Metabolic Changes Associated with Acquired Cisplatin Resistance

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UNIVERSITY OF MIAMI

METABOLIC CHANGES ASSOCIATED WITH ACQUIRED CISPLATIN RESISTANCE

By

Elizabeth J. Sullivan

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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METABOLIC CHANGES ASSOCIATED WITH ACQUIRED CISPLATIN RESISTANCE

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Although cisplatin is the drug of choice in treating a variety of different cancers, acquired resistance appears to be a common and serious drawback to its effectiveness in the clinic. Using three pairs of cisplatin-sensitive and resistant cancer cell lines, we have found significantly altered hexokinase (HK) I and II levels in cisplatin-resistant cancer cell lines. Based on our interest in utilizing glucose analogs to probe tumor metabolism, we investigated the role of HKI and HKII in cisplatin-resistant lung and ovarian cancer cell lines and asked whether these resistant cell lines could be targeted with sugar analogs under both normoxic and anaerobic conditions. Furthermore, we sought to determine if alterations in HKs would lead to metabolic reprogramming that could reveal targets in other metabolic pathways to be exploited for therapeutic gain in patients with cisplatin-resistant tumors.

In three different cisplatin-resistant cancer cell lines, we observed lower HKII protein than in the cisplatin-sensitive cancer cell lines from which they were derived. In cisplatin-resistant lung cancer cell lines HKI was also lower, and lower HK isoforms resulted in increased sensitivity to the glycolytic inhibitors 2-deoxyglucose (2-DG) and 2-fluorodeoxyglucose (2-FDG) under anaerobic and hypoxic conditions. This increased sensitivity to 2-DG and 2-FDG correlated with a significant decrease in lactate and ATP production in the presence of these glycolytic inhibitors. Knockdown of HKI or HKII
individually with siRNA in cisplatin-sensitive lung cancer cell lines lead to a significant increase in 2-FDG induced cell death under anaerobic conditions, however knockdown of both HKI and HKII with a single novel siRNA sequence yielded the greatest increase in cell death. Furthermore, blockage of alternate metabolic pathways, such as fatty acid oxidation and glutaminolysis, resulted in cell death under normal oxygen conditions in cisplatin-resistant lung cancer cell lines. When expanding our findings into a cisplatin-resistant ovarian cancer cell pair, we found decreased HKII expression in cisplatin-resistant A2780cis correlated with increased sensitivity to 2-DG under anaerobic conditions.

Overall, our results indicate that lowered hexokinase levels in two cisplatin-resistant lung cancer cell lines leads to increased sensitivity to glycolytic inhibition under anaerobic and hypoxic conditions. This increased sensitivity to glycolytic inhibitors correlates with a decrease in lactate and ATP production in the presence of 2-DG under anaerobic conditions. By lowering HK levels in cisplatin-sensitive cell lines, we observed a significant increase in 2-FDG mediated cell death under anaerobic conditions. Moreover, the cisplatin-resistant lung cancer cells with lower HK were sensitive to either fatty acid β-oxidation inhibition or glutamine deprivation under normoxia. When expanding the study to include cisplatin-resistant ovarian cancer cell lines, we found lower HKII levels correlated with increased sensitivity to 2-DG under anaerobic conditions. Thus, utilizing different metabolic inhibitors is effective in inducing cell death selectively in cisplatin-resistant cancer cells in vitro.
DEDICATION

This thesis is dedicated to my father Gerald Sullivan for all of his love and support throughout our 26 years together.
ACKNOWLEDGEMENTS

The following thesis was made possible by the support of numerous people in the Molecular Cell and Developmental Biology program. First, I would like to acknowledge my supervisor Dr. Theodore Lampidis for his support during my tenure in his laboratory as well as during my first year in the program. I would also like to thank Dr. Lampidis for providing me the opportunity to work on this particular project, and for allowing me the freedom to learn how to survive as an independent scientist. Additionally, I would like to thank my committee chair, Dr. Vinata Lokeshwar, for her guidance and encouragement during this process and for continuously pushing me to be a better scientist. Also, I would like to thank my committee members Dr. Diana Lopez and Dr. Mike Xu for their enthusiasm and support of both my work and myself. I could not have completed this thesis without any of their support and I am grateful for having the opportunity to learn from such a group of distinguished scientists. I would also like to thank past and present graduate directors, Dr. Pedro Salas and Dr. Nevis Fregien respectively, for their help and support during the highs and lows of my tenure in this program. I feel fortunate to have learned so much from them. Additionally, I would like to thank Maria Penton for all of her kindness and time talking with me throughout the last six years.

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Sullivan and my aunt Diane Orens, whom despite only knowing a short time provided me with so much knowledge and inspiration that I carry with me today. I cannot thank enough my friend, Vilma Rivera, who experienced the highs and lows of my work the last six years along side of me and helped me keep going when I was struggling.

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xi</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Cancer overview and treatment</td>
<td>1</td>
</tr>
<tr>
<td>1.2 The discovery of cisplatin and its structure</td>
<td>2</td>
</tr>
<tr>
<td>1.3 Metal compounds and medicine: laying the groundwork for metal</td>
<td>4</td>
</tr>
<tr>
<td>chemotherapy</td>
<td></td>
</tr>
<tr>
<td>1.4 Mechanism of cisplatin’s anti-tumor activity</td>
<td>7</td>
</tr>
<tr>
<td>1.5 The Warburg Effect and glucose metabolism in cancer</td>
<td>10</td>
</tr>
<tr>
<td>1.6 The role of hexokinase in cancer</td>
<td>12</td>
</tr>
<tr>
<td>1.7 Targeting HK: introduction to sugar analogues</td>
<td>17</td>
</tr>
<tr>
<td>1.8 Project rationale</td>
<td>18</td>
</tr>
<tr>
<td>2 MATERIALS AND METHODS</td>
<td>20</td>
</tr>
<tr>
<td>2.1 Materials</td>
<td>20</td>
</tr>
<tr>
<td>2.1.1 Cell lines</td>
<td>20</td>
</tr>
<tr>
<td>2.1.2 Compounds and antibodies</td>
<td>20</td>
</tr>
<tr>
<td>2.2 Methods</td>
<td>21</td>
</tr>
<tr>
<td>2.2.1 Models of hypoxia</td>
<td>21</td>
</tr>
<tr>
<td>2.2.2 Cytotoxicity assay</td>
<td>21</td>
</tr>
<tr>
<td>2.2.3 Immunoblot</td>
<td>22</td>
</tr>
<tr>
<td>2.2.4 Lactate assay</td>
<td>22</td>
</tr>
<tr>
<td>2.2.5 siRNA transfection</td>
<td>23</td>
</tr>
<tr>
<td>2.2.6 siRNA sequence selection</td>
<td>23</td>
</tr>
<tr>
<td>2.2.7 ATP assay</td>
<td>24</td>
</tr>
<tr>
<td>2.2.8 Statistics</td>
<td>24</td>
</tr>
<tr>
<td>3 LOWER HK LEVELS IN CISPLATIN-RESISTANT LUNG CANCER CELL LINES LEADS TO INCREASED SENSITIVITY TO GLYCOLYTIC INHIBITORS UNDER ANAEROBIC CONDITIONS</td>
<td>25</td>
</tr>
<tr>
<td>3.1 Cisplatin-resistant lung cancer cell lines that express low HK display increased sensitivity to 2-DG under chemically-induced anaerobic conditions</td>
<td>25</td>
</tr>
<tr>
<td>3.2 Cisplatin-resistant lung cancer cell lines that express low HK display increased sensitivity to 2-FDG under chemically induced anaerobic conditions</td>
<td>27</td>
</tr>
<tr>
<td>3.3 2-DG blocks lactate production more effectively in cisplatin-resistant lung cancer cell lines under anaerobic conditions</td>
<td>28</td>
</tr>
<tr>
<td>3.4 Glycolytic inhibitors 2-DG and 2-FDG block ATP production more efficiently under anaerobic conditions</td>
<td>29</td>
</tr>
</tbody>
</table>
3.5 Under anaerobic conditions reducing HK levels by siRNA in cisplatin sensitive cell lines induces 2-FDG mediated cell death. 31

