were incubated with $^{13}\text{C}_5,^{15}\text{N}_2$ -Gln tracer. As expected, $^{13}\text{C}_0,^{15}\text{N}_0$ -UDP-GlcNAc plateaued at a higher level in selenite-treated cells than in controls (Fig. 2.22A). In order to assess the rate of depletion in treated cells, the data were assumed to follow the simple exponential decay model:

$$y_t = \langle y \rangle + (y_0 - \langle y \rangle)^{-kt} \quad \text{Eq. 2.1}$$

As illustrated in table 2.2 the rate of $^{13}\text{C}_0,^{15}\text{N}_0$ -UDP-GlcNAc depletion in selenite-treated cells was actually greater than in controls suggesting a possible stimulation of UDP-GlcNAc synthesis or utilization initially, followed by an abrupt decrease as nitrogen and carbon from Gln are blocked from entry into these pathways.

Interestingly, $^{13}\text{C}_0,^{15}\text{N}_1$ -UDP-GlcNAc accumulated in selenite treated cells in contrast to controls, suggesting that at least one nitrogen-donating step in UDP-GlcNAc synthesis was not inhibited by treatment (Fig. 2.22B). $^{13}\text{C}_2,^{15}\text{N}_1$ -UDP-GlcNAc also accumulated with selenite treatment as shown in figure 2.22C, suggesting that malic enzyme decarboxylation of fully labeled malate to pyruvate

Figure 2.21. Effect of selenite on UDP-GlcNAc enrichment from glucose in NHBE and human tissue slices

NHBE cells were incubated with $^{13}\text{C}_6$ -glucose and treated with or without 6.25 μM Na_2SeO_3 for 24 hours. Nucleotides were extracted and $^{13}\text{C}_5$ (A), $^{13}\text{C}_6$ (B), $^{13}\text{C}_{13}$ (C), and $^{13}\text{C}_{15}$ (D) UDP-GlcNAc isotopologues were measured via FT-MS. Plots represent the intensity of the designated isotopologue divided by the total intensity as a percentage. E) Patient tissue slices were cultured in $^{13}\text{C}_6$ -glucose supplemented medium and treated with or without 6.25 μM Na_2SeO_3 for 24 hours. Nucleotides were extracted and UDP-GlcNAc isotopologue fractional distribution was measured via FT-MS. The fractional enrichment of selenite-treated isotopologue was then divided by the corresponding non-treated control value for each sample to obtain the fraction of control for each isotopologue. Data were plotted as fraction of control averaged from three separate patients.

Figure 2.22. Effect of selenite on UDP-GlcNAc enrichment from Gln in A549

A549 cells were pre-incubated for 2 hours with ¹³C₅, ¹⁵N₂-Gln and treated with or without 6.25 μM Na₂SeO₃ for 24 hours. Cells were harvested at 0, 1.5, 3, 6, 10, and 24 hours post treatment. Nucleotides were extracted and UDP-GlcNAc was measured via FT-MS. Plots represent the intensity of the designated isotopologue divided by the total intensity as a percentage.

6.25 μM Na ₂ SeO ₃	-	+
R ²	0.99	0.91
k	0.26	0.46
half-life (h)	2.62	1.50

Table 2.2. Effect of selenite on exponential decay of ¹³C₀, ¹⁵N₀-UDP-GlcNAc in A549

¹³C₀, ¹⁵N₀-UDP-GlcNAc FT-MS data were fit to the equation, $y_t = \langle y \rangle + (y_0 - \langle y \rangle)e^{-kt}$, in order to obtain the above variables. K values for the curves were significantly different with p values < 0.0001.

and then acetyl-CoA was functioning as described earlier. However, this result is puzzling given that flux through glutaminolysis and subsequently the TCA cycle is clearly impeded with selenite treatment. Perhaps there is an initial brief stimulation of glutaminolysis as suggested by table 2.2 followed by inhibition which results in accumulation of $^{13}\text{C}_2,^{15}\text{N}_1\text{-UDP-GlcNAc}$ as more fully labeled isotopologues are blocked. More highly labeled isotopologues, including $^{13}\text{C}_3,^{15}\text{N}_2$, $^{13}\text{C}_2,^{15}\text{N}_3$, $^{13}\text{C}_3,^{15}\text{N}_3$, and $^{13}\text{C}_5,^{15}\text{N}_3\text{-UDP-GlcNAc}$, were greatly attenuated by selenite, reinforcing that two out of the three nitrogen donating steps were inhibited along with incorporation of fully labeled Asp from labeled OAA transamination (Figs. 2.22D-F).

Surprisingly, when I measured UDP-GlcNAc isotopologues in NHBE cells grown with $^{13}\text{C}_5,^{15}\text{N}_2\text{-Gln}$ tracer nearly 100% were unlabeled, indicating little if any *de novo* synthesis from Gln. Furthermore, there was no effect from selenite on the distribution (Fig. 2.23A and B). As indicated in figure 2.17, UDP-GlcNAc represents a substantial portion of the hexosamine nucleotide pool. Taken together, these data suggest that *de novo* synthesis of UDP-GlcNAc in NHBE cells incorporates carbon and nitrogen primarily from glucose and/or endogenous Gln to maintain steady concentrations upon cell division. I then repeated these measurements in the tissue slice model with $^{13}\text{C}_5,^{15}\text{N}_2\text{-Gln}$ tracer from three different patients. I found that selenite significantly reduced $^{13}\text{C}_0,^{15}\text{N}_1$, $^{13}\text{C}_0,^{15}\text{N}_2$, and $^{13}\text{C}_0,^{15}\text{N}_3\text{-UDP-GlcNAc}$ in tumor tissue compared with paired benign lung tissue suggesting inhibition of the nitrogen donating steps in hexosamine biosynthesis (Fig. 2.23C).

Figure 2.23. Effect of selenite on UDP-GlcNAc enrichment from Gln human patient tissue slices

A and B) NHBE cells were incubated with $^{13}\text{C}_5, ^{15}\text{N}_2$ -Gln and treated with or without 6.25 μM Na_2SeO_3 for 24 hours. Nucleotides were extracted and UDP-GlcNAc was measured via FT-MS. Plots represent the intensity of the designated isotopologue divided by the total intensity as a percentage. C) UDP-GlcNAc isotopologue distribution from selenite-treated tumor and adjacent benign tissue slices. Data represent the average from three separate patients depicted as fraction of selenite over control. * $p < 0.05$; $n = 3$

In order to discriminate labeling differences in UDP-GlcNAc between uracil and the acetyl group caused by selenite, I then measured enrichment patterns in UTP. UTP contains the uracil ring and ribose of UDP-GlcNAc without *N*-acetylglucosamine. Therefore, by studying tracer incorporation into UTP one can make assumptions about which specific steps of UDP-GlcNAc synthesis were inhibited by selenite treatment. When A549 cells were given $^{13}\text{C}_6$ -glucose tracer, unlabeled UTP plateaued at a higher level with selenite treatment, again suggesting either decreased synthesis or utilization of UTP (Fig. 2.24A). Analogous to table 2.2, the rate of $^{13}\text{C}_0$ -UTP was modeled using equation 2.1 (Table 2.3). $^{13}\text{C}_0$ -UTP decreased at the same rate for both controls and selenite-treated cells, suggesting that selenite reduced the overall synthesis from glucose and/or utilization of UTP, but not the initial rate. Selenite delayed $^{13}\text{C}_5$ -UTP accumulation; however, $^{13}\text{C}_7$ -UTP was substantially more affected suggesting preferential inhibition in Asp label incorporation (Figs. 2.24B and C). When the experiment was repeated using $^{13}\text{C}_5, ^{15}\text{N}_2$ -Gln tracer, similar pathways appeared to be affected. Specifically, labeled Asp incorporation into the uracil ring was preferentially blocked by selenite treatment as evidenced by the depletion of $^{13}\text{C}_3, ^{15}\text{N}_1$ and $^{13}\text{C}_3, ^{15}\text{N}_2$ -UTP (Figs. 2.24F and G). The predominant isotopologue observed in the selenite-treated cells was fully unlabeled UTP (Fig. 2.24D), and all others were either absent or depleted for the duration of the experiment, suggesting that transamination of fructose-6-phosphate to glucosamine-6-phosphate during hexosamine biosynthesis is not as affected by selenite treatment as the other nitrogen-incorporating steps. When I measured UTP

Figure 2.24. Effect of selenite on UTP enrichment from glucose and Gln in A549

A-C) A549 cells were pre-incubated for 2 hours with $^{13}\text{C}_6$ -glucose and treated with or without 6.25 μM Na_2SeO_3 for 24 hours. D-G) A549 cells were pre-incubated for 2 hours with $^{13}\text{C}_5, ^{15}\text{N}_2$ -Gln and treated with or without 6.25 μM Na_2SeO_3 for 24 hours. Cells were harvested at 0, 1.5, 3, 6, 10, and 24 hours post treatment. Nucleotides were extracted and UTP was measured via FT-MS. Plots represent the intensity of the designated isotopologue divided by the total intensity as a percentage.

6.25 μM Na_2SeO_3	-/+
R^2	0.98/0.74
k	0.20
half-life (h)	3.4

Table 2.3. Effect of selenite on exponential decay of $^{13}\text{C}_0$ -UTP in A549

$^{13}\text{C}_0$ -UTP FT-MS data were fit to the equation, $y_t = \langle y \rangle + (y_0 - \langle y \rangle)e^{-kt}$, in order to obtain the above variables. K values for the curves were statistically the same.

isotopologues from both tracers in treated NHBE cells, no differences between treatments were noted. Again, I observed very little incorporation of Gln label (Fig. 2.25). Interestingly, the treated tissue slices recapitulated the selenite effects on tumor tissue, specifically with regards to Asp incorporation into the uracil ring (Fig. 2.26).

Finally, I measured the isotopologue distributions in ATP as a representation of purine metabolism using the experiments detailed above (Fig 2.27). Similar to UDP-GlcNAc and UTP, unlabeled ATP plateaued at a higher level in selenite-treated A549 cells as shown in figure 2.27A, suggesting decreased synthesis or utilization. The rates of both $^{13}\text{C}_0$ -ATP depletion and $^{13}\text{C}_5$ -ATP accumulation were modeled using equation 2.1 and the following model:

As shown in table 2.4, the depletion rate of unlabeled ATP was the same for both treated and untreated A549; however, the rate of $^{13}\text{C}_5$ -ATP was significantly reduced in selenite-treated cells. These data are consistent with an overall decrease in the synthesis from glucose and/or utilization of ATP with selenite treatment.

Consistent with the parameters determined in table 2.4, labeling of the ribose subunit was slightly delayed, while enrichment of the adenine ring was significantly slower as evidenced by figures 2.27B and C. Carbon in the adenine ring is derived from Gly, N^{10} -formyl-THF, and CO_2 . Interestingly, $^{13}\text{C}_1$ -Gly

Figure 2.25. Effect of selenite on UTP enrichment from glucose and Gln in NHBE

NHBE cells were incubated with either $^{13}\text{C}_6$ -glucose (A-C) or $^{13}\text{C}_5, ^{15}\text{N}_2$ -Gln (D and E) and treated with or without 6.25 μM Na_2SeO_3 for 24 hours. Nucleotides were extracted and UTP was measured via FT-MS. Plots represent the intensity of the designated isotopologue divided by the total intensity as a percentage.

Figure 2.26. Effect of selenite on UTP enrichment from glucose and Gln in human patient tissue slices

Tumor and adjacent benign tissue slices were incubated with $^{13}\text{C}_6$ -glucose (A) or $^{13}\text{C}_5, ^{15}\text{N}_2$ -Gln (B) tracers and treated with or without 6.25 μM Na_2SeO_3 for 24 h. Nucleotides were extracted and UTP was measured via FT-MS. Data represent the average from three separate patients depicted as fraction of selenite over control. * $p < 0.05$; $n = 3$

Figure 2.27. Effect of selenite on ATP enrichment from glucose and Gln in A549

A-C) A549 cells were pre-incubated for 2 hours with $^{13}\text{C}_6$ -glucose and treated with or without 6.25 μM Na_2SeO_3 for 24 hours. D-H) A549 cells were pre-incubated for 2 hours with $^{13}\text{C}_5, ^{15}\text{N}_2$ -Gln and treated with or without 6.25 μM Na_2SeO_3 for 24 hours. Cells were harvested at 0, 1.5, 3, 6, 10, and 24 hours post treatment. Nucleotides were extracted and ATP was measured via FT-MS. Plots represent the intensity of the designated isotopologue divided by the total intensity as a percentage.

6.25 μ M Na_2SeO_3	-/+	-	+
R^2	0.98/0.97	0.97	0.98
k	0.15	0.19	0.10
half-life (h)	4.76	3.60	6.91

Table 2.4. Effect of selenite on exponential decay of $^{13}\text{C}_0$ -ATP and accumulation of $^{13}\text{C}_5$ -ATP in A549

$^{13}\text{C}_0$ and $^{13}\text{C}_5$ -ATP FT-MS data were fit to the equations, $y_t = \langle y \rangle + (y_0 - \langle y \rangle)e^{-kt}$ and $y = y_0 + \langle y \rangle(1 - e^{-kt})$, respectively, in order to obtain the above variables. K values for the rate of $^{13}\text{C}_0$ -ATP depletion were statistically the same, but were significantly different for $^{13}\text{C}_5$ -ATP accumulation with p values < 0.05.

appeared to accumulate before $^{13}\text{C}_2$ -Gly in A549 control cells incubated with $^{13}\text{C}_6$ -glucose tracer, and enrichment was delayed by selenite treatment; however, the validity of these data were compromised due to noise (Fig. 2.28). Since the entire Gly backbone is incorporated into the adenine ring, one would expect to find $^{13}\text{C}_1$ followed by $^{13}\text{C}_2$ labeling in the adenine ring based upon the Gly enrichment pattern. This is likely why $^{13}\text{C}_1$ -adenine accumulates in A549 cells, since $^{13}\text{C}_6$ -ATP is presumably $^{13}\text{C}_5$ -ribose plus $^{13}\text{C}_1$ -adenine as $^{13}\text{C}_{\text{ribose}}$ -ATP is clearly and independently measurable from the NMR spectra shown in figure 2.4. However, ^{13}C could also be contributed from N^{10} -formyl-THF, which can itself be synthesized in part from Gly or Ser, so it is difficult to determine precisely where the adenine label is derived from.

Four out of the five nitrogen atoms in ATP can be traced back to Gln as their source, either directly or from Asp transamination as supported by a predominance of $^{13}\text{C}_0,^{15}\text{N}_4$ -ATP seen in the $^{13}\text{C}_5,^{15}\text{N}_2$ -Gln labeled controls of figure 2.27H. The predominant isotopologue observed for selenite-treated cells from this experiment was unlabeled ATP, again suggesting decreased purine synthesis and/or utilization (Fig. 2.27D). Interestingly, $^{13}\text{C}_0,^{15}\text{N}_1$ -ATP also accumulated in selenite treated A549 cells; however, due to the similarity of nitrogen donating steps during purine synthesis, it is likely a combination of $^{15}\text{N}_1$ -adenine isotopomers (Fig. 27E). Analysis of the isotopologue distribution in $^{13}\text{C}_6$ -glucose and $^{13}\text{C}_5,^{15}\text{N}_2$ -Gln labeled NHBE cells again yielded little if any changes induced by selenite treatment (Fig. 2.29). Interestingly, ATP also incorporated very little carbon or nitrogen from Gln over 24 h as evidenced by the

Figure 2.28. Effect of selenite on Gly enrichment from glucose in A549

A549 cells were pre-incubated for 2 hours with $^{13}\text{C}_6\text{-glucose}$ and treated with or without $6.25\ \mu\text{M}$ Na_2SeO_3 for 24 hours. Cells were harvested at 0, 1.5, 3, 6, 10, and 24 hours post treatment. Polar metabolites were extracted and Gly was measured via GC-MS and normalized to total protein. Plots represent the intensity of the designated isotopologue divided by the total intensity as a percentage.

Figure 2.29. Effect of selenite on ATP enrichment from glucose and Gln in NHBE

NHBE cells were incubated with either $^{13}\text{C}_6$ -glucose (A-C) or $^{13}\text{C}_5, ^{15}\text{N}_2$ -Gln (D) and treated with or without 6.25 μM Na_2SeO_3 for 24 hours. Nucleotides were extracted and ATP was measured via FT-MS. Plots represent the intensity of the designated isotopologue divided by the total intensity as a percentage.

Fig. 2.30. Effect of selenite on ATP enrichment from glucose and Gln in human patient tissue slices

Tumor and adjacent benign tissue slices were incubated with $^{13}\text{C}_6$ -glucose (A) or $^{13}\text{C}_5$, $^{15}\text{N}_2$ -Gln (B) tracers and treated with or without 6.25 μM Na_2SeO_3 for 24 h. Nucleotides were extracted and ATP was measured via FT-MS. Data represent the average from three separate patients depicted as fraction of selenite over control. * $p < 0.05$; $n=3$

predominance of unlabeled ATP with $^{13}\text{C}_5, ^{15}\text{N}_2\text{-Gln}$ tracers (Fig. 2.29D). Finally, when we applied fully labeled glucose tracer to the tissue slices, we found that selenite had little effect on enrichment of ribose (Fig. 2.30A). Nitrogen incorporation from $^{13}\text{C}_5, ^{15}\text{N}_2\text{-Gln}$ tracer into the adenine ring of ATP was significantly reduced with selenite treatment in the cancer tissues; however, benign tissues were also significantly affected as illustrated in figure 2.30B, and actually more so for $^{13}\text{C}_0, ^{15}\text{N}_2\text{-ATP}$.

2.3.6 Selenite decreases UDP-GlcNAc utilization

As discussed previously, UDP-GlcNAc decreased in selenite treated A549 cells substantially more so than in NHBE cells (Fig. 2.17). In order to determine if this decrease in UDP-GlcNAc was matched by a decrease in its utilization for protein modification, I examined the levels of O-GlcNAc glycosylation in selenite treated A549 cells. Interestingly, selenite induced a global reduction in O-GlcNAc modification as shown in figure 2.31. Glycosylation was also measured in NHBE cells and was less than in untreated A549 cells, analogous to the difference between tumor and benign tissue reported in the literature [45]. Furthermore, selenite did not have any effect on O-GlcNAc glycosylation in NHBE. I then attempted to isolate several putative targets of O-GlcNAc modification including the proto-oncogene protein c-MYC and mitochondrial transporter VDAC, both of which have been established as targets of OGT. Selenite has a significant effect on glutaminolysis and TCA cycle activity (see above); therefore, I chose to focus on c-MYC and VDAC due to their roles in Gln and mitochondrial metabolism, respectively [53, 68-70]. However, after immunoprecipitation, neither protein

Figure 2.31. Effect of selenite on UDP-GlcNAc utilization

A549 cells were treated with or without 6.25 μM Na_2SeO_3 for 24 hours and total protein was extracted. Lysates from treated A549 and untreated NHBE cells were probed for o-GlcNAc modification via WB. α -tubulin was also probed as loading control.

appeared to be modified by UDP-GlcNAc. We have since begun to focus on a proteomics approach combined with immunoprecipitation; this investigation is ongoing.

2.4 Conclusions

By utilizing tracer-based experimental design, this study was able to expand upon previous work by Fan et al. [60] on selenite-induced disruption of cancer cell metabolism. NSCLC tissue slices were demonstrated to be metabolically viable due to their uptake and incorporation of labeled tracers into metabolic intermediates. Furthermore, selenite induced massive necrosis in cancer tissue that was unmatched in the adjacent benign tissue, thereby illustrating its potency as an anti-cancer agent for this model. In order to expand upon these findings, I then pursued a thorough metabolic profile of selenite-perturbed metabolism by contrasting A549 human lung cancer cells with immortalized NHBE cells and NSCLC lung tissue.

During this investigation, I studied numerous selenite-induced disturbances in A549 metabolism. The driving force behind many of these effects seems to be a dramatic attenuation of glutaminolysis induced by selenite. Gln is one of the major anapleurotic substrates of the TCA cycle, and it contributes both carbon and nitrogen for the biosynthesis of amino acids, nucleotides, and Krebs' cycle intermediates to facilitate aerobic respiration. When A549 cells were labeled with $^{13}\text{C}_6$ -glucose, I found a dramatic reduction in the enrichment of doubly labeled TCA cycle intermediates citrate, fumarate, and malate along with

doubly labeled Asp suggestive of decreased entry of glucose into the cycle. Furthermore, when $^{13}\text{C}_5, ^{15}\text{N}_2\text{-Gln}$ tracer was applied, I found a severe depletion and accumulation of labeled Glu and Gln, indicating impaired glutaminolysis. Together, this indicates a decrease in flux through the TCA cycle that would presumably lead to an impaired ability to generate NADH and FADH_2 for oxidative phosphorylation, as well as provide intermediates for anabolic metabolism. As evidenced by the increase in $^{13}\text{C}_3\text{-lactate}$ and $^{13}\text{C}_3\text{-Ser}$ with selenite treatment, glycolytic flux is likely accelerated in order to augment the high cancer cell energy requirements through substrate level phosphorylation. Interestingly, I found that this effect on TCA flux was not reproduced in NHBE cells, suggesting that these effects are somehow cancer specific. Furthermore, selenite-induced Krebs' cycle flux attenuation was recapitulated in the tissue slice experiments, reinforcing this hypothesis within the actual tumor microenvironment.

I then examined labeling patterns in UDP-GlcNAc, UTP, and ATP from $^{13}\text{C}_6\text{-glucose}$ and $^{13}\text{C}_5, ^{15}\text{N}_2\text{-Gln}$ to determine if these disturbances in TCA cycle flux and anapleurosis were propagated into nucleotide synthesis. Through the analysis of $^{13}\text{C}_6\text{-glucose}$ labeled UDP-GlcNAc and subsequent GAIMS deconvolution, I showed that uracil and acetyl group enrichment were preferentially delayed by selenite treatment. Uracil synthesis requires incorporation of Asp into its ring, which itself is a product of Krebs' cycle intermediate OAA's transamination. Given that selenite reduces anapleurosis of the TCA cycle, it follows that Asp synthesis would also be reduced as well as its

incorporation into the uracil ring. In addition, uracil biosynthesis requires the activity of the mitochondrial enzyme dihydroorotate dehydrogenase, which requires reoxidation of the flavin by ubiquinone (cf complex II) and thus a functional respiratory chain [71]. Furthermore, selenite-induced stimulation of glycolysis and subsequent lactic acid fermentation to replenish NAD^+ could reduce flux through pyruvate dehydrogenase, limiting the acetyl-CoA substrate available for acetylation of glucosamine-6-phosphate during the glucosamine-6-phosphate *N*-acetyltransferase reaction. These blocked steps in hexosamine synthesis resulted in a significant reduction of total UDP-GlcNAc in A549 cells.

Examination of $^{13}\text{C}_5$, $^{15}\text{N}_2$ -Gln incorporation into UDP-GlcNAc and UTP reiterated these findings, illustrating that uracil synthesis from Asp was impaired by selenite. GFAT catalyzed transamination of fructose-6-phosphate to glucosamine-6-phosphate appeared to be the only nitrogen-donating reaction of UDP-GlcNAc synthesis that was not inhibited by selenite treatment, likely due to the increased pool of Gln available due to diminished glutaminolysis. When I repeated the experiments in NHBE cells, I found no effect from selenite on nucleotide synthesis. Furthermore, NHBE cells did not utilize exogenous Gln for nucleotide synthesis, reinforcing that Gln is a preferential nutrient of transformed cancer cells. Here again, analysis of tissue slice nucleotide synthesis from labeled precursors recapitulated the findings from the cell-based experiments illustrating a selenite-induced reduction in biosynthesis. Benign tissues were, however, affected as well by selenite treatment suggesting some effect on nucleotide synthesis in the surrounding lung tissue. Differences in the response

to selenite between tumor and adjacent benign tissue are likely to be smaller than in cell monoculture. The presence of stromal cells, in both the tumor and benign tissue along with inflammation, is partially responsible for reducing these differences.

Finally, I found that the reduction in UDP-GlcNAc biosynthesis caused by selenite was followed by a global reduction in its glycosylation of protein targets by OGT. Given the increasing recognition of the importance of O-GlcNAcylation in protein regulation[49-51], these selenite-induced downstream effects could have compounding implications for tumor cell survival and proliferation. However, further work is needed in order to determine which potential targets may be mediating these effects.

CHAPTER 3-SELENITE INHIBITS GLUTAMINOLYSIS BY DECREASING THE EXPRESSION OF GAC

3.1 Introduction

Selenium is a micronutrient that is incorporated into the 21st protein coding amino acid, selenocysteine [10]. It functions primarily as an anti-oxidant via its catalytic role in redox enzymes like thioredoxin reductase and glutathione peroxidase [72]. A selenium deficient diet leads to a constellation of syndromes including bone deformities, thyroid disorders, weakened immune response, and an endemic cardiomyopathy of the Keshan province in China [13-15]. Selenium also has a well-established history of anti-cancer and chemopreventive activity including its investigation in several large-scale human drug trials [20, 26, 27]. The anti-cancer or chemopreventive potential of selenium is highly dependent on the chemical species that is used, and a wide variety of organic and inorganic compounds have been studied. The discrepancy in efficacy of varying seleno-compounds may have been responsible for the failure of the SELECT trial (which used pure selenomethionone and vitamin E, and thus further research into selenium toxicity is needed [27]).

Selenite (SeO_3^{2-}) is one of the main inorganic forms of selenium that has shown promise as a chemopreventive and chemotherapeutic. Selenite has been shown to induce dose-dependent apoptosis in a variety of cancer types including

lung, prostate, glioma, and colorectal cancers [17, 24, 73, 74]. Recently, Park et al. uncovered evidence of ROS-mediated autophagy in A549 lung cancer cells eventually leading to cell death [22]. However, despite these findings, the exact mechanism of selenite-induced toxicity in cancer cells remains unclear. One of the main mechanistic theories involves the generation of ROS such as superoxide, hydrogen peroxide, and/or hydroxyl radical from the successive reduction of SeO_3^{2-} to elemental selenium (Se^0) [25]. The ROS generated can then induce DNA and protein damage that precipitates a cascade of events eventually leading to apoptosis and cell death. ROS accumulation and cell death are blocked in selenite-treated A549 cells by supplementation with *N*-acetylcysteine (NAC), a commonly used antioxidant, which supports this mechanistic theory [22].

Previously, using tracer-based experimental design Fan et al. showed that selenite treatment of A549 cells induces numerous metabolic alterations including disruption of the TCA cycle, glutaminolysis, and glycolysis [60]. The utility of stable isotope resolved metabolomics (SIRM) has also been demonstrated in establishing the differences between metabolic phenotypes of cancer and benign human lung tissues, the profiling of lipid biosynthesis in breast and lung cancer, and the non-steady state modeling of the hexosamine biosynthetic pathway in prostate [33, 55, 56]. Here, I employed SIRM as an approach towards mechanistic understanding of selenite toxicity in human lung cancer models, specifically disrupted glutamine (Gln) metabolism.

Gln is one of the main anapleurotic substrates of the TCA cycle and contributes in the synthesis of nucleotides, amino acids, hexosamines, and glutathione among other metabolites. Gln consumption is often increased in tumors and glutaminase (GLS) inhibition has been shown to induce apoptosis in kidney and lung cancer cells overexpressing *MYC*, the proto-oncogene that drives GLS expression [39]. We have also found that c-MYC expression is elevated in the majority of NSCLC tumors relative to adjacent benign tissue in our ongoing patient study. Since our previous findings indicated selenite disrupted Gln metabolism in A549 cells and the large body of literature surrounding Gln's importance in tumor nutrition, I have focused on Gln metabolism as a possible source of toxicity in A549 cells [23].

GLS exists as two main isoforms in mammals, GLS1 and GLS2 [75]. GLS2 or liver type GLS (LGA) is partially regulated through interaction with Tax interacting protein (TIP-1) via its C-terminal domain and has been implicated as a tumor suppressor [76, 77]. However, differences in the C-terminal domain of GLS1 prohibit interaction with TIP-1 [78]. GLS1 can be subdivided into two splice variants, kidney type GLS (KGA) and GLS C (GAC). GAC is a splice variant of KGA that has been shown to be upregulated at the mRNA level in a variety of cancers including those of brain, colorectal, and breast [79-82]. GAC was recently shown to be localized exclusively in the mitochondria and is activated by inorganic phosphate [83]. GAC expression is regulated at least in part via c-MYC and interestingly has also been shown to be a target of mitochondrial Lon protease mediated degradation when modulated by diphenylarsenic acid [84,

85]. These recent findings along with this study suggest an increasingly important role for GAC in tumor metabolism and selenite mediated anti-cancer activity.

3.2 Methods

Cell culture: A549 cells were grown in glucose-free, glutamine-free DMEM medium supplemented with 10% FBS, 0.2% glucose, 4 mM glutamine, and 100 I.U./mL penicillin 100 μ g/mL streptomycin at 37 °C and 5% CO₂. Na₂SeO₃ solutions were prepared immediately prior to use and sterilized using 0.22 μ m syringe filter. Cells were grown to 70% confluence for all experiments. Two hours prior to treatment, medium was aspirated and replaced with half volume of the tracer medium. The remaining medium was then added with doubled Na₂SeO₃ concentration in order for cells to be conditioned with the tracer medium prior to treatment.

Tissue slices: During NSCLC surgical resection, very thin slices (0.5-1 mm) were prepared from tumor and adjacent benign tissue by the operating surgeon (Dr. M. Bousamra II). Tissue slices were immediately placed in T-25 culture flasks with 8 mL DMEM medium supplemented with 10% FBS, 100 I.U./mL penicillin 100 μ g/mL streptomycin, 0.2% glucose, and 4 mM Gln. Either ¹³C₆-glucose or ¹³C₅¹⁵N₂-Gln was used for tracer experiments. Slice medium was then treated with or without 6.25 μ M Na₂SeO₃. Flasks were then placed on a rotator in a cell culture incubator at 37 °C and 5% CO₂ for 24 h. After 24 h, medium was collected

for downstream analysis, and tissue slices were washed with PBS before flash freezing in LN₂.