4 UNDER HYPOXIA, CISPLATIN-RESISTANT LUNG CANCER CELL LINES CAN BE TARGETED UTILIZING GLYCOLYTIC INHIBITORS 35
4.1 Glycolytic inhibitors are more cytotoxic in cisplatin-resistant lung cancer cell lines under hypoxic conditions. 35
4.2 Increased cell death in cisplatin-resistant lung cell lines under hypoxia correlates with decreased lactate and ATP production. 38

5 PRECLINICAL TESTING OF HK INHIBITOR GSK X IN SMALL CELL LUNG CANCER CELL LINES 41
5.1 GSK compound X is effective at targeting cisplatin-resistant lung cancer cell line SR2 under anaerobic conditions. 41
5.2 Under hypoxia, GSK compound X is effective at targeting cisplatin resistant lung cancer cell line SR2. 43

6 TARGETING ALTERNATE METABOLIC PATHWAYS IS TOXIC IN CISPLATIN-RESISTANT LUNG CANCER CELL LINES UNDER NORMOXIA 45
6.1 Inhibition of fatty acid β-oxidation correlates with cell death in cisplatin-resistant lung cancer cell line SR2. 45
6.2 Deprivation of glutamine leads to cell death in cisplatin-resistant non-small cell lung cancer cell line NSCLCSC. 47

7 ALTERED HK EXPRESSION IN OVARIAN CANCER CELLS CAN BE EXPLOITED WITH GLYCOLYTIC INHIBITORS UNDER ANAEROBIC CONDITIONS 50
7.1 Lower HKII expression in cisplatin-resistant ovarian cancer cell line A2780cis correlates with increased sensitivity to 2-DG and 2-FDG under anaerobic conditions. 50
7.2 2-DG and 2-FDG reduce ATP production more efficiently in cisplatin-resistant A2780cis under anaerobic conditions. 53

8 ELEVATED HIF AND HK EXPRESSION UNDER HYPOXIA CORRELATES WITH RESISTANCE TO 2-DG IN OVARIAN CANCER CELL LINES 54
8.1 Under hypoxia, restored HK levels in cisplatin-resistant A2780cis correlates with resistance to 2-DG. 54
8.2 Under hypoxia, 2-DG and 2-FDG do not significantly decrease ATP production in ovarian cancer cell lines. 56

9 TARGETING ALTERNATE METABOLIC PATHWAYS UNDER NORMOXIA IS INEFFECTIVE IN CISPLATIN-RESISTANT OVARIAN CANCER CELL LINE A2780CIS 57
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.1 Inhibition of fatty acid β-oxidation is not cytotoxic in cisplatin-resistant ovarian cancer cell lines</td>
<td>57</td>
</tr>
<tr>
<td>9.2 Glutamine deprivation does not lead to cell death in ovarian cancer cell lines</td>
<td>58</td>
</tr>
<tr>
<td>9.3 Transfection of A2780cis with siRNA targeting HKI yields increased cell death after 72 hours</td>
<td>59</td>
</tr>
<tr>
<td>10 DISCUSSION</td>
<td>61</td>
</tr>
<tr>
<td>WORKS CITED</td>
<td>66</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure Content</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2.1 Structure of Magnus’ Salt compared to cisplatin</td>
<td>2</td>
</tr>
<tr>
<td>1.2.2 Structures of cis- and transplatin</td>
<td>3</td>
</tr>
<tr>
<td>1.4 The activity of cisplatin in vivo</td>
<td>8</td>
</tr>
<tr>
<td>1.6.1 Description of the HK family of enzymes</td>
<td>13</td>
</tr>
<tr>
<td>1.6.2 Description of pathways that require glucose</td>
<td>14</td>
</tr>
<tr>
<td>1.6.3 The effects of blocking glycolysis with 2-DG under normoxia and hypoxia</td>
<td>16</td>
</tr>
<tr>
<td>1.7 The metabolism of 2-DG and 2-FDG compared to glucose in a cell</td>
<td>18</td>
</tr>
<tr>
<td>3.1 Cisplatin-resistant lung cancer cell lines that express low HK display increased sensitivity to 2-DG under chemically induced anaerobic conditions</td>
<td>26</td>
</tr>
<tr>
<td>3.2 Cisplatin-resistant lung cancer cell lines display increased sensitivity to 2-DG under chemically induced anaerobiosis</td>
<td>27</td>
</tr>
<tr>
<td>3.3 2-DG blocks lactate production more effectively in cisplatin-resistant lung cancer cell lines under anaerobic conditions</td>
<td>28</td>
</tr>
<tr>
<td>3.4 Glycolytic inhibitors 2-DG and 2-FDG block ATP production more efficiently under anaerobic conditions</td>
<td>30</td>
</tr>
<tr>
<td>3.5 Under anaerobic conditions reducing HK levels by siRNA in cisplatin sensitive cell lines induces 2-FDG mediated cell death</td>
<td>33</td>
</tr>
<tr>
<td>4.1 Glycolytic inhibitors are more cytotoxic in cisplatin-resistant lung cancer cell lines under hypoxia</td>
<td>36</td>
</tr>
<tr>
<td>4.2 Increased cell death in cisplatin-resistant cells under hypoxia correlates with decreased lactate and ATP production</td>
<td>39</td>
</tr>
<tr>
<td>5.1 GSK X is cytotoxic in cisplatin-resistant SR2 under anaerobic conditions</td>
<td>42</td>
</tr>
<tr>
<td>5.2 Under hypoxia, GSK X effectively targets cisplatin-resistant cell line SR2</td>
<td>44</td>
</tr>
<tr>
<td>6.1 Inhibition of fatty acid β-oxidation correlates with cell death in cisplatin-resistant lung cancer cell line SR2</td>
<td>46</td>
</tr>
<tr>
<td>6.2 Deprivation of glutamine leads to cell death in cisplatin-resistant lung cancer cell line NSCLCSC</td>
<td>49</td>
</tr>
<tr>
<td>7.1 Lower HKII expression in cisplatin-resistant ovarian cancer cell line A2780cis correlates with increased sensitivity to 2-DG and 2-FDG under anaerobic conditions</td>
<td>52</td>
</tr>
<tr>
<td>7.2 Under anaerobiosis, 2-FDG is effective at targeting cisplatin-resistant ovarian cancer cell line A2780cis</td>
<td>53</td>
</tr>
<tr>
<td>8.1 Under hypoxia, restored HK levels in cisplatin-resistant ovarian cancer cell line A2780cis correlates with resistance to 2-DG</td>
<td>55</td>
</tr>
<tr>
<td>8.2 Under hypoxia, 2-DG and 2-FDG do not significantly decrease ATP production in ovarian cancer cell lines</td>
<td>56</td>
</tr>
<tr>
<td>9.1 Inhibition of fatty acid β-oxidation is not cytotoxic in cisplatin-resistant ovarian cancer cell line A2780cis</td>
<td>58</td>
</tr>
<tr>
<td>9.2 Glutamine deprivation does not lead to cell death in A2780cis</td>
<td>59</td>
</tr>
<tr>
<td>9.3 Transfection of A2780cis with siRNA targeting HKI yields increased cell death after 72 hours</td>
<td>60</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Content</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Table depicting the name and drug sensitivity of cell lines used in this thesis</td>
<td>20</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-DG</td>
<td>2-Deoxy-d-Glucose</td>
</tr>
<tr>
<td>2-DG-6-P</td>
<td>2-Deoxy-d-Glucose-6-Phosphate</td>
</tr>
<tr>
<td>2-FDG</td>
<td>2-Deoxyfluoro-d-Glucose</td>
</tr>
<tr>
<td>2-FDG-6-P</td>
<td>2-Deoxyfluoro-d-Glucose-6-Phosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>CPT-1a</td>
<td>Carnitine Palmitoyltransferase 1 Isoform a</td>
</tr>
<tr>
<td>CPT-1b</td>
<td>Carnitine Palmitoyltransferase 1 Isoform b</td>
</tr>
<tr>
<td>CPT-1c</td>
<td>Carnitine Palmitoyltransferase 1 Isoform c</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>G-6-P</td>
<td>Glucose-6-Phosphate</td>
</tr>
<tr>
<td>GLS</td>
<td>Glutaminase</td>
</tr>
<tr>
<td>GLS2</td>
<td>Glutaminase Isoform 2</td>
</tr>
<tr>
<td>GLUTs</td>
<td>Glucose Transporters</td>
</tr>
<tr>
<td>GK</td>
<td>Glucokinase or Hexokinase Isoform IV</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia Inducible Factor 1</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia Inducible Factor 1 Subunit α</td>
</tr>
<tr>
<td>HK</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>HKI</td>
<td>Hexokinase Isoform I</td>
</tr>
<tr>
<td>HKII</td>
<td>Hexokinase Isoform II</td>
</tr>
<tr>
<td>HKIII</td>
<td>Hexokinase Isoform III</td>
</tr>
<tr>
<td>HKIV</td>
<td>Hexokinase Isoform IV or Glucokinase</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non Small Cell Lung Cancer</td>
</tr>
<tr>
<td>OxPhos</td>
<td>Oxidative Phosphorylation</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PGI</td>
<td>Phosphoglucoisomerase</td>
</tr>
<tr>
<td>Pt</td>
<td>Platinum</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SCLC</td>
<td>Small Cell Lung Cancer</td>
</tr>
<tr>
<td>si-Dual</td>
<td>siRNA sequence targeting both HKI and HKII</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic Acid Cycle or Kreb’s Cycle</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage Dependent Anion Channel</td>
</tr>
<tr>
<td>VDAC1</td>
<td>Voltage Dependent Anion Channel Isoform 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Cancer overview and treatment

Every year in the United States there are over 500,000 estimated deaths from cancer, with over 1.5 million new diagnoses (1). Lung cancer is the third most common cancer in both men and women in the United States, and more people die of lung cancer than from breast, colon, and prostate cancer combined (2). Lung and bronchus cancer represents approximately 13.7% of new diagnoses in the United States each year (2). Despite decades of biomedical research aimed at understanding the mechanisms underling the transition from normal somatic cells to transformed malignancies and in developing viable therapies to eradicate these abnormal cells, researchers and clinicians still struggle to find new strategies that mediate durable clinical responses. Standard of care treatment options for this deadly disease include a combination of surgical, radiation and chemotherapy regimens. While in select cases these options can be curative, a typical oncology treatment protocol places selection pressure on the tumor cell and supporting stromal populations such that surviving cells are those that have elaborate traits that render them resistant to treatment. Biomedical research is tasked with the challenge to both identify means by which the efficacy of the current gold standard treatment modalities can be enhanced as well as to uncover new mechanisms of malignancy and resistance to cancer treatment that can be nodes for therapeutic intervention. Despite these efforts the treatment options for inoperable cancers that exhibit intrinsic or acquired resistance to one treatment mainstay, chemotherapy, remain substantially limited in nature. One particular first line treatment for cancer in which resistance is observed or
induced is the chemotherapy drug cisplatin, a member of the widely used platinum (Pt) family of chemotherapeutics.

1.2 The discovery of cisplatin and its structure

Cisplatin (cis-diamminedichloroplatinum II) is used as a first line treatment in a multitude of cancers, including small cell (SCLC) and non-small cell lung cancer (NSCLC), bladder, testicular, ovarian, head and neck cancers (3). The history of cisplatin is an interesting one: beginning with the first documentation of its synthesis by the Italian chemist named Michel Peyrone in 1844, who named the compound “Peyrone’s Chloride” (4-7). Peyrone’s chloride was synthesized by Peyrone after he began an exhaustive line of work with platinum (Pt) compounds in an agricultural lab at the University of Geissen in Germany, focusing in particular on the compound called Magnus’ green salt (4-7). Magnus’ green salt was the first discovered platinum ammine compound (4-7). Peyrone’s chloride was synthesized when Peyrone added excess ammonia to the solution while synthesizing Magnus’ green salt. Here he noted two products, the green salt and a second unexpected yellow product with properties different from the green salt, yet for intellectual reasons refused to consider the possibility that they were isomers (Fig 1.2.1).

![Structural formulas](image)

**Fig 1.2.1 Structure of Magnus’ Salt compared to cisplatin. Adapted from (4).**
Peyrone’s discovery was before the advent of modern coordination chemistry, and while he had an idea the compound was related to Magnus’ green salt, he was not completely able to interpret his results due to limited techniques. After decades of debate regarding the structure of Peyrone’s chloride, in 1890 Alfred Werner elucidated its square planar configuration and differentiated cisplatin from trans-diamminedichloroplatinum, or transplatin (Fig 1.2.2) (5-8). Werner ultimately won the Nobel Prize in 1913 for his work on “the linkage of atoms… especially in inorganic chemistry” (9). After these discoveries, cis and transplatin isomers were relegated to obscurity.

![Fig 1.2.2 Structures of cis- and trans- platin (\([\text{Pt } \text{II} \text{Cl}_2 (\text{NH}_3)_2]\)) and schematic representations of DNA adducts. From (10).](image)

The story of cisplatin in biomedical research next picks up in the 1960s, where platinum was initially used as an electrode in a continuous culture system in a laboratory at the new biophysics department at Michigan State University to investigate how electrical impulses influenced cell growth. Platinum was widely believed to be inert in biological systems at the time, making it a suitable substrate for conducting electricity in order to address this question. Initial tests of this culturing system used the relatively easy to grow \(E. \text{coli}\) as a cell model instead of the more costly, challenging and adherent mammalian cell culture systems, and it was in 1965 that Barnett Rosenberg observed a
decline in bacteria density using this system when a voltage drop was applied across the electrodes (11). Interestingly, once the electrodes were turned off the bacterial density rapidly recovered, indicating a resumption of bacterial growth. Rosenberg was able to determine that the growth inhibitory responses he observed in bacteria, *i.e.* filamentation and growth arrest as assessed by optical density, were due to electrolysis from the platinum electrodes used in this system. It appeared that some compound in the nutrient broth requisite for bacterial growth was interacting with the platinum electrodes since broth exposed to electrodes through which a current had been run was also cytotoxic to bacteria in the absence of persistent current. After isolating and testing these electrolytes, Rosenberg and his group determined the +2 Pt compounds cisplatin and transplatin were among a group of platinum compounds with interesting biological activity. The choice of platinum electrodes for Rosenberg’s experiments turned out to be a fortuitous one.