Extraction of metabolites: At the time of harvest, medium was collected before all plates were washed three times with ice cold PBS, and PBS was removed as much as possible by aspiration. The cells' metabolism was then quenched with 1 ml of CH₃CN kept at -20°C before 0.75 ml of nanopure water was added and cells were harvested by scraping. The process was repeated once before CHCl₃ addition to a final CH₃CN:H₂O:CHCl₃ ratio of 2:1.5:1 to obtain the polar, non-polar and insoluble proteinaceous interface fractions for downstream analysis [65, 66]. For extraction of metabolites from human tissue slices, frozen tissue was first pulverized to powder in a liquid nitrogen freezer mill. The frozen powder was then extracted using the same ratio of CH₃CN:H₂O:CHCl₃ detailed above.

Glutaminase assays: For assaying whole cell glutaminase activity, cells were washed with PBS, trypsinized, and flash frozen in LN₂. Cell pellets were resuspended in and then sheared via aspiration through a 28.5 gauge syringe. The assay solution consisted of cell lysate, 5 mM alpha-ketoglutarate, 200 mM KH₂HPO₄, 0.2 mM EDTA, 48 U/mL glutamate dehydrogenase (GDH), 0.3 mM NADH, and 20 mM Gln. The reaction was initiated with Gln and the absorbance was measured continuously at 340 nm for 20 min. Specific activity was then calculated from the change in the amount of NADH produced (derived from A₃₄₀ and molar absorptivity) over time. For mitochondrial activity assays, mitochondria

preparations described below were used. The assay solution consisted of 0.2 mM Tris acetate pH 8.6, 20mM K₂HPO₄, 2mM NAD⁺, 15U/mL GDH, and 50mM Gln, Reaction was initiated with Gln and 5 µg total protein was used per reaction. Absorbance was then measured, and specific activity calculated as above.

Mitochondrial isolation: Cells were washed with PBS, trypsinized, and pelleted before resuspending in 200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES pH 7.5. A protease and phosphatase inhibitor cocktail was added immediately prior to isolation as follows: HALT protease inhibitor cocktail (Pierce Biotechnology), 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 2 mM imidazole, and 1.15 mM Na molybdate. Cells were homogenized with 28.5 g needles and then centrifuged at 1000 g for 10min. Supernatant was collected and spun at 10,000-15,000 g for 15 min. Pellet was washed with 1 mL isolation buffer, centrifuged at 15,000 g for 15 min, and suspended in 25 mM HEPES, 150 mM NaCl, 1 mM EDTA, and 0.01% triton X-100.

NMR experiments: All NMR experiments were conducted on a Varian Inova 14.1 T instrument equipped with 5 mm HCN cold probe. Cell extracts were dissolved in D₂O with 30 nmol d₆-DSS, respectively, as internal standard. Shigemi tubes were used for polar extracts to minimize sample volume and thus to concentrate the metabolites. 1D proton spectra were recorded with an acquisition time of 2 seconds after initial presaturation of the H₂O signal with a recycle time of 5 seconds. 1D ¹³C-filtered HSQC spectra were measured using an acquisition time

of 150 ms. 1D proton spectra were processed using a 1 Hz exponential line broadening function and a zero fill of 128 k points. 1D ^{13}C -filtered HSQC spectra were processed with a 6 Hz line-broadening exponential and 0.07 Hz Gaussian functions and zero filled to 16 k points. Both types of spectra were processed manually for baseline and phase corrections. 1D proton spectra were referenced using the methyl signal of D₆-DSS at 0 ppm, and 1D ^{13}C -filtered HSQC spectra were referenced to the C4-Gln peak at 2.43 ppm. Metabolites were identified in reference to an in-house standard database and quantified by calibration with the methyl resonance of the DSS internal standard using the MestReNova NMR processing software (Mestrelab Research, Santiago de Compostela, Spain) [59].

GC-MS analysis: An aliquot of the lyophilized extracts was derivatized in 50% *N*-*tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% *tert*-Butyldimethylchlorosilane (MTBSTFA + 1% TBDMSCI) in CH₃CN before GC-MS analysis. All GC-MS data were acquired on a ThermoFinnigan PolarisQ GC-Ion trap MSn instrument (ThermoScientific, Rockford IL). One μL of the derivatized sample was separated on a SGE Forte (Victoria, Australia) 5% phenyl capillary column with the following dimensions: 50 m x 0.15 mm; 0.25 μm film thickness. The GC parameters were as follows: injector and transfer line, 280 °C; oven, 60 °C for 2 min, 60 to 150 °C at 20 °C/min ramp, 150 to 300 °C at 6 °C/min ramp; He carrier gas with flow rate of 1.5 mL/min. Spectra were acquired in segscan mode with ranges of 140-206, 209-280, and 283-650 m/z with an acquisition time of 0.97 s. Spectra were processed using Xcalibur MS software and quantified

using a mixture of 63 standards at 50 μ M each except for glutamate at 500 μ M. All samples and standards were spiked with 1 μ M norleucine as internal standard.

Western Blot: At time of harvest cells were lysed directly on the plate using 62.5 mM Tris, 2% SDS buffer at pH 6.8 before harvest by scraping, and protein concentration was measured via the BCA method (Pierce). All protein extracts were diluted in 4X loading buffer (50% glycerol, 4% SDS, 150 mM Tris pH 6.8, 1 mM DTT, 0.02% bromophenol blue, 10% β -mercaptoethanol) to 1X strength and then boiled for 10 minutes prior to electrophoresis. Approximately 30 μ g protein was loaded for each sample onto a denaturing SDS 10 % acrylamide gel with a 6% stacking gel. Protein extracts were stacked for 30 min at 100 V and then resolved for 1.5 h at 200 V using commercially available Tris/Gly/SDS buffer (National Diagnostics, Atlanta, GA). Proteins were then transferred onto a PVDF membrane (Immobilon-P, Millipore, Bedford, MA) at 25 V for 2 h at room temperature in an Invitrogen transfer apparatus. All primary antibodies were incubated with membrane at 4 °C on a rocker overnight as follows: KGA (c-terminus specific, Abnova), 1:2000 in PBST with 5% non-fat powdered milk; GAC (generously donated from Dias) 1:3300 in PBST; α -tubulin (Epitomics), 1:50,000 in PBST with 5% non-fat powdered milk. Membranes were then washed 3 times with PBST before incubating 1 hour at room temperature with an HRP-conjugated secondary antibody (Pierce, 1:2000 in PBST with 5% non-fat powdered milk). Blots were then incubated in Pierce supersignal extended

duration chemiluminescent reagent before exposure to x-ray film (SCBT) for visualization. Expression was then quantified using ImageJ software.

RT-qPCR: At time of harvest, total RNA was extracted from cells using Trizol reagent (Invitrogen) and protocol. RNA concentration and quality were determined via absorbance on a Nanodrop instrument (Thermo Fisher), and integrity was verified via agarose gel electrophoresis. cDNA was synthesized using Invitrogen Superscript VILO cDNA synthesis kit. Primers for GAC and KGA were designed and purchased through IDT and β -actin through Qiagen. SYBR Green qPCR mastermix was purchased through Applied Biosystems. Primer and template concentrations were optimized for amplification efficiencies >90% before continuing with experimental assays. For quantification the $\Delta\Delta C_t$ method was applied with β -actin as the housekeeping gene.

Microscopy: A549 cells were grown to ~70% confluence in 6-well plates containing sterilized cover slips before treating with 6.25 mM Na_2SeO_3 . At 3, 6, 24 and 48 hours medium was aspirated. For ROS measurement 10 mM $\text{H}_2\text{-DCFDA}$ (Molecular Probes) in 1 mL fresh medium was added to each well and incubated at room temperature for 20 min. Medium was then replaced with 3 mL fresh medium and cells were incubated for another 30 min at room temperature to allow dye to hydrolyze. Cover slips were then washed with fresh medium, and slides were placed face down onto slides pre-loaded with 20 mL PBS. Cover slip edges were then sealed with fingernail polish. For mitochondrial staining 25-100

nM MitoTracker Red (Molecular Probes) in 1 mL fresh medium was added to each well and incubated at 37 °C for 0.5-1 hour. Coverslips were then washed with fresh medium before placing on slide and sealing as above. Images were recorded on an Olympus BX51 fluorescence microscope using a 100x /1.3 Ph3, oil immersion lens (Olympus, Melville, NY) and appropriate filters (Omega Optical, Brattleboro, VT).

Proliferation assays: Proliferation was measured in A549 according to the protocol by Raspotnig et al. [64]. Briefly, at the time of measurement cell medium was aspirated and replaced with minimal volume of 50% ethanol in H₂O for 30 min. Ethanol was then aspirated and replaced with minimal volume of 0.2% Janus green dye in PBS for 5 min. Dye was aspirated and plates were rinsed in RO H₂O and then air-dried. Dye was then dissolved in 0.5 N HCl and absorbance was measured at 595 nm. 6.25 μ M Na₂SeO₃ and 5mM Glu was used for all proliferation assays. For rescue experiments, Glu and/or selenite were introduced to media simultaneously and growth was quantified as the fraction of absorbance over day 0.

Statistics: All metabolite amounts were normalized to total protein measured via the BCA method. All error bars represent the standard deviation of an average of experiments (the specific number of experiments is provided in appropriate figure legends). For statistical significance of experiments where only a single variable was measured (i.e., enzyme activity assay) the two-tailed Student paired t-test

was used to calculate p values, and p values <0.05 were considered significant. For experiments involving multiple measurements (i.e., measurement of metabolites from NMR and MS analysis) the false discovery rate method described by Benjamini and Hochberg was applied [67]. Briefly, naïve p -values for all measured metabolites were calculated. These values were then ranked in order of decreasing significance and assigned a rank number. A q_i value was then calculated according to the equation $q_i = kp_i/i$ where i =rank of the p value among the k tests. The false discovery rate (FDR) was then defined for every q_i value such that $FDR_i = \min(q_i, \dots, q_k)$. Tests with p values <0.05 and an FDR value <0.05 were then accepted as significant. For cell-based experiments, only Glu, Gln, GSG, and GSH were measured for statistical differences among treatments. For tissue experiments Glu, fumarate, malate, and Asp were measured.

3.3 Results and Discussion

3.3.1 Selenite disrupts Gln utilization in human lung cancer models

3.3.1.1 Cell culture

In order to probe SeO_3^{2-} perturbed metabolism in cancerous and non-cancerous lung models, A549 human lung cancer cells and non-transformed NHBE cells were treated with or without 6.25 μM Na_2SeO_3 , and metabolites in polar extracts were measured using NMR and MS. An immediately obvious feature of the polar metabolic profiles from SeO_3^{2-} treated A549 cells was the buildup and depletion of Gln and Glu, respectively, as illustrated in figure 3.1. Quantitatively, Gln was elevated ~2.5-fold and Glu decreased ~3-fold (Fig. 3.2A).

Figure 3.1. Effect of selenite on Gln metabolism.

1D proton spectra were recorded of polar extracts of A549 and NHBE cells treated for 24 hours with 6.25 μM Na_2SeO_3 . Spectra were superimposed and normalized to total protein to assess differences between treatments. Selected region displays the C4 protons from both Gln and Glu.

Figure 3.2. Gln and Glu quantification from selenite treated A549 cells.

A) 1D proton spectra were recorded from the polar extracts of A549 cells treated with 6.25 μM Na_2SeO_3 for 24 hours. C4-Glu and Gln proton peaks at 2.34 and 2.44 ppm were quantified and normalized to total protein. * $p < 0.001$; $n = 3$. B) Total Gln and Glu normalized to total protein were measured from GC-MS spectra of polar extracts recorded at 0, 1.5, 3, 6, and 24 hours of selenite treatment.

However, little to no effect on Gln or Glu was apparent in the corresponding spectra of NHBE cells treated with the same concentration of SeO_3^{2-} (Fig. 3.1).

Time course GC-MS measurements taken from SeO_3^{2-} treated A549 cells indicated that Gln and Glu levels began to increase and decrease, respectively, at ~3 h of treatment and these changes continued for the duration of the experiment (Fig. 3.2B). By ~6 h of selenite treatment, Glu depletion had plateaued with a half-life of ~2 h, while Gln continued to accumulate suggesting inhibition of the glutaminase reaction that converts Gln to Glu while maintaining Gln import.

In order to further probe altered glutaminolysis in cell experiments, $^{13}\text{C}_5^{15}\text{N}_2$ -Gln was utilized as a tracer in time course experiments to directly measure the conversion of Gln to Glu and downstream metabolites. 1D ^{13}C -filtered HSQC spectra were recorded of the polar extracts of SeO_3^{2-} treated A549 cells to follow the time course changes in ^{13}C -labeled metabolites. Consistent with findings from the unlabeled experiments, the selenite-induced ^{13}C -Gln buildup/Glu depletion began at ~3 h and the same trend continued thereafter (Fig. 3.3). Selenite also elicited a depletion of ^{13}C -labeled glutathione beginning at ~6 h of the treatment and a depletion of ^{13}C -labeled Asp and citrate beginning at ~3 h of treatment, as illustrated in figure 3.3. Furthermore, the level of downstream products of $^{13}\text{C}_5^{15}\text{N}_2$ -Gln via glutaminase and the Krebs cycle, i.e. $^{13}\text{C}_5^{15}\text{N}_1$ -Glu, $^{13}\text{C}_4$ -fumarate, $^{13}\text{C}_4$ -malate, and $^{13}\text{C}_4^{15}\text{N}_1$ -Asp showed a progressive decrease with selenite treatment, which began at ~3h to near non-detectable by 24h (Fig. 3.4). The corresponding metabolites in the controls

Figure 3.3. Time course effect of selenite on Gln, Glu, and glutathione

1D $^1\text{H}[33]$ HSQC spectra were recorded from polar extracts of A549 cells pre-incubated for 2 hours with $^{13}\text{C}_5, ^{15}\text{N}_2$ -Gln tracer and treated with or without $6.25 \mu\text{M Na}_2\text{SeO}_3$ for 0, 1.5, 3, 6, 11, and 24 hours. Control and selenite spectra were superimposed and normalized to total protein to visualize differences between treatments in ^{13}C -4-glutathione, ^{13}C -4-Gln, and ^{13}C -4-Glu at 2.54, 2.44, and 2.34 ppm, respectively.

Figure 3.4. Time course quantitation of selenite effects on Gln-derived metabolites in A549
GC-MS spectra were recorded from A549 cells treated pre-incubated for 2 hours with $^{13}\text{C}_5, ^{15}\text{N}_2\text{-Gln}$ tracer and treated with or without $6.25 \mu\text{M Na}_2\text{SeO}_3$ for 0, 1.5, 3, 6, 11, and 24 hours. $^{13}\text{C}_5, ^{15}\text{N}_1\text{-Glu}$, $^{13}\text{C}_4\text{-malate}$, $^{13}\text{C}_4\text{-fumarate}$, and $^{13}\text{C}_4, ^{15}\text{N}_1\text{-Asp}$ were quantified and normalized to total protein.

continued to increase for the duration of the experiment, possibly because the TCA cycle had not yet reached isotopic steady state. Fluctuations at 2 h may be due to equilibration of metabolism to medium change.

3.3.1.2 Human Tissue Slices

Thin (0.5-1 mm) slices of tumor and adjacent benign lung tissue from human non-small cell lung cancer (NSLSC) surgical patients were used as an individualized model for human lung cancer. These tissue slices are analogous to those described by Warburg, but are placed in medium rather than on wet blotting paper, thereby giving much greater control over nutrient supply and removal of waste products [29]. Such slices represent better models than cultured cells as they contain much of the in situ microenvironment that is lost with cell cultures. Including the original tissue architecture and the stromal cells of the tumor. These tissue slices were treated with or without 6.25 μM Na_2SeO_3 in the presence of $^{13}\text{C}_5, ^{15}\text{N}_2\text{-Gln}$ for 24 h. As shown in the 1D ^{13}C -filtered HSQC spectra of figure 3.5A, the ^{13}C -labeled Gln/Glu buildup/depletion observed in the cell experiments was recapitulated in the lung tumor slices but much less so for the benign counterpart. Quantification of the ^{13}C -Gln and Glu from the spectra yielded significantly elevated Gln/Glu ratios induced by selenite in the cancerous tissues over the benign tissues, consistent with impaired glutaminolysis (Fig. 3.5B). Downstream metabolites of $^{13}\text{C}_5, ^{15}\text{N}_2\text{-Gln}$ ($^{13}\text{C}_5, ^{15}\text{N}_1\text{-Glu}$, $^{13}\text{C}_4\text{-fumarate}$, $^{13}\text{C}_4\text{-malate}$, and $^{13}\text{C}_4, ^{15}\text{N}_1\text{-Asp}$) were also measured in cancerous and benign human tissue slices treated with selenite for 24 h. The reduction of labeled Glu, fumarate, malate, and Asp in treated cancerous tissues relative to controls was

Figure 3.5. Effect of selenite on Gln/Glu ratio in NSCLC surgical patient tissue slices

A) 1D ¹H[33] HSQC spectra recorded from polar extracts of benign and cancerous human tissue slices labeled with ¹³C₅, ¹⁵N₂-Gln tracer and treated with or without 6.25 μM Na₂SeO₃ for 24 hours. Control and selenite spectra were superimposed and normalized to total protein in order to visualize differences between treatments. B) Gln/Glu peak area ratios were calculated. *p<0.001, #p<0.05 for the increase in Gln/Glu in selenite treated cancer tissue slices with respect to non-treated controls and selenite treated benign tissue slices, respectively.

not as dramatic as in the cell experiments (Fig. 3.6). Benign tissues also responded to selenite treatment, albeit to a lesser extent, suggesting that there is some impact on glutaminolysis in the surrounding non-cancerous tissue. Smaller differences, however, are to be expected between tumor and benign tissue than in pure cell culture due to the presence of stromal cells in both tissue environments and inflammation in the lung.

3.3.2 Selenite inhibits GLS activity by decreasing its expression

Since the tracer-based metabolic characterization of SeO_3^{2-} treated lung cancer models strongly suggested inhibited glutaminolysis, I then probed the glutaminase (GLS) activity directly by utilizing a coupled GLS-GDH colorimetric enzyme assay (Fig. 3.7). GLS activity was significantly reduced under selenite treatment by 6 h, which continued for 24 hours. In order to determine if enzyme activity was modulated through direct or allosteric means, I measured GLS activity in crude A549 lysates subsequently treated with $6.25 \mu\text{M Na}_2\text{SeO}_3$ for various times. However, only a weak dose response decrease in activity was observed (Fig. 3.8). We then measured the specific activity of purified GAC isoform treated with $6.25 \mu\text{M Na}_2\text{SeO}_3$ and again noted no change in activity (Fig. 3.9).

In order to determine if selenite exerted its inhibitory effect via enzyme expression, I then measured levels of the kidney (KGA) and GAC isoforms of GLS from selenite-treated A549 cells via western blot (Fig. 3.10). Interestingly, KGA levels remained largely unchanged while GAC expression was greatly attenuated with the selenite treatment and undetected by 24 h, which traced the

Figure 3.6. Quantitation of selenite effects on Gln derived metabolites in NSCLC surgical patient tissue slices

Polar metabolites were extracted and quantified from tumor and adjacent benign tissue slices incubated with $^{13}\text{C}_5, ^{15}\text{N}_2$ -Gln and treated with or without 6.25 μM Na_2SeO_3 for 24 hours. Downstream metabolites of Gln were quantified via GC-MS and normalized to total protein before determining ratios. *'s represent a significant reduction in the selenite-treated cells compared to non-treated controls for the designated tissue type. * $p < 0.05$; $n = 3$

Figure 3.7. Time course changes in GLS activity of control and SeO_3 treated A549 cells

A549 cells were treated with or without 6.25 μM Na_2SeO_3 and harvested at 0, 1.5, 3, 6, 11, and 24 h after treatment. Mitochondria were isolated and assayed for GLS activity as described. Activity was normalized to total protein determined via BCA. * $p < 0.05$; $n = 3$

Figure 3.8. Time course changes in GLS activity of selenite-treated A549 cell lysate

A549 lysates were pooled and treated with increasing concentrations of Na_2SeO_3 for 0, 3, 6, 10, and 24 h before assaying for GLS activity. Activity was normalized to total protein determined via BCA. Single experiment.

Figure 3.9. Effect of selenite on purified GAC activity

5 mg of purified GAC were treated with increasing concentrations of Na_2SeO_3 for 0, 3, 6, 10, and 24 h before assaying for activity as described. Single experiment.

Figure 3.10. Effect of selenite on GAC and KGA expression in A549

A549 cells were treated with or without 6.25 μM Na_2SeO_3 and harvested 0, 1.5, 3, 6, 9, and 24 h after treatment. Protein was extracted and processed for GLS expression as described. A) Representative western blot from selenite-treated A549 time course experiment illustrating GAC and KGA expression. B) Quantification of GAC and KGA normalized to α -tubulin and expressed as selenite/CTL ratio from three separate experiments.

activity measurements shown in Figure 3.7. GAC and KGA expression was then measured in selenite-treated NHBE cells to determine if the effect matched that seen in A549 (Fig. 3.11). In agreement with the NMR spectra from figure 3.1 GAC expression was unchanged by selenite; however, baseline expression was decreased relative to A549, suggesting that cancer cells may require increased levels of GAC for proliferation. Furthermore, the data also imply that reduction of GAC expression may be partly responsible for the cancer-specific cytotoxicity observed with selenite treatment. Finally, I then examined GAC and KGA expression in selenite-treated tumor and adjacent benign tissue slices. Interestingly, the GAC expression was dramatically reduced in both tumor and adjacent benign tissue by selenite, recapitulating the A549 response and suggesting that GAC loss and/or glutaminolysis in the benign tissue is not as damaging as it is in tumor (Fig. 3.12). Furthermore, KGA expression was essentially unchanged by selenite treatment as in A549 cells. Despite unchanged KGA expression, the increased Gln/Glu ratio in figure 3.5 suggests that adequate glutaminase activity was not being supplied.

Total RNA was then harvested from parallel selenite-treated A549 time course experiments in order to quantify KGA and GAC mRNA via RT-qPCR and to determine if the decrease in GAC expression was transcriptionally regulated (Fig. 3.13). However, no difference in KGA or GAC mRNA relative to housekeeping gene β -actin was noted between control and selenite-treated cell samples.

Figure 3.11. Effect of selenite on GAC and KGA expression in NHBE

NHBE and A549 cells were treated with or without 6.25 μ M Na₂SeO₃ and harvested 24 h after treatment. Protein was extracted and processed for GLS expression as described. A) Representative western blot from selenite-treated A549 and NHBE cells illustrating GAC and KGA expression. B) Quantification of GAC and KGA normalized to α -tubulin and expressed as selenite/CTL ratio from three separate experiments.

3.3.3 Selenite-induced glutathione perturbations and ROS damage

As selenite is known to induce oxidative stress, GSSG/GSH ratios were also measured from the NMR spectra of selenite-treated A549 polar extracts as indicators of oxidative stress. The GSSG/GSH ratio was ~4-fold higher than controls after 24 h of selenite treatment in A549 cells (Fig. 3.14). Mitochondrial and ROS fluorescence were also measured in micrographs of selenite-treated A549 cells as another indicator of oxidative damage (Fig 3.15). ROS accumulation was evident as early as 3 h and increased dramatically by 24h.

3.3.4 Glu rescue experiment

We then postulated that the decrease in GAC expression and consequent depletion of Glu and downstream metabolites induced by selenite was responsible for the decreased total glutathione levels, increased GSSG/GSH ratios, and increased ROS production. In order to test this hypothesis we treated A549 cells with 6.25 μM Na_2SeO_3 in the presence or absence of 5 mM Glu. By supplying the cells with the direct product of the GAC reaction we expected to mitigate the anti-proliferative effects of selenite. Indeed, in the presence of Glu, the proliferation of selenite-treated A549 cells was substantially rescued, as measured via Janus green proliferation assay (Fig. 3.16). Interestingly, Glu appeared to stimulate control growth above baseline.

Figure 3.12. Effect of selenite on GAC and KGA expression in NSCLC surgical patient tissue slices

A) Representative western blot from selenite-treated tumor (T) and adjacent benign (B) NSCLC patient tissue. B) Quantification of GAC and KGA normalized to tubulin and expressed as SeO₃²⁻/CTL ratio from four separate patients.

Figure 3.13. Relative fold change of KGA and GAC mRNA.

A549 cells were treated with or without 6.25 μM Na_2SeO_3 and harvested at 0, 1.5, 3, 6, 11, and 24 h after treatment. Total RNA was extracted and measured as described. ddC_t values were calculated with b-actin as housekeeping gene. Fold changes were then calculated and are shown as $\text{SeO}_3^{2-}/\text{CTL}$ relative changes.

Figure 3.14. Quantitation of GSSG/GSH in selenite treated A549 cells

1D proton spectra were recorded from the polar extracts of A549 cells treated with or without 6.25 μM Na_2SeO_3 for 24 hours. GSSG and GSH were quantified, and GSSG/GSH was calculated. * $p < 0.01$; $n = 3$

Figure 3.15. Effect of selenite on ROS in A549 cells

A549 cells were treated with or without 6.25 μM Na_2SeO_3 and prepared for ROS measurement as described at 3, 6, 24, and 48 h after treatment as described. Red stain indicates mitochondria and green stain indicates DCHDFA oxidation as a measure of ROS production.

Figure 3.16. Glu rescues the proliferative phenotype in selenite-treated A549 cells

A549 cells were treated with or without 6.25 μM Na_2SeO_3 +/- 5 mM Glu and measured for growth via Janus green assay 0, 1, 2, and 3 days after treatment as described. Fractional growth was calculated as the absorbance at day n divided by absorbance at day 0. Fractional growth is plotted as selenite-treated cells +/- Glu divided by the fractional growth of non-treated controls.

* $p < 0.001$; $n = 5$

3.4 Conclusions

These experiments illustrate a novel mechanism for selenite-induced ROS accumulation in A549 cells. Examination of the metabolic profiles generated from selenite-treated A549 showed significantly elevated Gln and depleted Glu consistent with decreased glutaminase activity. This effect on glutaminase was selective towards cancer cells as Gln and Glu levels in non-transformed NHBE cells were substantially less affected. By utilizing a tracer-based experimental design with $^{13}\text{C}_5, ^{15}\text{N}_2\text{-Gln}$ we then showed that anapleurosis of the TCA cycle through glutaminolysis was dramatically attenuated by selenite treatment. Depletion of labeled glutaminolytic metabolites from $^{13}\text{C}_5, ^{15}\text{N}_2\text{-Gln}$ occurred rapidly within 3 hours of treatment. We then repeated these experiments in an individualized lung cancer tissue slice model, which recapitulated impaired glutaminolysis substantially more so in the cancer than in the adjacent benign tissue. Treatment with selenite caused a time-dependent decrease in GAC protein expression without impacting transcription levels or enzyme specific activity indicating that selenite affects either the translation or degradation rate. Interestingly, KGA was unchanged by selenite treatment suggesting that the GAC splice variant is vital for cancer growth and viability. Furthermore, GAC expression was also decreased in selenite treated tissue slices from four separate NSCLC patients.

Gln is one of the main anapleurotic substrates contributing towards anabolism including glutathione synthesis. We showed that selenite treatment substantially decreased newly synthesized glutathione as well as increased the

ratio of GSSG/GSH. Presumably, the decrease in glutathione synthesis stems from impaired glutaminolysis which is needed to supply glutamate, and could contribute towards increased GSSG/GSH as reduced glutathione is consumed. As A549 cells rapidly proliferate, GSH must be synthesized continually in order to maintain steady-state levels and replenish stores lost through non-oxidative means; however, selenite blocks this process by inhibiting glutaminolysis. Increased ROS production in the presence of selenite overwhelms the capacity of the GPX/GR/NADPH systems, leading to increased GSSG/GSH. The resulting high ROS may initiate autophagic and apoptotic pathways, further increasing ROS [22]. Preventing this cascade of events would require providing the necessary reducing equivalents for the surge in ROS. Park et al. [22] showed that exogenous NAC supplied these reducing equivalents; however, we found that supplying the cells with Glu restored proliferation as well. This suggests that impaired glutaminolysis via decreased GAC expression is one of the primary sources for escalating ROS production and selenite mediated cell death.

CHAPTER 4-GLYCOGEN IS AN IMPORTANT FUEL

SOURCE FOR LUNG CANCER

4.1 Introduction

Glycogen is a polymeric storage form of glucose in mammalian tissues, particularly in liver and skeletal muscle. It is a highly branched polymer, comprising linear chains of α -1,4 linked glucose subunits, with α -1,6 branch points, and is stored in granules in the cytoplasm. Glycogen synthesis consumes an ATP equivalent (via the activation step of $G1P + UTP \rightarrow UDPGlc + Pi$). Glycogen is mobilized through glycogenolysis via glycogen phosphorylase (PYG) and the debranching enzyme, which can liberate glucose-1-phosphate (G1P) during fasted or exerted states when blood glucose is low. G1P is readily isomerized to G6P without the consumption of ATP. Thus, glycogenolysis followed by glycolytic transformation to pyruvate generates 3 ATP molecules per molecule of glucose oxidized.

Glycogen metabolism is generally tightly regulated through both insulin and epinephrine-dependent and independent mechanisms acting reciprocally through glycogen synthase and glycogen phosphorylase [86]. There is a well-characterized clinical history of disorders in glycogen metabolism typically classified as the glycogen storage diseases. Glycogen storage diseases typically manifest with varying clinical severity and a pathophysiology stemming from

mutations in the three main isoforms of glycogen phosphorylase (GPase), which are the brain or fetal (PYGB), liver (PYGL), and muscle (PYGM) variants [87, 88].