1.3. **Metal compounds and medicine: laying the groundwork for metal chemotherapy**

The use of metal compounds in medicine has long been performed but only recently been documented and rigorously studied, particularly in regards to the treatment of bacterial infections (12). Briefly, Paul Erhlich made significant contributions to the field and has long been considered the founder of chemotherapy. By Erhlich’s definition, chemotherapy utilizes a well-defined molecular species with affinity for a pathogen or deleterious agent to induce its selective attenuation without causing injury to the host. One aspect of Ehrlich’s prolific scientific career was devoted to screening small molecule compounds obtained from the German dye industry in order to diagnose and treat
bacterial pathogens (12). Some of Erhlich’s studies focused on African trypanosomes as the target and trypan red as the chemotherapeutic. Indeed, this model system establish the proof of principle for the “magic bullet” theory of treatment, i.e. the creation of a drug only targeting a specific pathogen (12,13). Erhlich also worked with numerous arsenic-containing compounds, particularly compound 606, that was used to target syphilis, a significant threat to public health in Europe at the time due to its immediate (e.g. blindness) and delayed (e.g. neurosyphilis) effects. Once debilitating side effects were documented with compound 606, several modifications were made to this molecule in order to maintain efficacy and decrease problematic consequences of arsenic therapy (12, 13).

Building on Ehrlich’s work, Francis P. Dwyer conducted numerous studies in the 1950s on the effects of inorganic metal compounds, including platinum compounds, on biological systems (14). However, it was not until 1965 that Rosenberg’s lab was testing the effect of simpler metal compounds on bacteria. By 1968, Rosenberg and his lab were able to synthesize and test the cisplatin isomer, which was used as a model for numerous metal compounds with this bacteriocidal activity. Work completed not only by Rosenberg’s lab but others detailed that complexes that were potent inducers of lysis in bacteria also were active as potent anticancer agents against cultured mammalian tumor cells (5-7, 15). The results obtained from this simple bacteriocidal model system allowed biomedical research to focus on a bigger problem in medicine: the lack of potent and effective chemotherapeutics to specifically target cancer cells, i.e. the cancer “magic bullet” first envisioned by Ehrlich.
This work culminated with a paper publication in Nature detailing the potential antitumor effects of platinum compounds in two different animal models of cancer in 1969 (15). With cisplatin as the lead compound for platinum-based chemotherapy, extensive in vitro and in vivo testing of this drug in numerous tumor models systems was conducted. Cisplatin was active against a number of models including transplantable, viral and chemical carcinogen tumor models in mice (6, 7, 15). Such in vivo studies provided sufficient preclinical evidence to justify a significant clinical trial that ultimately led to Food and Drug Administration (FDA) approval for use in patients in 1978, at which time the measure of success of a drug was tumor response rate. The original FDA approval in 1978 was for limited use in testicular and ovarian cancer, but over time this has expanded to include several other cancers, including bladder cancer, lung cancer and osteosarcomas, based on clinical efficacy. The initial successes of cisplatin in patients and the subsequent approval by the FDA lead to a whole new field of chemotherapeutic compounds, and the search for compounds with less potent side effects. Cisplatin over time has been shown to cause some dose-limiting toxicities, including ototoxicity and nephrotoxicity, necessitating investigation into safer derivative compounds (16-19). More recently approved platinum compounds named carboplatin and oxaliplatin, approved in 1989 and 2002 respectively, are both potent platinum-based chemotherapeutics that are used clinically today with milder side effects than cisplatin (16-19).

While overall response to cisplatin is initially positive in certain forms of cancer, cisplatin resistance is frequently observed and remains a substantial clinical problem. Cancer cells resistant to cisplatin are typically slow growing and are not sensitive to other commonly employed chemotherapeutics that target rapidly dividing cells. Currently, the
therapeutic options for platinum resistant cancer are severely limited, representing an attractive opportunity to discover novel approaches to target this resistant population of cells. Extensive research is continuing to identify not only the mechanisms of resistance to this drug, but also to further elucidate cisplatin’s mechanism of action upon entering the cell.

1.4 Mechanism of cisplatin’s anti-tumor activity

Based on the initial experiments briefly described and its profound effects observed upon bacterial growth, cisplatin was hypothesized to interact with and interfere with the function of the most abundant negatively charged molecule inside cells: deoxyribonucleic acid (DNA). With the advent of molecular biology, cisplatin was shown to directly make adducts with DNA (18-23). Thus, cisplatin is classically viewed as a DNA damaging agent that interferes with DNA synthesis, which occurs rapidly in dividing tumor cells and is the basis for a major therapeutic window between normal and malignant cells. When administered to patients, cisplatin is suspended in a saline solution in order to maintain maximum efficacy (5-7). Indeed, it has been shown that if cisplatin is solubilized in the polar solvent dimethylsulfoxide (DMSO), its effectiveness is reduced because of the formation of cisplatin-DMSO adducts (23). Furthermore, cisplatin-DMSO adducted compounds have different pharmacodynamic and pharmacokinetic properties than cisplatin solubilized in saline. This highlights the importance of utilizing saline for patients receiving cisplatin clinically, and the importance of proper suspension of cisplatin for use in preclinical studies.
Clinically, cisplatin is administered by a slow intravenous injection and in systemic circulation it retains its chloride ions in the bloodstream, since the chloride content of blood is sufficiently high to favor this equilibrium (5-7). Cisplatin then enters the cell through a poorly elucidated process. In addition to passive diffusion it is believed that cell surface copper transporters facilitate cisplatin entry, most likely due to similar molecular diameters and charges between cisplatin and copper atoms (24). Upon entering a cell, where the chloride ion concentration is much lower than systemic circulation, the cisplatin molecule undergoes a process called aquation, where the two chlorine atoms are replaced with hydrogen bonded water molecules, thereby leaving the platinum with a +2 charge (Fig 1.4). The Pt +2 charge facilitates interaction with DNA due to the negatively charged phosphodiester backbone of this polymer. Most commonly, intra-strand adducts between adjacent guanine residues on DNA have been documented (17-22).

Fig 1.4 The activity of cisplatin in vivo. Upon entering the cell, cisplatin loses each Cl molecule through aquation. This renders a +2 charge on the Pt molecule, which then can interact with the negatively charged cellular DNA. Adapted from (5).
Although cisplatin is initially effective at tumor debulking, resistance to cisplatin is observed after continued treatment, representing a significant clinical challenge for patients receiving this drug as a cancer therapy. Understanding the cellular mechanisms that confer primary and metastatic tumors with resistance to cisplatin may aid in the identification of new molecular targets for the development of clinically relevant and synergistic approaches for chemotherapy. Interestingly, more recent studies have expanded cisplatin’s interactions to include adducts with a variety of cellular components, including but not limited to: phospholipids, ribonucleic acid (RNA), cellular thiol compounds such as glutathione, cytosolic membranes, mitochondrial proteins and mitochondrial DNA (18-22, 25). In fact, the greatest amount of cisplatin adduct formation was observed with the mitochondrial pore protein VDAC1 (Voltage Dependent Anion Channel 1) than with other cellular components (25). Furthermore, knockdown of VDAC1 in cells by either siRNA or genetic methods renders them resistant to cisplatin and if VDAC1 is re-expressed in VDAC1-null cells they become re-sensitized to cisplatin’s cytotoxic effects in vitro (26).

The voltage dependent anion channel is an outer mitochondrial membrane protein that plays roles in the mechanisms of both mitochondrial and cellular metabolism, as well as in the initiation of apoptosis (25, 26). The two isoforms of the glycolytic enzyme hexokinase (HK) localize at the VDAC, and utilize ATP released from the mitochondria to phosphorylate glucose in order to confer a negative charge to it in order to trap this sugar in the cell for further processing (27, 28). Additional research has detailed the effects of cisplatin on glucose metabolism in the cell, finding a reduction in the activity of the enzyme HK upon cisplatin exposure (29, 30). Collectively, these results highlight the
fact that cisplatin treatment has multiple effects on a cell beyond nuclear DNA adduct formation, and that cellular metabolism may be greatly affected by cisplatin treatment. Although interaction of cisplatin with molecules other than DNA would, in earlier times, be interpreted as an off-target effect, this more promiscuous binding may be important for therapeutic efficacy and is now an area of interest for biomedical researchers. This is a salient point because differences in metabolic processes have been documented in cancer cells compared to normal tissue, and if cellular metabolism is fundamentally altered or affected by cisplatin treatment this may afford a new strategy for clinical exploitation using combinatorial strategies.