Glycogenolysis is also of burgeoning interest in the context of cancer metabolism. PYGB was the main isoform expressed in 59 cancer cell lines including A549, whose growth was reduced via pharmacological inhibition of PYGB [89]. Furthermore, glycolytic models have indicated that glycogen degradation is an integral process for controlling flux through glycolysis under glucose-deprived tumor environments [90]. Glycogenolysis also seems to be closely tied to UDP-GlcNAc regulation as was illustrated by a glycogen-derived increase in O-GlcNAc modification of proteins in response to glucose deprivation [50]. Furthermore, the brain isoform of GPase is less sensitive to regulation by phosphorylation than either the liver or muscle isoforms, but is strongly activated by AMP, making it an energy-sensing enzyme [29]

In order to further elucidate the anti-cancer potential of glycogen phosphorylase inhibition and obtain detailed understanding of its metabolic basis I utilized SIRM-based experimental design to evaluate the metabolic effect of PYGB suppression in A549 cells. This target was also chosen to follow up on the reduction of glycogen synthesis that was observed in selenite-treated lung cancer tissue. I chose to focus on PYGB because it is the isoform that is primarily expressed in A549 cells and its relevance with regards to growth inhibition in cancer cells [91]. Finally, its role in UDP-GlcNAc metabolism is unclear and thus a worthwhile target for further investigation.

4.2 Methods

Cell culture: A549 and 293T cells were grown in glucose-free, glutamine-free DMEM medium supplemented with 10% FBS, 0.2% (A549) or 0.45% (293T) glucose, 4 mM glutamine, and 100 I.U./mL penicillin 100 µg/mL streptomycin at 37 °C and 5% CO₂. For tracer experiments, cells were grown to ~70% confluence, and medium was aspirated and replaced with the tracer medium containing 0.2% ¹³C₆-glucose (10.8 mM) (Sigma Isotec, St. Louis. MO). For western blots, cells were lysed directly on the plate using 62.5 mM Tris, 2% SDS buffer at pH 6.8 before harvest by scraping and protein concentration was measured via the BCA method (Pierce).

Lentiviral PYGB suppression: 293T cells generously donated by Dr. Mariia Yuneva were grown to ~50% confluence in 6-well plates before transfecting with shRNA constructs. One h prior to transfection, medium was replaced with fresh medium. 0.3 µg pMDG (Yuneva), 0.6 µg pCMV (Yuneva), 1.2 µg shRNA, and 10 µL X-tremeGENE HP transfection reagent (Roche, Mannheim, Germany) were mixed with OPTIMEM (Invitrogen) medium to a total volume of 110 µL per well (6-well plate) and incubated for 20 min at room temperature before applying to wells dropwise. shPYGB's and the empty vector (shEV, Mission® shRNA, Sigma, St. Louis, MO.) (Sigma) had the following sequences: sh339, CCGGGCTATGGAATCCGCTATGAATCTCGAGATTCATAGCGGATTCCATAG CTTTTTTG; sh479, CCGGGATCGTGAAACAGTCGGTCTTCTCGAGAAGACCG ACTGTTTCACGATCTTTTTTTG; sh010, CCGGCACGAAGAAGACCTGTGCATA

CTCGAGTATGCACAGGTCTTCTTCGTGTTTTTTG. About 12 h after applying transfection reagents, medium was again replaced with fresh medium. Transduction of A549 cells occurred ~48 h after transfection of 293T cells. In preparation, A549 cells were grown to ~50% confluence and medium was replaced immediately prior to transduction. Viral medium from 293T cells was collected and filtered using a 0.22 μm syringe filter before adding 16 $\mu\text{g/mL}$ polybrene (Sigma). The filtered medium was then applied to wells dropwise (polybrene final concentration, 8 $\mu\text{g/mL}$). Fresh medium was added to transfected 293T cells for repeating the transduction procedure 24 h later. After 24 h of the final transduction, A549 medium was replaced with fresh medium. 48-72 h after transduction, A549 cells were selected using medium supplemented with 1 $\mu\text{g/mL}$ puromycin (Sigma).

Proliferation assays: Proliferation of A549 cells was measured in 96-well plates according to the protocol by Raspotnig et al. [64]. Briefly, at the time of measurement, cell medium was aspirated and replaced with 50% ethanol in H_2O for 30 min. Ethanol was then aspirated and replaced with minimal volume of 0.2% Janus green dye in PBS for 5 min. Dye was aspirated and plates were rinsed in tap H_2O and drained by gravity. Dye was then dissolved in 0.5 N HCl and absorbance was measured at 595 nm at 0, 24, 48, and 72 h of growth. Growth was reported as the fraction of the absorbance at a given time point divided by the absorbance at 0 h.

Colony formation assay: One % agarose in H₂O was melted in a microwave to sterilize and then mixed with equal volumes of pre-warmed 2X DMEM cell culture medium. One mL of the mixture was then quickly added to each well of a 6-well culture plate and allowed to solidify at room temperature. After bottom layer had solidified, 3% agarose in PBS was melted in a microwave and allowed to partially cool. Cells to be assayed were trypsinized, counted, and diluted to 11,000 cells/mL in pre-warmed DMEM culture medium. The cell suspension was then quickly mixed with 3% agarose at a ratio of 10:1 suspended cells:agarose, and 1 mL of the resulting mixture was immediately added on top of the bottom agarose layer without disruption of the bottom layer or bubble formation. The top layer was then allowed to solidify for 30-60 min at room temperature. After solidification, 1 mL of DMEM culture medium was then carefully added to each well to avoid disturbing the agarose. Once a week, medium was carefully replaced via aspiration, again to avoid agarose disruption. After ~2 weeks or when colonies had grown sufficiently, medium was aspirated and wells were stained with 0.005% crystal violet in PBS for >1 h. Colonies (>8 aggregated cells) were digitally documented for colony size and shape and counted for 20 random fields of view.

Extraction of metabolites: Transduced A549 cells were seeded in 10 cm plates and grown until the shEV-transduced cells reached ~90% confluence for harvest. At the time of harvest, medium was collected before all plates were washed three times with ice cold PBS, and PBS was removed as much as possible by

aspiration. The cells' metabolism was then quenched with 1 ml of CH₃CN kept at -20°C before 0.75 ml of nanopure water was added and cells were harvested by scraping. The process was repeated once before CHCl₃ addition to a final CH₃CN:H₂O:CHCl₃ ratio of 2:1.5:1 to obtain the polar, non-polar and insoluble proteinaceous interface fractions for downstream analysis [65, 66]. For metabolite extraction from the medium, protein was first precipitated in ice-cold 10% trichloroacetic acid (TCA), and supernatant was lyophilized immediately to remove the acid. Since some metabolites such as glutamine are sensitive to prolonged storage in acidic conditions, metabolite analysis of medium samples by NMR or GC-MS was performed immediately after lyophilization.

NMR experiments: All NMR experiments were conducted on a Varian Inova 14.1 T instrument equipped with 5 mm HCN cold probe. Cell and medium extracts were dissolved in D₂O with 30 and 50 nmol d₆-DSS, respectively, as internal standard. Shigemi tubes were used for polar extracts to minimize sample volume and thus to concentrate the metabolites. 1D proton spectra were recorded with an acquisition time of 2 seconds after initial presaturation of the H₂O signal with a recycle time of 5 seconds. 1D ¹³C-filtered HSQC spectra were measured using an acquisition time of 150 ms. 1D proton spectra were processed using a 1 Hz exponential line broadening function and a zero fill of 128 k points. 1D ¹³C-filtered HSQC spectra were processed with a 6 Hz line-broadening exponential and 0.07 Hz Gaussian functions and zero filled to 16 k points. Both types of spectra were processed manually for baseline and phase corrections. 1D proton spectra were

referenced using the methyl signal of D₆-DSS at 0 ppm, and 1D ¹³C-filtered HSQC spectra were referenced to the C3-lactate peak at 1.32 ppm and 1.41 ppm for cell and medium extracts, respectively. Metabolites were identified in reference to an in-house standard database and quantified by calibration with the methyl resonance of the DSS internal standard using the MestReNova NMR processing software (Mestrelab Research, Santiago de Compostela, Spain) [59].

GC-MS analysis: An aliquot of the lyophilized extracts was derivatized in 50% *N*-*tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% *tert*-Butyldimethylchlorosilane (MTBSTFA + 1% TBDMSCl) in CH₃CN before GC-MS analysis. All GC-MS data were acquired on a ThermoFinnigan PolarisQ GC-Ion trap MSn instrument (ThermoScientific, Rockford IL). One µL of the derivatized sample was separated on a SGE Forte (Victoria, Australia) 5% phenyl capillary column with the following dimensions: 50 m x 0.15 mm; 0.25 µm film thickness. The GC parameters were as follows: injector and transfer line, 280 °C; oven, 60 °C for 2 min, 60 to 150 °C at 20 °C/min ramp, 150 to 300 °C at 6 °C/min ramp; He carrier gas with flow rate of 1.5 mL/min. Spectra were acquired in segscan mode with ranges of 140-206, 209-280, and 283-650 m/z with an acquisition time of 0.97 s. Spectra were processed using Xcalibur MS software and quantified using a mixture of 63 standards at 50 µM each except for glutamate at 500 µM. All samples and standards were spiked with 1 µM norleucine as internal standard.

Nucleotide processing for FT-ICR-MS (Fourier transform-ion cyclotron resonance-mass spectrometry) analysis: Nucleotides were ion-paired with hexylamine and extracted from polar extracts as described previously [57]. Briefly, dried extract aliquots (e.g. 1/16 of one 10 cm plate) were dissolved in 5 mM hexylamine in H₂O (buffer A) with the pH adjusted to 6.3 with acetic acid. Dissolved nucleotides were adsorbed onto C18 ZipTips (Millipore, Billerica, MA) pre-conditioned with MeOH and buffer A. Bound nucleotides were then washed with buffer A followed by elution with 70% buffer A/30% buffer B (90% methanol and 10% 10mM ammonium acetate adjusted to pH 8.5 with aqueous ammonia). Eluent was then lyophilized and reconstituted in 2:1 MeOH:buffer B for FT-ICR-MS analysis.

FT-ICR-MS analysis: FT-ICR-MS analysis was performed as described previously [57]. Briefly, data were recorded on a hybrid linear ion trap–FT-ICR mass spectrometer (Finnigan LTQ FT, Thermo Electron, Bremen, Germany) using an automated nanospray ion source (TriVersa NanoMate, Advion BioSciences, Ithaca, NY) set with an “A” chip run at 1.5 kV and 0.5 psi head pressure. Measurements were recorded for 15 min, which consisted of 14 min of high resolution FT-ICR scans, followed by 30 s of low resolution ion trap scans (LTQ). FT-ICR-MS scans were recorded from 150-800 Da with a mass resolving power set to 400,000 at 400 m/z. Each transformed spectrum consisted of 5 summed transients at 2 s per transient for a cycle time of 10 s. All FT-ICR-MS scans were summed to produce the final spectrum. Spectra were then exported

via Xcalibur MS software and peaks were corrected and assigned using an in-house software (PREMISE [56]).

Western Blot: At time of harvest cells were lysed directly on the plate using 62.5 mM Tris, 2% SDS buffer at pH 6.8 before harvest by scraping, and protein concentration was measured via the BCA method (Pierce). All protein extracts were diluted in 4X loading buffer (50% glycerol, 4% SDS, 150 mM Tris pH 6.8, 1 mM DTT, 0.02% bromophenol blue, 10% β -mercaptoethanol) to 1X strength and then boiled for 10 minutes prior to electrophoresis. Approximately 30 μ g protein was loaded for each sample onto a denaturing SDS 10 % acrylamide gel with a 6% stacking gel. Protein extracts were stacked for 30 min at 100 V and then resolved for 1.5 h at 200 V using commercially available Tris/Gly/SDS buffer (National Diagnostics, Atlanta, GA). Proteins were then transferred onto a PVDF membrane (Immobilon-P, Millipore, Bedford, MA) at 25 V for 2 h at room temperature in an Invitrogen transfer apparatus. The transferred membrane was then incubated in the following primary antibody at 4 °C on a rocker overnight: PYGB (LSBio, Seattle, WA), 1:1000 in PBST with 5% BSA; α -tubulin (epitomics), 1:50,000 in PBST with 5% non-fat powdered milk. Membranes were then washed 3 times with PBST before incubating 1 hour at room temperature with an HRP-conjugated secondary antibody (Pierce, 1:2000 in PBST with 5% non-fat powdered milk). Blots were then incubated in Pierce supersignal extended duration chemiluminescent reagent before exposure to x-ray film (SCBT) for visualization.

4.3 Results and discussion

4.3.1 PYGB suppression inhibits growth and colony formation in A549 cells

In order to determine if glycogen metabolism is important for supporting cancer cell proliferation, PYGB suppression in A549 cells was achieved using lentiviral transduction system and shPYGB vectors. Three different vectors were tested for their efficiency in PYGB suppression and effect on cell growth as shown in figure 4.1A. Sh339 and sh479 had the greatest effect on PYGB expression, while sh010 had much less impact (Fig. 4.1B). However, all three vectors had similar effects on proliferation, and by 72 h growth was attenuated by about 100%. Interestingly, sh010 exerted the strongest effect on proliferation despite having a slight effect on PYGB expression suggesting an off target mechanism of growth reduction. Colony formation assay was also performed as a surrogate analysis for tumorigenesis with these three vectors (Fig. 4.2). Sh479 but not the other two vectors had a small but significant effect on colony formation, reducing the number of colonies by ~30% suggesting that PYGB may also be involved in tumorigenesis but has a greater effect on growth.

4.3.2 PYGB-suppressed A549 cells accumulate glycogen and glucose

The metabolic perturbations in these suppressed cells were investigated using $^{13}\text{C}_6$ -glucose as a tracer. The NMR spectra of polar metabolites for shPYGB and shENV samples showed a noticeable accumulation of unlabeled glycogen (5.4 ppm) and glucose (5.22 ppm) in sh339 and sh479-suppressed cells relative to controls as shown in figure 4.3. Newly synthesized glucose (e.g.

Figure 4.1. PYGB suppression inhibited A549 cell growth

A549 cells were transduced with three different lentiviral shPYGB constructs along with an empty vector (shENV) as described in Methods. Growth was measured via Janus green assay five passages after transduction. Growth was plotted as fraction relative to day 0. B) PYGB expression in A549 cells 5 passages after transduction.

Figure 4.2. PYGB suppression reduced A549 colony formation

ShPYGB transduced A549 cells were assayed for colony formation after five passages. Bars represent the average number of colonies from 20 field views measured in biological duplicates.

Figure 4.3. PYGB suppressed A549 cells accumulated glycogen and glucose

5 passages after shPYGB suppression, A549 cells were incubated with $^{13}\text{C}_6$ -glucose for 24 h. Polar metabolites were extracted and measured via 1D proton NMR. Spectra were normalized to total protein for visualizing differences in glycogen and glucose content.