1.5 The Warburg Effect and glucose metabolism in cancer

One of the first major steps in understanding tumor cell metabolism and the mechanisms by which it differs from normal cells was accomplished by the Nobel Laureate Otto Warburg. Otto Warburg was a German biochemist who held degrees in both chemistry and medicine and devoted his life toward the study of cancer, particularly in the area of cellular metabolism (31, 32). His work culminated in the discovery of elevated lactic acid production exhibited by several different tumor cells types in both normoxic and hypoxic conditions (31, 32). This finding was in complete contrast to the well established Pasteur effect, where yeast cells consume less sugar when O₂ is present and as a result will produce less lactic acid. Interestingly, the respiratory rate of these tumor cells was not compromised by this elevated lactic acid production. This work resulted in Otto Warburg’s seminal publication detailing the Hallmark of Cancer now commonly referred to as the Warburg Effect (31, 32).
Warburg’s publication opened up a new field of research: exploring metabolic changes that occur during oncogenic transformation. With the advent of molecular biology, the field of tumor metabolism has undergone a renewal of interest with particular attention devoted to alterations in glucose metabolism. More recently, changes in glucose metabolism have been attributed to cellular oncogene activation and this metabolic shift recognized as requisite for cancer development (33, 34). In their first publication in 2000, Hanahan and Weinberg detailed the following characteristics displayed by cancer: 1) cellular immortality, 2) evasion of programmed cell death, 3) angiogenic capabilities, 4) uncontrolled proliferation, 5) resistance to microenvironment growth inhibitory signaling, 6) invasion and metastasis (35). In addition to these accepted characteristics, an updated list of Hanahan and Weinburg’s Hallmarks of Cancer in 2011 now includes altered glucose metabolism, i.e. The Warburg Effect, as a fundamental characteristic of cancer cells in general (36).

It is now well appreciated that cancer cells, unlike normal non-transformed tissues, rely on glycolysis, or sugar breakdown, to generate ATP to fuel cellular processes, and this fundamental difference serves as the basis for a commonly used and informative clinical test, the positron emission tomography (PET) scan. The PET scan exploits this Hallmark of Cancer by utilizing a bolus of labeled sugar molecules administered by intravenous injection into a patient. A substantial portion of this material will enrich in cancerous cells relative to their normal counterparts. In the most commonly applied application of the PET scan, a sugar analog containing a radioactive fluorine isotope, 2-deoxy-2-(\(^{18}\)F)fluoro-D-glucose (2-\(^{18}\)FDG), is removed from systemic circulation by tumor cells by active transport via glucose transporters (GLUTs) and phosphorylated by
the first enzyme in glycolysis, HK. HK effectively traps glucose into the cell by utilizing ATP as a donor for the negative charge conferred by phosphate to phosphorylate the carbon at the sixth position of the 6-carbon sugar ring, thereby preventing the glucose molecule from freely diffusing in and out the cell. Sugar analogues such as 2-FDG or 2-deoxy-D-glucose (2-DG) are recognized and phosphorylated by HK, but are not able to further proceed down the glycolytic pathway.

Therefore these sugar analogues build up in a cell and ultimately disrupt glycolysis. In the PET scan, $2^{18}$FDG builds up in the cell and releases positrons from the fluorine isotope that are used to determine both primary tumor size and the presence of distant metastatic lesions. This powerful technology relies on active HK enzymes in cancer cells for its efficacy. Interestingly, after a regimen of cisplatin chemotherapy for cancer treatment, radiolabeled $2^{18}$FDG trapping in tumor lesions was decreased, indicative of either a direct or indirect effect of cisplatin on cellular metabolism (37, 38).

1.6 The role of hexokinase in cancer

While there are many steps involved in glucose metabolism, a critical enzymatic reaction required to retain an uncharged glucose molecule intracellularly is accomplished by the previously mentioned HK family of enzymes. There are four isoforms of HK identified in normal tissue: HKI, HKII, HKIII and HKIV (Fig 1.6.1). Briefly, HKIV is also called glucokinase and is the 50kDa liver isoform. It is thought that the other three isoforms of HK arose from the duplication and fusion of glucokinase, since HKI, HKII and HKIII are each 100 kDa in size (27, 28). HKI is considered the ubiquitous isoform and is particularly important in the brain, while HKII and HKIII are more specifically
expressed in certain tissues (Fig 1.6.1) (28). Of these isoforms, only HKI and HKII localize near the outer mitochondrial membrane (28).

<table>
<thead>
<tr>
<th>Human gene locus</th>
<th>HKI</th>
<th>HKII</th>
<th>HKIII</th>
<th>HKIV (GK)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW (kDa)</td>
<td>10k22</td>
<td>2p13</td>
<td>5q35.2</td>
<td>7p15.1–3</td>
</tr>
<tr>
<td>Kₘ for Glic (mM)</td>
<td>~100</td>
<td>~100</td>
<td>~100</td>
<td>~50</td>
</tr>
<tr>
<td>Kₘ for ATP (mM)</td>
<td>0.030</td>
<td>0.300</td>
<td>0.003a</td>
<td>6</td>
</tr>
<tr>
<td>Glc-6-P inhibition (Kᵢ – mM)</td>
<td>0.5</td>
<td>0.7</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Pᵢ, Attenuation of Glc-6-P inhibition</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td>–</td>
</tr>
<tr>
<td>Insulin regulation</td>
<td>–</td>
<td>+</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>Mitochondrial binding</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Major tissue expression</td>
<td>Brain, kidney</td>
<td>Muscle, adipose</td>
<td>Lung, spleen</td>
<td>Liver, pancreas</td>
</tr>
</tbody>
</table>

**Fig 1.6.1 Description of the hexokinase family of enzymes, and where they are located in normal tissue. From 28.**

It is not surprising that HKI and HKII have been found to be located in association with the outer membrane of mitochondria, the organelles responsible for oxidative phosphorylation (Oxphos), since ATP is required by HKs to catalyze the first step in intracellular glucose metabolism and is produced in large quantities by the mitochondria via OxPhos (28, 39). Specifically, HKI and HKII have been shown to localize with the protein VDAC on the outer mitochondrial membrane (28, 39). This is thought not only to aid in the phosphorylation of glucose by providing HKs with physical access to ATP, but also to help prevent cancer cell apoptosis by inhibiting cytochrome C release and thereby blocking the association of proapoptotic signals such as Bax and Bak with mitochondria (39-43).

As previously mentioned, HK is the first enzyme in the glycolytic pathway, and is responsible for trapping the freely diffusible glucose inside the cell by attaching a phosphate group to the sixth carbon of the molecule (Fig 1.6.2). Once glucose is phosphorylated to form glucose-6-phosphate (G-6-P), not only can it be broken down in the glycolytic pathway to generate ATP, but it can also be utilized by the pentose phosphate shunt to facilitate the synthesis of the 5 carbon ribose rings comprising DNA
and RNA bases. Furthermore, glucose is also crucial for post-translational glycosylation of proteins, which are important not only for rapidly cycling cells but also for generating cell-surface receptors and secreted proteins suitable to interact with their cognate receptors such as cytokines and growth factors.

**Fig 1.6.2** The phosphorylation of glucose by hexokinase supplies not only glycolysis, but also the pentose phosphate shunt, glyconeogenesis and n-linked glycosylation. From 28.