^{13}C 1-glucose at 5.22 ppm) and glycogen (^{13}C 1-glycogen at 5.4 ppm) also accumulated in sh339 and sh479-transduced cells relative to the control cells as shown in the protein normalized HSQC spectra, which suggests increased glycogen synthesis and/or decreased utilization (Fig. 4.4). The latter is expected from the block of GPase activity from the PYGB knockdown. However, the NMR-observable glycogen peak only represents the freely mobile reducing ends of glycogen, which may not fully represent the status of bulk glycogen. Namely, some of these C1-protons may be immobilized within the interior of the molecule and are not observable in ^1H NMR analysis.

Thus, to better visualize the total glycogen pool in these crude extracts, the extracts were hydrolyzed with 1M HCl at 65 °C for 10 min. Total glycogen was hydrolysed to free glucose, which was quantified by the peak at 5.22 ppm in the ^1H NMR spectra. As shown in figures 4.5 and 4.6, there was an increase in the H1 glucose peak at 5.22 ppm in both the 1D proton and 1D ^{13}C -filtered HSQC spectra of sh339 and sh479-suppressed cells, indicating glycogen buildup as expected with PYGB suppression. However, the effect of sh010 transduction differed, i.e. a significant increase in the level of acid-liberated ^{12}C -glucose but not in that of ^{13}C -glucose. This pattern suggests a buildup of unlabeled glycogen but not newly synthesized ^{13}C -labeled glycogen consistent with a block in both glycogen hydrolysis and synthesis. Altogether, these data demonstrate selective suppression of PYGB by sh339 and sh479, while sh010 suppressed PYGB less while having non-targeted suppression of glycogen synthesis.

Figure 4.4. PYGB suppressed A549 cells accumulated newly synthesized glycogen in A549 5 passages after shPYGB suppression, A549 cells were incubated with $^{13}\text{C}_6$ -glucose for 24 h. Polar metabolites were extracted and measured via ^{13}C -filtered 1D HSQC NMR. Spectra were normalized to total protein in order to visualize differences in glycogen and glucose.

Figure 4.5. Hydrolysis of polar extracts from shPYG suppressed A549 cells liberated unlabeled glucose units from glycogen

5 passages after shPYGB suppression, A549 cells were incubated with $^{13}\text{C}_6$ -glucose for 24 h. Polar metabolites were extracted and glycogen was hydrolyzed as described. HCl-liberated glucose was measured via 1D proton NMR, and spectra were normalized to total protein in order to visualize differences.

Figure 4.6. Hydrolysis of polar extracts from shPYG suppressed A549 cells liberated labeled glucose units from glycogen

5 passages after shPYGB suppression, A549 cells were incubated with $^{13}\text{C}_6$ -glucose for 24 h. Polar metabolites were extracted and glycogen was hydrolyzed as described. HCl-liberated glucose was measured via ^{13}C -filtered 1D HSQC NMR, and spectra were normalized to total protein in order to visualize differences.

Because there was an increase in intracellular glucose levels with PYGB suppression, lactate production and glucose consumption were also measured in the medium, as shown in figures 4.7A and B, respectively. $^{13}\text{C}_6$ -glucose levels remained higher in the media of the PYGB-suppressed cells, particularly cells with sh339 and sh479 constructs. However, ^{13}C -lactate released into the medium was comparable for all experimental groups. The lower glucose consumption elicited by PYGB suppression can be attributed to slower proliferation as well as the block in glycogen utilization leading to the accumulation of newly synthesized glycogen (Fig. 4.6). In the latter case, glycogen synthesis could be a substantial sink for glucose utilization in A549 cells. Taken together, glycogen is an important fuel for supporting cancer cell growth.

4.3.3 PYGB suppression reduces Krebs' cycle activity

In order to determine the effect of PYGB suppression on Krebs' cycle metabolism in A549 cells, Krebs' cycle metabolites and their ^{13}C labeling patterns from the $^{13}\text{C}_6$ -glucose tracer experiments were quantified using GC-MS. PYGB suppression, particularly with the sh479 vector, attenuated the total levels of citrate, fumarate, malate, and succinate as shown in figure 4.8. In addition, ^{13}C incorporation from labeled glucose into doubly labeled ($^{13}\text{C}_2$) Asp, fumarate, malate, and succinate were significantly reduced primarily in sh479-suppressed cells. These data are consistent with a reduced flux through the Krebs' cycle in sh479-transduced cells (Fig. 4.9). A similar reducing trend was also observed in the levels of many amino acids as shown in figure 4.10, again with the sh479 transduction having the greatest effect (2-3 fold changes). This effect of PYGB

Figure 4.7. PYGB suppression reduces glucose consumption

5 passages after shPYGB suppression, A549 cells were incubated with $^{13}\text{C}_6$ -glucose for 24 h. Polar metabolites of the medium were extracted and measured via 1D proton NMR. Medium $^{13}\text{C}_3$ -lactate (A) and $^{13}\text{C}_1$ -glucose (B) resonances were quantitated and normalized to total protein.

Figure 4.8. PYGB suppression decreases the level of Krebs' cycle intermediates

5 passages after shPYGB suppression, A549 cells were incubated with $^{13}\text{C}_6$ -glucose for 24 h. Polar metabolites were extracted and measured via GC-MS, and Krebs' cycle intermediates were quantified and normalized to total protein. Data are represented as the fraction of metabolite in the designated shPYGB construct over that in shENV.

Figure 4.9. PYGB suppression decreases flux through the TCA cycle

5 passages after shPYGB suppression, A549 cells were incubated with $^{13}\text{C}_6$ -glucose for 24 h. Polar metabolites were extracted and measured via GC-MS, and TCA cycle isotopologues were quantified and normalized to total protein. Data are represented as the fraction of $^{13}\text{C}_2$ -metabolite in the designated shPYGB construct over that in shENV.

Figure 4.10. PYGB suppression reduces amino acid level

5 passages after shPYGB suppression, A549 cells were incubated with $^{13}\text{C}_6$ -glucose for 24 h. Polar metabolites were extracted and measured via GC-MS, and amino acids were quantified and normalized to total protein. Data are represented as the fraction of metabolite in the designated shPYGB construct over that in shENV.

suppression on amino acids could be related to reduced synthesis of non-essential amino acids (Fig. 4.11).

4.3.4 PYGB suppression blocks nucleobase synthesis in A549 cells

^{13}C incorporation into nucleotides was also measured by FT-ICR-MS to determine the effect of PYGB suppression on nucleotide synthesis. The $^{13}\text{C}_5$ -UTP isotopologue corresponds to the fully ^{13}C labeled ribose subunit while isotopologues with >5 ^{13}C atoms ($^{13}\text{C}_{6-8}$ in Fig. 4.12) represent additional ^{13}C incorporation into the uracil ring via the aspartate precursor derived from Krebs' cycle OAA. Labeling of the ribose subunit was unaffected by the knockdown, whereas labeling of the uracil ring was significantly attenuated by PYGB suppression, especially for 2 and 3 ^{13}C atoms entering the uracil ring, which showed a 30-40% reduction in PYGB suppressed cells compared with control cells. Again, the sh479 construct had the greatest effect (Fig. 4.12). The percentage of unlabeled UTP ($^{13}\text{C}_0$ -UTP) was also increased by PYGB suppression, consistent with reduced incorporation of ^{13}C into the uracil ring and with decreased synthesis of its precursor Asp from $^{13}\text{C}_6$ -glucose (cf. Fig. 4.9). A related sugar nucleotide UDP-N-acetylglucosamine (UDP-GlcNAc) showed reduced fractional enrichment in the more extensively labeled isotopologues (e.g. $^{13}\text{C}_{14-16}$ -UDP-GlcNAc in Fig. 4.13) but increased fractional enrichment in the less extensively labeled isotopologues (e.g. $^{13}\text{C}_5$, $^{13}\text{C}_6$, and $^{13}\text{C}_{11}$ -UDP-GlcNAc). Unlabeled UDP-GlcNAc was absent by 24 h. $^{13}\text{C}_5$ and $^{13}\text{C}_6$ -UDP-GlcNAc isotopologues most likely each corresponds to complete enrichment of the ribose and glucosamine subunit, respectively, while $^{13}\text{C}_{11}$ -UDP-GlcNAc likely represents

Figure 4.11. PYGB suppression reduces amino acid synthesis

5 passages after shPYGB suppression, A549 cells were incubated with $^{13}\text{C}_6\text{-glucose}$ for 24 h. Polar metabolites were extracted and measured via GC-MS, and amino acids were quantified and normalized to total protein. Data are represented as the fraction of unlabeled isotopologue divided by the total amount.

Figure 4.12. PYGB suppression reduces uracil synthesis

5 passages after shPYGB suppression, A549 cells were incubated with $^{13}\text{C}_6\text{-glucose}$ for 24 h. Nucleotides were extracted and measured via FT-ICR-MS. Fractional distribution of ^{13}C isotopologues of UTP were quantified and are represented as the fraction of UTP isotopologue in the designated shPYGB construct over that in shENV.

Figure 4.13. Effect of PYGB suppression on UDP-GlcNAc enrichment

5 passages after shPYGB suppression, A549 cells were incubated with $^{13}\text{C}_6$ -glucose for 24 h. Nucleotides were extracted and measured via FT-ICR-MS. UDP-GlcNAc isotopologues were quantified and are represented as the fraction of UDP-GlcNAc isotopologue in the designated shPYGB construct over shENV.

labeling in both subunits. $^{13}\text{C}_{14-16}$ -UDP-GlcNAc isotopologues then reflect labeling in the uracil ring and/or the acetyl unit [92]. Therefore, the reduced labeling in the $^{13}\text{C}_{14-16}$ isotopologues indicates decreased enrichment of the uracil and/or acetyl subunits, consistent with attenuated UTP ring synthesis and Krebs' cycle activity (Fig. 2.18). Finally, $^{13}\text{C}_6$ -glucose incorporation into ATP showed selective attenuation in the fractional enrichment of $^{13}\text{C}_{6-7}$ -ATP relative to $^{13}\text{C}_5$ -ATP with PYGB suppression, again suggesting decreased enrichment in the adenine ring (Fig. 4.14). Carbon in the adenine ring is derived from either Gly or N^{10} -formyl tetrahydrofolate (THF), the latter of which can be synthesized from Gly or Ser and ultimately from glucose (Fig. 4.15). Gly and Ser levels were reduced by PYGB suppression (cf. Figs. 4.10, 4.16), which is consistent with a decrease in their incorporation into the adenine ring and thus, purine synthesis. Taken together, all of the above nucleotide data point to no effect from PYGB suppression on ribose incorporation.

4.4 Conclusions

This study has shown that lung cancer cells have the capacity to synthesize and utilize glycogen in the presence of abundant glucose supply. The expression of the fetal form of glycogen phosphorylase (PYGB) and the phenotypic consequences of its knockdown on A549 cells demonstrated the importance of glycogen utilization for lung cancer cell proliferation, and to some extent tumorigenesis. Parallel SIRM investigations reveal perturbations in the Krebs cycle activity, nucleobase synthesis, and amino acid metabolism that may

Figure 4.14. Effect of PYGB suppression on ATP enrichment

5 passages after shPYGB suppression, A549 cells were incubated with $^{13}\text{C}_6\text{-glucose}$ for 24 h. Nucleotides were extracted and measured via FT-ICR-MS. ATP isotopologues were quantified and are represented as the fraction of ATP isotopologue in the designated shPYGB construct over shENV.

Figure 4.15. Origin of carbon and nitrogen in ATP

Figure 4.16. PYGB suppression reduces Gly and Ser synthesis

5 passages after shPYGB suppression, A549 cells were incubated with $^{13}\text{C}_6$ -glucose for 24 h. Polar metabolites were extracted and measured via GC-MS, and amino acids were quantified and normalized to total protein. Data are represented as the fraction of unlabeled isotopologue divided by the total amount.

underlie the phenotypic changes. These results highlight the need for continued investigation into the role of glycogen metabolism and PYGB in particular in other cancer models and the notion that PYGB is worthy to pursue as a new target for cancer chemotherapeutics. Specifically, the individualized lung cancer models detailed in chapter 2 are ideal candidates for probing the effects of glycogen inhibitors of lung cancer metabolism. We are currently utilizing this approach in our ongoing patient study.

It is worth noting that sh479 had the clearest effect of metabolic disruption linked to A549 growth attenuation and reduced colony formation. This effect was smaller with sh339 suppression and in all regards anomalous with sh010 as its suppression of PYGB was minimal. Furthermore, formation of colonies was reduced by PYGB suppression via sh479, suggesting that PYGB is partially necessary for tumorigenesis, but likely more important in the setting of tumor cell growth and/or survival.

CHAPTER 5-CONCLUSIONS

The central thesis at the core of this study was that the anti-cancer mechanism of selenite could be elucidated by utilizing a metabolic approach. In order to approach this aim SIRM was extensively used to generate a comprehensive profile of the metabolic perturbations that follow when human lung cancer is exposed to selenite by using a combination of patient tissue and cell-based models. These findings were then followed up with complementary methods in order to gain a more finely detailed understanding of the specific pathways that may be targeted by selenite. Indeed a number of pathways were rigorously examined simultaneously using SIRM experimental design, which underscored the viability and necessity of tracer-based design for this level of metabolic detail.

A principal finding in the initial studies was that the activity of the TCA cycle is dramatically reduced by selenite with a concomitant increase in glycolytic rate in a cancer-specific manner. This increase in flux through glycolysis is accompanied by an increase in lactic fermentation and decrease in flux through the pentose phosphate pathway in an effort to meet the compromised energy demands of the rapidly growing cancer cells caused by disruption of aerobic respiration. These alterations had far-reaching effects in nucleotide and hexosamine nucleotide synthesis and utilization. Purine and pyrimidine base

synthesis was profoundly impacted by selenite treatment and, to a lesser extent, ribose synthesis. Decreased synthesis of the bases was traced back to decreased incorporation of Asp, Gly, and Gln carbon and/or nitrogen into the purine or pyrimidine rings due primarily to a compromised Krebs' cycle. This attenuation was propagated in the synthesis of UDP-GlcNAc, which in turn has downstream implications for the regulation of specific protein targets. Indeed, O-GlcNAc modification of proteins was extensively decreased in selenite-treated cells.

Unfortunately, efforts to isolate and further characterize a specific target were met with difficulty. By isolating a protein target one could then reassess the metabolic profile generated from selenite treatment in order to evaluate possible downstream effects of this altered regulatory modification. As O-GlcNAcylation is an area of burgeoning interest and relatively few targets (there are many) have been thoroughly studied, this approach would be a worthwhile continuation of this study, especially in the context of the individualized lung cancer models.

A likely source for the observed selenite-induced perturbations in cancer metabolism is decreased glutaminolysis. Glutaminolysis was rapidly and dramatically inhibited with selenite treatment, and decreased expression of GAC was isolated as a probable source. A plausible mechanism for selenite-induced cytotoxicity was then postulated that underscored the pivotal role of GLS in glutathione synthesis, especially when the cell is compromised by increased ROS and the reduction of GSSG becomes overwhelmed. Restoration of A549 growth upon supplementation with a product of GAC, Glu, was crucial towards

solidifying this hypothesis. Furthermore, GAC expression was unchanged in non-cancer cells, which alludes to a possible mechanism for the cancer-specificity of selenite. Examining whether or not Glu supplementation also reverses ROS accumulation would be a useful corollary for these experiments. Furthermore, assessing the state of ROS with or without Glu supplementation in the individualized cancer models would also generate a more compelling story. Also what is indicated from this investigation is a thorough dissection of the mechanism behind the decrease in GAC expression and why this effect is absent in non-transformed cells.

Finally, an alternative pathway that was affected by selenite treatment, glycogenolysis, was also investigated, albeit less so. Using a combination of shRNA PYGB suppression and tracer-based design, I concluded that glycogen was an important fuel source for lung cancer, and disruption of its utilization led to decreased proliferation as well as tumor formation to a smaller extent. These effects of suppression were measured in the context of a host of other metabolic alterations that included a decrease in TCA cycle activity as well as nucleobase synthesis. These experiments could be greatly augmented by implementation of the individualized lung cancer models and a PYGB inhibitor, which is ongoing.

The use of individualized lung cancer models was integral towards illustrating the translational capabilities of these experiments. A more thorough inclusion of specimens from the ongoing patient study would be an exciting project as there is a wealth of clinical data associated with these models. This clinical information includes factors like the tumor grade, subtype, and

environmental history of the patient from whom it was derived and correlating these data to the metabolic profile would be a worthy endeavor.

Furthermore, these experiments illustrate the need for continued investigation of selenite supplementation for chemopreventive or adjunctive chemotherapeutic potential. These efforts are particularly important in the context of the negative press generated from the failed SELECT trial. Recent analyses of traditional Inuit diets have underscored the potential health benefits of selenium-high diets despite the high levels of mercury and organic pollutants inherent in these food sources [93, 94]. The *in vivo* efficacy of selenite has already been demonstrated by the reduction of tumor growth in rodents (reviewed in [20]). Clearly, these results point towards exciting future prospects for selenium research. Continued accrual and analyses of NSCLC patients as demonstrated would likely bolster these overall conclusions. In the future, it is imperative that the focus of more targeted studies and/or trials involves seleno-compounds with known anti-cancer efficacy over ineffective species, such as selenomethionine.

Finally, exposure to the breadth and quality of scientific analyses possible by taking advantage of NMR, MS, and molecular biological methods has itself been integral for the fruition of this thesis. The opportunities possible for metabolic research in the context of tumor biology and beyond are numerous and exciting, and this study comprises a small portion of that potential.

REFERENCES

1. Howlader N, N.A., Krapcho M, Garshell J, Neyman N, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds), *SEER Cancer Statistics Review, 1975-2010, National Cancer Institute. Bethesda, MD.* 2013.
2. Donna L. Hoyert, J.X., *Deaths: Preliminary Data for 2011.* National Vital Statistics Reports, 2012. **61**(6).
3. Society, A.C., *American Cancer Society: Cancer Facts and Figures 2013.* 2013.
4. Bain, C., et al., *Lung Cancer Rates in Men and Women With Comparable Histories of Smoking.* Journal of the National Cancer Institute, 2004. **96**(11): p. 826-834.
5. Institute, N.C., *Cancer Trends Progress Report-2011/2012 Update.* 2012.
6. Strauss, G.M.J., Ahmedin; McKenna, Michael B.; Strauss, Jordan A.; Cummings, K M., *Lung Cancer Survival in Relation to Histologic Subtype: An Analysis based upon Surveillance Epidemiology and End Results (SEER) Data: B4-06.* Journal of Thoracic Oncology, 2007. **2**(8): p. S345-S346.
7. Wender, R., et al., *American Cancer Society lung cancer screening guidelines.* CA: A Cancer Journal for Clinicians, 2013. **63**(2): p. 106-117.
8. Goldstraw, P., et al., *The IASLC Lung Cancer Staging Project: Proposals for the Revision of the TNM Stage Groupings in the Forthcoming (Seventh) Edition of the TNM Classification of Malignant Tumours.* Journal of Thoracic Oncology, 2007. **2**(8): p. 706-714
9. Shirish M. Gadgeel, S.S.R., Gregory P. Kalemkerian, *Treatment of Lung Cancer.* Radiologic Clinics of North America, 2012. **50**(5).

10. Chambers I, F.J., Goldfarb P, Affara N, McBain W, and Harrison PR, *The structure of the mouse glutathione peroxidase gene: the selenocysteine in the active site is encoded by the 'termination' codon*. EMBO J 1986. **5**: p. 1221-1227.
11. U.S. Department of Agriculture, A.R.S., *USDA National Nutrient Database for Standard Reference, Release 25*. 2012.
12. Ross AC, C.B., Cousins RJ, Tucker KL, Ziegler TR, *Selenium*. Modern Nutrition in Health and Disease, 2012: p. 225-37.
13. Vanderpas, J.B., et al., *Iodine and selenium deficiency associated with cretinism in northern Zaire*. The American Journal of Clinical Nutrition, 1990. **52**(6): p. 1087-93.
14. Taylor, E.W., *Selenium and cellular immunity. Evidence that selenoproteins may be encoded in the +1 reading frame overlapping the human CD4, CD8, and HLA-DR genes*. Biological Trace Element Research, 1995. **49**: p. 85-95
15. Cheng, Y.Y., & Qian, P. C., *The effect of selenium-fortified table salt in the prevention of Keshan disease on a population of 1.05 million*. Biomedical and Environmental Sciences, 1990. **3**: p. 422-428.
16. Sutter, M.E., et al., *Selenium Toxicity: A Case of Selenosis Caused by a Nutritional Supplement*. Annals of Internal Medicine, 2008. **148**(12): p. 970-971.
17. Sung Dae Cho, C.J., Barbara Malewicz, Yan Dong, Charles Y.F. Young, Kyung-Sun Kang, Yong-Soon Lee, Clement Ip, and Junxuan Lu, *Methyl Selenium Metabolites Decrease Prostate-Specific Antigen Expression by Inducing Protein Degradation and Suppressing Androgen-Stimulated Transcription*. Mol Cancer Ther, 2004. **3**(5): p. 605-611.
18. Yan Dong, H.Z., Allen C. Gao, James R. Marshall, and Clement Ip, *Androgen Receptor Signaling Intensity is a Key Factor in Determining the Sensitivity of Prostate Cancer Cells to Selenium Inhibition of Growth and Cancer-Specific Biomarkers*. Mol Cancer Ther, 2005. **4**(7): p. 1047-1055.

19. Yan Dong, S.O.L., Haitao Zhang, James Marshall, Allen C. Gao, and Clement Ip, *Prostate Specific Antigen Expression is Down-Regulated by Selenium Through Disruption of Androgen Receptor Signaling*. Cancer Research, 2004. **64**: p. 19-22.
20. Brozmanová, J., et al., *Selenium: a double-edged sword for defense and offence in cancer*. Archives of Toxicology, 2010. **84**(12): p. 919-938.
21. Ramoutar, R.R. and J.L. Brumaghim, *Effects of inorganic selenium compounds on oxidative DNA damage*. Journal of Inorganic Biochemistry, 2007. **101**(7): p. 1028-1035.
22. Park, S.-H., et al., *Induction of apoptosis and autophagy by sodium selenite in A549 human lung carcinoma cells through generation of reactive oxygen species*. Toxicology Letters, 2012. **212**(3): p. 252-261.
23. Fan, T.W.M., R.M. Higashi, and A.N. Lane, *Integrating Metabolomics and Transcriptomics for Probing Se Anticancer Mechanisms**. Drug Metabolism Reviews, 2006. **38**(4): p. 707-732.
24. Cherukuri, D.P. and M.A. Nelson, *Role of reactive oxygen species (ROS) and JNKs in selenite-induced apoptosis in HepG2 cells*. Cancer Biology & Therapy, 2008. **7**(5): p. 697-698.
25. Ramoutar, R. and J. Brumaghim, *Antioxidant and Anticancer Properties and Mechanisms of Inorganic Selenium, Oxo-Sulfur, and Oxo-Selenium Compounds*. Cell Biochemistry and Biophysics, 2010. **58**(1): p. 1-23.
26. L.C. Clark, B.D., A. Krongrad, G.F. Combs Jr, B.W. Turnbull, E.H. Slate, R. Witherington, J.H. Herlong, E. Janosko, D. Carpenter, C. Borosso, S. Falk and J. Rounder, *Decreased incidence of prostate cancer with selenium supplementation: results of a double-blind cancer prevention trial*. British Journal of Urology, 1998. **81**: p. 730-734.
27. Dunn, B.K., et al., *A Nutrient Approach to Prostate Cancer Prevention: The Selenium and Vitamin E Cancer Prevention Trial (SELECT)*. Nutrition and Cancer, 2010. **62**(7): p. 896 - 918.
28. Zeng, H., M. Wu, and J.H. Botnen, *Methylselenol, a Selenium Metabolite, Induces Cell Cycle Arrest in G1 Phase and Apoptosis via the Extracellular-Regulated Kinase 1/2*

- Pathway and Other Cancer Signaling Genes*. The Journal of Nutrition, 2009. **139**(9): p. 1613-1618.
29. Warburg O, P.K., Negelein E, *Über den Stoffwechsel der Tumoren*. Biochem Z, 1924(152): p. 319-344.
 30. Dwarakanath, B.S. and V. Jain, *Targeting glucose metabolism with 2-deoxy-D-glucose for improving cancer therapy*. Future Oncology, 2009. **5**(5): p. 581-585.
 31. Ganapathy-Kannikappan, S., R. Kunjithapatham, and J.-F. Geschwind, *Anticancer Efficacy of the Metabolic Blocker 3-Bromopyruvate: Specific Molecular Targeting*. Anticancer Research, 2013. **33**(1): p. 13-20.
 32. Clem, B.F., et al., *Targeting 6-Phosphofructo-2-Kinase (PFKFB3) as a Therapeutic Strategy against Cancer*. Molecular Cancer Therapeutics, 2013.
 33. Fan, T., et al., *Altered regulation of metabolic pathways in human lung cancer discerned by ¹³C stable isotope-resolved metabolomics (SIRM)*. Molecular Cancer, 2009. **8**(1): p. 41.
 34. Puzio-Kuter, A.J.L.a.A.M., *The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes*. Science, 2010. **330**: p. 1340.
 35. Semenza, G.L., *Targeting HIF-1 for cancer therapy*. Nature Reviews Cancer, 2003. **3**(10): p. 721.
 36. Chiacchiera, F., et al., *p38[alpha] blockade inhibits colorectal cancer growth in vivo by inducing a switch from HIF1[alpha]- to FoxO-dependent transcription*. Cell Death Differ, 2009. **16**(9): p. 1203-1214.
 37. Christofk, H.R., et al., *Pyruvate kinase M2 is a phosphotyrosine-binding protein*. Nature, 2008. **452**(7184): p. 181-186.
 38. Ralph J. DeBeradinis, A.M., Evgueni Daikhin, Ilana Nissim, Marc Yudkoff, Suzanne Wehrli, Craig B. Thompson, *Beyond aerobic glycolysis: Transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis*. PNAS, 2007. **104**: p. 19345.

39. Yuneva, M.O., et al., *The Metabolic Profile of Tumors Depends on Both the Responsible Genetic Lesion and Tissue Type*. Cell Metabolism, 2012. **15**(2): p. 157-170.
40. Linehan, W.M. and T.A. Rouault, *Molecular Pathways: Fumarate Hydratase-Deficient Kidney Cancer—Targeting the Warburg Effect in Cancer*. Clinical Cancer Research, 2013. **19**(13): p. 3345-3352.
41. Martin G. Belinsky, L.R., and Margaret von Mehren, *Succinate dehydrogenase deficiency in pediatric and adult gastrointestinal stromal tumors*. frontiers in oncology, 2013. **3**: p. 117.
42. Milewski, S., I. Gabriel, and J. Olchowy, *Enzymes of UDP-GlcNAc biosynthesis in yeast*. Yeast, 2006. **23**(1): p. 1-14.
43. O'Donnell, N., et al., *Ogt-Dependent X-Chromosome-Linked Protein Glycosylation Is a Requisite Modification in Somatic Cell Function and Embryo Viability*. Mol. Cell. Biol., 2004. **24**(4): p. 1680-1690.
44. Kreppel, L.K. and G.W. Hart, *Regulation of a Cytosolic and Nuclear O-GlcNAc Transferase*. Journal of Biological Chemistry, 1999. **274**(45): p. 32015-32022.
45. Mi, W., et al., *O-GlcNAcylation is a novel regulator of lung and colon cancer malignancy*. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 2011. **1812**(4): p. 514-519.
46. Caldwell, S.A., et al., *Nutrient sensor O-GlcNAc transferase regulates breast cancer tumorigenesis through targeting of the oncogenic transcription factor FoxM1*. Oncogene, 2010. **29**(19): p. 2831-2842.
47. Wang, Z., A. Pandey, and G.W. Hart, *Dynamic Interplay between O-Linked N-Acetylglucosaminylation and Glycogen Synthase Kinase-3-dependent Phosphorylation*. Molecular & Cellular Proteomics, 2007. **6**(8): p. 1365-1379.
48. Copeland, R.J., J.W. Bullen, and G.W. Hart, *Cross-talk between GlcNAcylation and phosphorylation: roles in insulin resistance and glucose toxicity*. Am J Physiol Endocrinol Metab, 2008. **295**: p. E17-28.

49. Yi, W., et al., *Phosphofructokinase 1 Glycosylation Regulates Cell Growth and Metabolism*. Science, 2012. **337**(6097): p. 975-980.
50. Kang, J.G., et al., *O-GlcNAc Protein Modification in Cancer Cells Increases in Response to Glucose Deprivation through Glycogen Degradation*. Journal of Biological Chemistry, 2009. **284**(50): p. 34777-34784.
51. Zachara, N., et al., *The dynamic stress-induced "O-GlcNAc-ome" highlights functions for O-GlcNAc in regulating DNA damage/repair and other cellular pathways*. Amino Acids, 2011. **40**(3): p. 793-808.
52. Zhang, S., et al., *Modification of histones by the sugar N-acetylglucosamine (GlcNAc) occurs on multiple residues, including H3 serine 10, and is cell cycle regulated*. Journal of Biological Chemistry, 2011.
53. Chou, T.-Y., G.W. Hart, and C.V. Dang, *c-Myc Is Glycosylated at Threonine 58, a Known Phosphorylation Site and a Mutational Hot Spot in Lymphomas*. Journal of Biological Chemistry, 1995. **270**: p. 18961-18965.
54. Yang, W.H., et al., *Modification of p53 with O-linked N-acetylglucosamine regulates p53 activity and stability*. Nat Cell Biol, 2006. **8**(10): p. 1074-83.
55. Moseley, H.N.B., et al., *A novel deconvolution method for modeling UDP-N-acetyl-D-glucosamine biosynthetic pathways based on ¹³C mass isotopologue profiles under non-steady-state conditions*. BMC Biology, 2011. **9**(1): p. 37-52.
56. Lane, A.N., et al., *Isotopomer analysis of lipid biosynthesis by high resolution mass spectrometry and NMR*. Analytica Chimica Acta, 2009. **651**(2): p. 201-208.
57. Lorkiewicz, P., et al., *High information throughput analysis of nucleotides and their isotopically enriched isotopologues by direct-infusion FTICR-MS*. Metabolomics, 2012. **8**(5): p. 930-939.
58. Teresa W.-M. Fan, A.N.L., and Richard M. Higashi, *The Promise of Metabolomics in Cancer Molecular Therapeutics*. Current Opinion in Molecular Therapeutics, 2004. **6**(6): p. 584-592.