The most important HK isoform that has been reported to be involved in increased glucose metabolism in tumor cells is HKII (39, 44-48). HKII is the only isoform of the HK family to have two catalytically active sites to phosphorylate glucose, and is typically upregulated in cancer cells while HKI has only one catalytic site, and its role in cancer cell metabolism is less well understood (28, 49). HKII has been extensively studied and characterized in a hepatocellular carcinoma model of cancer (39, 44-48).
What is unique about hepatocellular carcinomas is that they express amplified levels of HKII, and to some extent HKI, while normal hepatocytes express HKIV (glucokinase) but not HKII (39, 44-48). This fact, combined with the ease with which primary tumor cells can be isolated from a large, easily accessible organ such as the liver, provided a unique and robust system to study this enzyme and determine its importance in cancer. It was ultimately discovered that indeed mitochondria associated HKs, particularly HKII, played multi-faceted roles in oncogenesis (43-48). HKII protein expression is altered by several known oncogenes and tumor suppressors, such as AKT and p53 (28, 45). Furthermore, HKI and HKII’s interaction with the VDAC is now thought to be a major part of not only metabolic signaling cascades, but also in apoptosis.

Interestingly, HKI and HKII also can be upregulated in low oxygen tumor microenvironments by the transcription factor hypoxia inducible factor or HIF (50, 51). As tumor cells proliferate in vivo, the need for increased access to vasculature for nutrients becomes critical. While tumor cells activate pathways such as vascular endothelial growth factor (VEGF) in order to increase angiogenesis to facilitate oxygen delivery from systemic circulation in response to a decrease in oxygen partial pressure due to the biophysical constraints of an increased tumor burden, cells in energy and oxygen restricted environments also can activate a family of transcription factors known as hypoxia inducible factors, or HIFs (51, 52). HIF transcription factors target genes that aid in survival under nutrient and oxygen deprived conditions. Cells living under hypoxic conditions will predominantly rely on glycolysis for ATP, because the electron transport chain in the mitochondria utilizes O₂ to generate ATP (Fig 1.6.3). HKs are important for cell survival under low oxygen tension, when other metabolic sources cannot be utilized,
and are well-known target genes of the transcription factor HIF due to HIF binding sites in the promoter region of these genes. In this regard, it was previously reported that HK, specifically HKII, may have a role in HIF-1 mediated resistance to glycolytic inhibition under hypoxia (50).

Fig 1.6.3. The effects of blocking glycolysis at the level of HK with 2-DG under normoxia and hypoxia. From 53.
1.7 Targeting HK: introduction to sugar analogues

Since HK plays an important role in tumor cell metabolism, it may be an attractive target for chemotherapy. Two such compounds demonstrated to inhibit HK are the glucose analogs 2-DG and 2-FDG. 2-DG was first identified in the 1950s as a compound that is indeed related to glucose, and ultimately discovered to block glucose metabolism at the level of HK as well as the downstream glycolytic enzyme, phosphoglucoisomerase or PGI (53, 54). Upon entering the cell, 2-DG and 2-FDG can be recognized by HK and phosphorylated to 2-DG-6-phosphate (2-DG-6-P) and 2-FDG-6-phosphate (2-FDG-6-P) respectively (Fig 1.7). 2-DG-6-P and 2-FDG-6-P are not recognized by PGI and accumulate in the cell. Most of the early studies involving this compound utilized it as a tool to study the biological effects of insulin, a hormone secreted by beta cells of the pancreas in response to elevated levels of circulating glucose (54, 55). Initial reports focused on 2-DG-6-P competing with glucose-6-phosphate, or G-6-P, for access to PGI in the cell but additional research also found that 2-DG-6-P can inhibit HK (55).

Fig 1.7 The metabolism of 2-DG and 2-FDG compared to glucose in a cell. Upon entering a cell, 2-DG, 2-FDG as well as glucose are phosphorylated by hexokinase to yield 2-DG-6-phosphate (2DG-6-P), 2-FDG-6-phosphate (2FDG-6-P), and glucose-6-phosphate (G-6-P) respectively. While G-6-P is utilized by the cell, 2DG-6-P and 2FDG-6-P cannot be broken down further. Adapted from 56.
Indeed, 2-DG differs only from glucose in that it lacks a hydroxyl (-OH) group at the number 2 carbon position. In 2-FDG, the –OH group is replaced with an electronegative fluorine atom, which more closely matches the electronegative characteristics of the native hydroxyl group. Because of this property, 2-FDG is a better glycolytic inhibitor compared to 2-DG, functionally reducing lactate levels approximately threefold more efficiently than 2-DG (53). Interestingly, another name for 2-DG is 2-deoxymannose and is therefore able to block oligosaccharide formation by interfering with N-linked glycosylation (57, 58). The functional consequences of 2-DG interference with N-linked glycosylation have been described (57, 58). The focus of this dissertation work is on the impact of the glycolytic inhibitors of 2-DG and 2-FDG on cisplatin resistance.

1.8 Project rationale

The overall purpose of this dissertation is to investigate metabolic changes that have been induced by acquired cisplatin resistance in lung and ovarian cancer cells that potentially may be exploited for therapeutic gain clinically. While screening cisplatin-
resistant cells for metabolic reprogramming, we have identified several sensitivities that distinguished the resistant cells from their cisplatin-sensitive counterparts. Given that many tumors elaborate resistance to standard of care chemotherapeutics as a consequence of persistant treatment regiments, there is a pressing clinical need for alternative or complementary ways of both enhancing the efficacy of such therapies as well as exploiting different sensitivities to achieve durable cures. Utilizing a variety of metabolic inhibitors as tools targeting different pathways, we investigated these changes and have shown that it is possible to target cisplatin-resistant cancer cell lines under three different conditions: normal oxygen tension, anaerobic and hypoxia conditions.
Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Cell lines

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>Cisplatin-sensitive Cell Line</th>
<th>Cisplatin-resistant Cell Line</th>
<th>Adriamycin Resistant Cell Line</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCLC</td>
<td>SCLC1</td>
<td>SR2</td>
<td>None</td>
</tr>
<tr>
<td>NSCLC</td>
<td>NSCLCS</td>
<td>NSCLCSC</td>
<td>None</td>
</tr>
<tr>
<td>OVCAR</td>
<td>A2780</td>
<td>A2780cis</td>
<td>A2780adr</td>
</tr>
</tbody>
</table>

Table 2.1 Table depicting the name and drug sensitivity of cell lines used in this thesis.

The SCLC, SCLC1 and SR2, and NSCLC, NSCLCS and NSCLCSC, cell pairs were a kind gift from Dr. Niramol Savaraj and have previously been characterized (59). Briefly, cisplatin-resistant SR2 and NSCLCSC were generated by exposure of SCLC1 and NSCLCS respectively to increasing concentrations of cisplatin *in vitro*. The OVCAR cell lines were purchased from Sigma Aldrich, and the resistant lines were generated by repeated exposure to increasing concentrations of either cisplatin or adriamycin. Resistant cell lines were exposed to adriamycin and cisplatin weekly to maintain resistance.

2.1.2 Compounds and antibodies

2-DG, 2-FDG, oligomycin, and etomoxir were all purchased from Sigma Aldrich. RPMI 1640 media and Trypsin 0.25% were purchased from Invitrogen. Anti-HKI and anti-
HKII primary antibodies were purchased from Cell Signaling (Danvers, MA). β-actin primary mouse antibody (AC-15) was purchased from Sigma Aldrich (St. Louis, MO). Anti-HIF-1α primary antibody was purchased from BD Biosciences (San Jose, CA). Anti-CPT1a, GLS, and GLS2 primary antibodies were purchased from Abcam (Cambridge, MA).