59. Teresa W.-M. Fan, A.N.L., *Structure-Based Profiling of Metabolites and Isotopomers by NMR*. Progress in Nuclear Magnetic Resonance Spectroscopy, 2007.
60. Teresa W.-M. Fan, L.L.B., Richard M. Higashi, and Andrew N. Lane, *Metabolomics-Edited Transcriptomics Analysis of Se Anticancer Action in Human Lung Cancer Cells*. Metabolomics, 2005.
61. Yehezkel, G., et al., *O-Linked α -N-Acetylglucosaminylation (O-GlcNAcylation) in Primary and Metastatic Colorectal Cancer Clones and Effect of N-Acetyl- α -d-glucosaminidase Silencing on Cell Phenotype and Transcriptome*. Journal of Biological Chemistry, 2012. **287**(34): p. 28755-28769.
62. Wellen, K.E., et al., *The hexosamine biosynthetic pathway couples growth factor-induced glutamine uptake to glucose metabolism*. Genes & Development, 2010. **24**(24): p. 2784-2799.
63. Donadio, A.C., et al., *Antisense glutaminase inhibition modifies the O-GlcNAc pattern and flux through the hexosamine pathway in breast cancer cells*. Journal of Cellular Biochemistry, 2008. **103**(3): p. 800-811.
64. Raspotnig, G., et al., *Colorimetric Determination of Cell Numbers by Janus Green Staining*. Analytical Biochemistry, 1999. **275**(1): p. 74-83.
65. Le, A., et al., *Glucose-Independent Glutamine Metabolism via TCA Cycling for Proliferation and Survival in B Cells*. Cell metabolism, 2012. **15**(1): p. 110-21.
66. Fan, T.W.-M., *Sample Preparation for Metabolomics Investigation*, in *The Handbook of Metabolomics: Pathway and Flux Analysis, Methods in Pharmacology and Toxicology*. T.W.-M. Fan, A.N. Lane, and R.M. Higashi, Editors. 2012, Springer Science: New York. p. 7-27.
67. Benjamini, Y., Hochberg, Y., *Controlling the false discovery rate: A practical and powerful approach to multiple testing*. Journal of the Royal Statistical Society B, 1995. **57**(1): p. 289-300.

68. Vervoorts, J.r., J. Loescher-Firzlaff, and B. Loescher, *The Ins and Outs of MYC Regulation by Posttranslational Mechanisms*. Journal of Biological Chemistry, 2006. **281**:: p. 34725-34729.
69. Ngoh, G.A., et al., *Non-canonical glycosyltransferase modulates post-hypoxic cardiac myocyte death and mitochondrial permeability transition*. Journal of Molecular and Cellular Cardiology, 2008. **45**(2): p. 313-325.
70. Shoshan-Barmatz, V., et al., *VDAC, a multi-functional mitochondrial protein regulating cell life and death*. Molecular Aspects of Medicine, 2010. **31**(3): p. 227-285.
71. Hines, V. and M. Johnston, *Analysis of the kinetic mechanism of the bovine liver mitochondrial dihydroorotate dehydrogenase*. Biochemistry, 1989. **28**(3): p. 1222-1226.
72. David H Holben, A.M.S., *The diverse role of selenium within selenoproteins: A review*. Journal of the American Dietetic Association, 1999. **99**(7).
73. Kenya Yamaguchi, R.G.U., Julia Pimkina, Peter Makhov, Konstantin Golovine, Paul Crispen, and Vladimir M Kolenko, *Methylseleninic acid sensitizes prostate cancer cells to TRAIL-mediated apoptosis*. Oncogene, 2005. **24**: p. 5868-5877.
74. Shannon Reagan-Shaw, M.N., Haseeb Ahsan, Hasan Mukhtar, and Nihal Ahmad, *Combination of Vitamin E and Selenium Causes an Induction of Apoptosis of Human Prostate Cancer Cells by Enhancing Bax/Bcl-2 Ratio*. The Prostate, 2008. **68**: p. 1624-1634.
75. Aledo, J.C., et al., *Identification of two human glutaminase loci and tissue-specific expression of the two related genes*. Mammalian Genome, 2000. **11**(12): p. 1107-1110.
76. Olalla, L., et al., *Expression of the scaffolding PDZ protein glutaminase-interacting protein in mammalian brain*. Journal of Neuroscience Research, 2008. **86**(2): p. 281-292.
77. Szeliga, M., et al., *Transfection of a human glioblastoma cell line with liver-type glutaminase (LGA) down-regulates the expression of DNA-repair gene MGMT and sensitizes the cells to alkylating agents*. Journal of Neurochemistry, 2012. **123**(3): p. 428-436.

78. Chung-Bok, M.I., et al., *Rat hepatic glutaminase: identification of the full coding sequence and characterization of a functional promoter*. Biochem. J., 1997. **324**(1): p. 193-200.
79. Szeliga, M., et al., *Relative Expression of mRNAs Coding for Glutaminase Isoforms in CNS Tissues and CNS Tumors*. Neurochemical Research, 2008. **33**(5): p. 808-813.
80. Szeliga, M., et al., *Lack of expression of the liver-type glutaminase (LGA) mRNA in human malignant gliomas*. Neuroscience Letters, 2005. **374**(3): p. 171-173.
81. Turner, A. and J.D. McGivan, *Glutaminase isoform expression in cell lines derived from human colorectal adenomas and carcinomas*. Biochem. J., 2003. **370**(2): p. 403-408.
82. ELGADI, K.M., et al., *Cloning and analysis of unique human glutaminase isoforms generated by tissue-specific alternative splicing*. Physiological Genomics, 1999. **1**(2): p. 51-62.
83. Cassago, A., et al., *Mitochondrial localization and structure-based phosphate activation mechanism of Glutaminase C with implications for cancer metabolism*. Proceedings of the National Academy of Sciences, 2012. **109**(4): p. 1092-1097.
84. Gao, P., et al., *c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism*. Nature, 2009. **458**(7239): p. 762-765.
85. Kita, K., T. Suzuki, and T. Ochi, *Diphenylarsinic Acid Promotes Degradation of Glutaminase C by Mitochondrial Lon Protease*. Journal of Biological Chemistry, 2012. **287**(22): p. 18163-18172.
86. Jensen, T.E. and E.A. Richter, *Regulation of glucose and glycogen metabolism during and after exercise*. The Journal of Physiology, 2012. **590**(5): p. 1069-1076.
87. Burwinkel, B., et al., *Mutations in the Liver Glycogen Phosphorylase Gene (PYGL) Underlying Glycogenosis Type VI (Hers Disease)*. The American Journal of Human Genetics, 1998. **62**(4): p. 785-791.
88. Nogales-Gadea, G., J. Arenas, and A.L. Andreu, *Molecular genetics of McArdle's disease*. Current Neurology and Neuroscience Reports, 2007. **7**(1): p. 84-92.

89. Schnier, J.B., et al., *Inhibition of glycogen phosphorylase (GP) by CP-91,149 induces growth inhibition correlating with brain GP expression*. Biochemical and Biophysical Research Communications, 2003. **309**(1): p. 126-134.
90. Marín-Hernández, A., et al., *Modeling cancer glycolysis*. Biochimica et Biophysica Acta (BBA) - Bioenergetics, 2011. **1807**(6): p. 755-767.
91. Newgard, C.B., et al., *Human brain glycogen phosphorylase. Cloning, sequence analysis, chromosomal mapping, tissue expression, and comparison with the human liver and muscle isozymes*. Journal of Biological Chemistry, 1988. **263**(8): p. 3850-3857.
92. Moseley, H., et al., *A novel deconvolution method for modeling UDP-GlcNAc biosynthetic pathways based on ¹³C mass isotopologue profiles under non steady-state conditions*. BMC Biology, 2011. **9**(1): p. 37.
93. Gagne D, B.R., Lauziere J, Vaissiere E, Vezina C, Ayotte P, Dery S, Turgeon O'Brien H., *Traditional food consumption is associated with higher nutrient intakes in Inuit children attending childcare centres in Nunavik*. Int J Circumpolar Health, 2012. **71**: p. 18401.
94. Bjerregaard P, M.G., *The best of two worlds: how the Greenland Board of Nutrition has handled conflicting evidence about diet and health*. Int J Circumpolar Health, 2012. **71**: p. 18588.

APPENDIX

List of abbreviations

TCA cycle: tricarboxylic acid cycle

NMR: nuclear magnetic resonance

MS: mass spectrometry

GC-MS: gas chromatography-mass spectrometry

FT-ICR-MS: Fourier transform-ion cyclotron resonance-mass spectrometry

NSCLC: non-small cell lung cancer

UDP-GlcNAc: uridine diphospho-*N*-acetylglucosamine

NHBE: normal human bronchial epithelial

KGA: glutaminase, kidney isoform

GAC: glutaminase C

ROS: reactive oxygen species

shRNA: small hairpin ribonucleic acid

PYGB: glycogen phosphorylase, brain isoform

COPD: chronic obstructive pulmonary disease

3BP: 3-bromopyruvate

PET: positron emission tomography

mTOR: mammalian target of rapamycin

PTEN: phosphatase and tensin homolog

HIF-1 α : hypoxia-inducible factor-1 α GLS: glutaminase
FH: fumarate hydratase
SQR: succinate dehydrogenase
HLRCC: hereditary leiomyomatosis and renal cell cancer
GIST: gastrointestinal stromal tumors
HBP: hexosamine biosynthetic pathway
OGT: O-GlcNAc transferase
PFK: phosphofructokinase
SIRM: stable isotope-resolved metabolomics
NLST: national lung cancer screening trial
LDCT: low-dose computed tomography
SCLC: small-cell lung cancer
ACS: American Cancer Society
RT: radiotherapy
SBRT: stereotactic body radiotherapy
MSA: methylseleninic acid
SeM: selenomethionine
SELECT: selenium and vitamin E cancer prevention trial

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Education

BS	University of Louisville Graduated <i>Summa Cum Laude</i> Chemistry; Biochemistry concentration Thesis: <i>Metabolomic investigation of selenium interaction with androgen sensitivity in human prostate cancer cells</i> Advisor: Teresa Fan	2008
MD/PhD	University of Louisville	2008-present

Awards

First Place among first years	University of Louisville Research Louisville! Poster session	2008
Third Place	University of Louisville Brown Cancer Center retreat poster session	2011
Hypercube award	University of Louisville Chemistry department award	2008
Honors Scholar award	University of Louisville Successful completion of honors program and defense of thesis	2008

Tuition Award	2011-present
University of Louisville	
Interdisciplinary Programs in Biomedical	
Science scholarship	

Publications

A novel deconvolution method for modeling UDP-GlcNAc biosynthetic pathways based on ¹³C mass isotopologue profiles under non steady-state conditions. Hunter N. B. Moseley, Andrew N. Lane, Alex C. Belshoff, Richard M. Higashi, Teresa W.-M. Fan. BMC Systems Biology. 2011

Sensitivity to sodium arsenite in human melanoma cells depends upon susceptibility to arsenite-induced mitotic arrest. McNeely SC, Belshoff AC, Taylor BF, Fan TW, McCabe MJ Jr, Pinhas AR, States JC. Toxicol Appl Pharmacol. 2008

Poster Abstracts

MD/PhD regional meeting	2012
Louisville, KY	
<i>Sodium Selenite alters metabolic pathways involved in UDP-GlcNAc synthesis in human lung cancer models.</i> Alex Belshoff, Michael Bousamra II, Richard Higashi, Hunter Moseley, Andrew Lane, Teresa Fan.	

Brown Cancer Center retreat	2011
Louisville, KY	
<i>An Investigation of UDP-GlcNAc Synthesis in Human Lung Cancer with Perturbations by Selenium Compound Treatment.</i> <u>Alex Belshoff</u> , Andrew Lane, Hunter Moseley, Michael Bousamra II, Richard Higashi, Teresa Fan.	

Metabolism and cancer conference	2011
Baltimore, MD	
American Association for Cancer Research	
<i>Stable isotope-resolved metabolomic investigations reveal anti-cancer potential of select Se agents vs. the failure of selenomethionine as a chemopreventive agent.</i> <u>Teresa W.-M. Fan</u> , Pawel Lorkiewicz, Alex Belshoff, Richard Higashi, and Andrew N. Lane	

Research Louisville!	2009
Louisville, KY	
<i>Generation of ANAP11 recombinant mutant to investigate As induced mitotic arrest.</i> <u>Alex Belshoff</u> , Christopher States, Apsara Wickramasinghe, Sam States, Matt Zajack	

Summer Research Opportunity Program	2008
Louisville, KY	

Arsenic Detoxification: The Effects of Glutathione Production Inhibition on Cellular Accumulation and the Significance of Arsenic-thiol Conjugates. Alex Belshoff, Sam McNeely, Ben Taylor, Teresa Fan, Alan Pinhas, Christopher States

Research Experience

Graduate student	2010-present
University of Louisville Department of Pharmacology and Toxicology Advisor: Teresa Fan	
Graduate student rotation	2010
University of Louisville Department of Pharmacology and Toxicology Advisor: Christopher States	
Graduate student rotation	2009
University of Louisville Department of Biochemistry and Molecular Biology Advisor: Andrew Lane	
Undergraduate research assistant	2007-2008
University of Louisville Department of Chemistry Advisor: Teresa Fan	
Undergraduate research assistant	2006-2007
University of Louisville Cancer prevention program Department of Pharmacology and Toxicology Advisor: Christopher States	

Extra-curriculars

Phlebotomy lesson	2010
University of Louisville Instructor	
Supplies over seas	2010
University of Louisville Volunteer	
HOPE student clinic	2009-2010
University of Louisville Director	
H3 alliance private practice committee	2009

University of Louisville Volunteer	
Gross Notes student manual University of Louisville Editor	2008
Society of Undergraduate Chemistry Students University of Louisville Secretary	2006-2008
Honors organic lab I and II University of Louisville Undergraduate teaching assistant	2006-2007
National Chemistry Week Louisville Science Museum Volunteer	2005, 2006
Science Olympiad Ballard high school Judge	2006
Freshman guides program University of Louisville Mentor	2006