2.2 Methods

2.2.1 Models of hypoxia

Two models of hypoxia, chemical and environmental, were utilized and previously described by our laboratory [60, 61]. The chemical model of anaerobiosis uses oligomycin at 0.1 µg/mL and the environmental model uses a Pro-Ox in vitro hypoxia chamber set to 0.5% O₂ (Reming Bio-instruments).

2.2.2 Cytotoxicity assay

Cells were seeded in 24-well plates, and cultured in normal oxygen tension for 24 hours. Cells were then treated with escalating doses of 2-DG or 2-FDG and placed in either normal oxygen conditions or anaerobic/hypoxic conditions as indicated for 24 hours. For etomoxir and glutamine deprivation experiments, cells were incubated for 72 hours under normal oxygen conditions. After incubation, the cells were trypsinized, combined with respective media and centrifuged at 400g. The pellets were then re-suspended in Hanks’ Balanced Sal Solution (HBSS) and analyzed with a Vi-cell cell
viability analyzer (Beckman Coulter, Fullerton CA) based on trypan blue exclusion. All cytotoxicity graphs are representative experiments that were repeated in triplicate.

2.2.3 Immunoblot

Cells were seeded in 6 well plates under normal oxygen tension for 24 hours and then cultured under the indicated treatment conditions for another 24 hours until cells were 70-75% confluent. The cells were then harvested and collected to prepare whole cell lysates using lysis buffer (100 mM Tris-HCL at pH 7.4, 1% SDS, phosphatase inhibitor cocktail 2 and protease inhibitor cocktail-Sigma Aldrich). Protein concentrations of cell lysates were determined using the MicroBCA Protein Assay according to manufacturer’s directions (Pierce Biotechnology). Equal amounts of protein were loaded per well into 4-15% gradient Tris-HCL gels (BioRad), and lysates were resolved as previously described (50). All western blot images are representative images of triplicate experiments unless indicated.

2.2.4 Lactate assay

Cells were seeded for 24 hours in 24-well culture dishes and then treated with the indicated drugs for either 6 or 24 hours. Following treatment, 0.5 mL of medium from each sample was removed and combined with 1 mL of perchlorate (8%) to remove proteins from medium. Each sample was centrifuged 3x at 1500 x g for 10 minutes. Lactic acid concentration was measured by adding 0.025 mL of the final clear supernatant to a reaction mixture as previously described (50). The OD of each sample in was individually recorded at 340 nm. Cells were trypsinized, combined with the
remaining medium, and centrifuged at 400g. All lactate measurements were standardized to the viable cell count, which was assessed by trypan blue exclusion.

2.2.5 siRNA transfection

Cells were seeded in 12-welled culture plates for 24 hours in antibiotic free RPMI. Cell were transfected using a control pGL3 (Gaussia luciferase) or ON-TARGETplus SMARTpool siRNA (Dharmacon Co., Lafayette, CO) against human HKI or HKII using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer’s protocol. 50 nM of siRNA was transfected in both experimental and control conditions. 48 hours post transfection, cells were treated as indicated for 24 hours and collected for cytotoxicity experiments and immunoblot. Immunoblot was used to verify efficacy of siRNA transfection for each experiment.

2.2.6 siRNA sequence selection

An siRNA sequence targeting both HKI and HKII mRNA transcripts was designed as previously described (62). Briefly, at least two different siRNA design algorithms from Thermo/Dharmacon and siRNA scales were queried and 19-mer antisense sequences with complete homology to HKI and HKII were identified. The most promising candidate from both algorithms was ordered from IDT as a 19-mer with 3’ 2’ nucleotide overhangs where nucleotides are modified at the 2’ position with an O-methyl group. The candidate sequence (si-Dual) was then tested for efficacy by transfection and immunoblotting for HKI and HKII compared to a similar modified control siRNA sequence against luciferase.
2.2.7 ATP assay

Cells were seeded into a 96 well plate and allowed to attach for 24 hours. Media was then removed and cells were then treated with indicated compounds for 6 hours. To measure cellular ATP levels, the Promega CellTiter-GLO kit was used according to manufacturer’s instructions (Madison, WI). ATP levels were quantified in a Wallac Victor2 Multilabel Counter luminometer. Triplicate samples were averaged, and treated conditions were graphed as percent of untreated controls.

2.2.8 Statistics

For each experiment, triplicate samples were used and averages. Error bars indicate standard deviation from the mean. To determine statistically significant differences between experimental and control conditions an unpaired Student’s t test was used (p<0.05). Data were graphed using GraphPad Prism.
Chapter 3: Lower HK levels in cisplatin-resistant lung cancer cell lines leads to increased sensitivity to glycolytic inhibitors under anaerobic conditions

3.1 Cisplatin-resistant lung cancer cell lines that express low HK display increased sensitivity to 2-DG under chemically-induced anaerobic conditions

While evaluating the metabolic profile of cisplatin-resistant lung cancer cell lines we observed lower HKI and HKII protein levels in cisplatin-resistant small cell (SCLC) and non small cell (NSCLC) lung cancer cell lines in vitro as compared to their respective parental cell lines from which they were derived that were initially sensitive to cisplatin (Fig 3.1.A). Previously we reported that tumor cells under three distinct models of anaerobiosis are hypersensitive to glycolytic inhibitors such as 2-DG and 2-FDG, which have been shown to interact with HK (60). Here we investigated whether the lower HK found in cisplatin-resistant cells rendered them sensitive to glycolytic inhibition under normoxic or anaerobic conditions. To mimic anaerobic conditions favoring glycolysis without activating HIF, we used the Complex V/ATP synthase inhibitor oligomycin. Treatment with oligomycin alone did not alter HK expression (Fig 3.1.B). Under normoxia, when other metabolites are available for ATP besides glucose, there was no significant cytotoxicity observed in either SCLC or NSCLC cell lines (Fig 3.1.C and 3.1.D). For the cisplatin-sensitive SCLC1 and cisplatin-resistant SR2, a 24 hour treatment with the glycolytic inhibitor 2-DG induced marked cytotoxicity in SR2 under anaerobic conditions at 6 mM and 12 mM doses while displaying no significant toxicity in the parental cell line (Figure 3.1.C.).
Fig 3.1 Cisplatin-resistant lung cancer cell lines express lower HKI and HKII protein under (A) normoxia and (B) anaerobic conditions. Immunoblot showing HKII and HKI protein levels in cisplatin-sensitive and resistant cell lines under normoxia. β-Actin was used as a loading control. (C) 2-DG is selectively cytotoxic in cisplatin-resistant SR2 under anaerobic conditions at 6 mM and 12 mM. *, p=0.010; **, p<0.0001. (D) 2-DG is cytotoxic in cisplatin-resistant NSCLCSC at 12 mM under anaerobic conditions. #, p=0.0002.

Similarly, sensitivity to 2-DG under anaerobic conditions was found in a cisplatin-resistant non-small cell lung cancer line, NSCLCSC, at 12 mM, albeit somewhat less than what was found at this dose in SR2 (Fig 3.1.D). Nonetheless, finding the same pattern of sensitivity in a second uniquely derived cisplatin-sensitive/resistant cell pair indicates that this phenomenon is less likely to be an artifact of clonal selection of the
SCLC cell pair and more likely to be a common collateral metabolic alteration in response to cisplatin.

3.2 Cisplatin-resistant lung cancer cell lines that express low HK display increased sensitivity to 2-FDG under chemically induced anaerobic conditions

Using the more potent inhibitor of glycolysis, 2-FDG, we found that significant cell death was induced in both cisplatin-resistant cell lines at 6 mM and 12 mM under anaerobic conditions again with little or no cell death in the respective cisplatin-sensitive parental cell lines (Fig 3.2.A and 3.2.B). Moreover, as anticipated, 2-FDG induced cell death at lower concentrations than 2-DG in SR2 (3 mM) and in NSCLCSC (6 mM) (Fig 3.2.C and 3.2.D). Under normoxia no significant death was observed in either cell pair when treated with either 2-DG or 2-FDG. Collectively these results indicate that low HK protein expression renders cisplatin-resistant lung cancer cells sensitive to glycolytic inhibition under anaerobic conditions, which suggests an effective means of targeting difficult to kill chemoresistant tumor cells with analogs of glucose.

Fig 3.2  (A) 2-FDG effectively targets cisplatin-resistant SR2 under anaerobic conditions at 3 mM, 6mM, and 12 mM. *, p=0.006; **, p<0.0001. (B) 2-FDG
effectively targets cisplatin-resistant NSCLCSC under anaerobic conditions at 6 mM and 12 mM. #, p=0.0001; ##, p <0.0001.

3.3 2-DG blocks lactate production more effectively in cisplatin-resistant lung cancer cell lines under anaerobic conditions

To measure the consequences of 2-DG treatment in cisplatin-resistant cells, a lactate assay was used as a functional readout of glycolytic activity. We hypothesized that 2-DG would effectively block lactate production more in cell lines expressing low HK, since 2-DG is a competitive inhibitor of glucose for the catalytic site(s) in this enzyme. A sub-lethal dose of 2-DG (3 mM) was used to avoid any artifacts observed from dying cells. Indeed, under normoxic and anaerobic conditions, 2-DG was found to inhibit lactate production more in the cisplatin-resistant SR2 cell line than in its cisplatin-sensitive parental counterpart SCLC1 after 6 and 24 hours (Fig 3.3.A and 3.3.B).
Fig 3.3 2-DG inhibits lactate production in cisplatin-resistant cells more efficiently than in cisplatin-sensitive cell lines under anaerobic conditions. (A) After 6 hours, 2-DG effectively blocks lactate production in cisplatin-resistant SR2 under normoxia and anaerobiosis. *, p =0.0023; **, p =0.005. (B) After 24 hours, 3 mM of 2-DG blocks lactate production in cisplatin-resistant SR2 under normoxic and anaerobic conditions. *, p=0.0029; **, p = 0.0180. (C) 6 hour treatment of 2-DG significantly blocks lactate production in cisplatin-resistant NSCLCSC under normoxia. #, p = 0.014. (D) 24 hour treatment with 3 mM 2-DG attenuates lactate production under anaerobic conditions in cisplatin-resistant NSCLCSC. #, p = 0.0226.

In the NSCLC pair, under anaerobic conditions it took 24 hours before a difference in inhibition of lactate could be detected between cisplatin-sensitive and resistant cells (Fig 3.3.C and 3.3.D). The slightly higher HKII levels in the NSCLCSC cell line compared to cisplatin-resistant SR2 might explain this result. Moreover, these data are in accordance with the higher doses of 2-DG required to kill this cisplatin-resistant cell line than SR2 under anaerobic conditions as noted above. Our data showing that 2-DG is more effective at blocking lactate production, and therefore glycolysis, in both cisplatin-resistant cell lines correlate well with increased sensitivity to 2-DG under anaerobic conditions in these cell lines.

3.4 Glycolytic inhibitors 2-DG and 2-FDG block ATP production more efficiently under anaerobic conditions

To support the previously presented data showing significantly decreased lactate production in cisplatin-resistant cell lines exposed to glycolytic inhibitors under
anaerobic conditions, an assay was used to measure ATP production. First, all four lung cancer cell lines were exposed to either 2-DG or 2-FDG under normoxia for 6 hours and then ATP production was measured. Similarly to the lactate experiment, a sublethal dosage of 2-DG and 2-FDG was used to avoid any artifacts in the experiment. Under normoxia, when alternate metabolic substrates such as fats or proteins are present in the media, blocking glycolysis with 3 mM of 2-DG and 2-FDG did not significantly effect on ATP production in cisplatin-resistant lung cancer cell lines expressing low levels of HK protein (Fig 3.4.A). This may indicate that these cell lines utilize other substrates, such as fatty acids or glutamine, for ATP production. Interestingly, ATP production was decreased more in cisplatin-sensitive cell lines. This may be a result of activation of the unfolded protein response pathway (UPR) induced by 2-DG. 2-DG is a mannose mimetic as previously stated, and as a result can also interfere with N-linked glycosylation, activating UPR under normoxia (56, 57).

Fig 3.4 (A) Under normoxia, 2-DG and 2-FDG lowers ATP levels more effectively in SCLC1 and NSCLCS, but not in cisplatin-resistant SR2 and NSCLCSC. **, p <0.0001, ##, p<0.0001, *, p= 0.0002, #, p= 0.0492. (B). 2-DG and 2-FDG block ATP
production more effectively in cisplatin-resistant SR2 and NSCLCSC under anaerobic conditions, **, p <0.0001, #, p = 0.0065, *, p = 0.0009 ##, p = 0.0001.

To further probe the functional consequences of lower HK expression in cisplatin-resistant cells, ATP production was measured in these four lung cancer cell lines under anaerobic conditions. We observed significantly decreased ATP production in cisplatin-resistant cell lines in the presence of 2-DG and 2-FDG under anaerobic conditions compared with the parental cisplatin-sensitive cell lines that express higher levels of HKI and HKII (3.4.B). Furthermore, the more potent glycolytic inhibitor, 2-FDG, was more effective than 2-DG in lowering ATP levels. These data correlate with our previously observed decrease in lactate production and increased cell death under anaerobic conditions in these cell lines.

3.5 Under anaerobic conditions reducing HK levels by siRNA in cisplatin-sensitive cell lines induces 2-FDG mediated cell death

To directly assess the importance of HKI and HKII levels in sensitivity to glycolytic inhibition under anaerobic conditions, cells were transfected with small interfering RNA (siRNA) targeting either HKI or HKII and evaluated 2-FDG cytotoxicity compared to cells transfected with a control siRNA targeting luciferase. Luciferase siRNA was used as a control because luciferase is a protein not normally expressed in mammalian cells. 2-FDG was used to address this question since it is a more potent inhibitor of glycolysis compared to 2-DG. In the parental cisplatin-sensitive cell line, SCLC1, knockdown of either HKII or HKI (Fig 3.5.A) lead to a significant increase in cytotoxicity under anaerobic conditions in cells treated with 2-FDG compared to those
transfected with a control siRNA against luciferase after 24 hours (Fig 3.5.B). There was no detectable 2-FDG induced cell death under normoxia in SCLC1 cells transfected with either HKI or HKII siRNA, which correlates to what is observed in the cisplatin-resistant SR2 cell line. Furthermore, when this experiment was repeated using the cisplatin-sensitive parental cell line NSCLCS, siRNA-mediated reduction of either HKI or HKII similarly resulted in 2-FDG induced cytotoxicity under anaerobic conditions (Fig 3.5.C and 3.5.D). Taken together, these data support the hypothesis that lower HK levels render cancer cells more sensitive to glycolytic inhibition under anaerobic conditions.

However, the amount of 2-FDG induced cytotoxicity in cisplatin-sensitive lines transfected with either HKI or HKII siRNA was not as great as what was observed in cisplatin-resistant cell lines in which both isoforms of HK were reduced. This result led to the idea that since there is similar sequence homology of HKI and HKII it was likely that a single siRNA duplex (si-Dual) could be designed against conserved genetic regions of the mRNA transcripts for these isoforms to determine if dual inhibition of HKI and HKII would enhance the cytotoxicity of glycolytic inhibitors. Indeed, transfection of SCLC1 and NSCLCS with the si-Dual, downregulated HKI and HKII expression to levels comparable to what is shown with the siRNAs targeting either alone (Fig 3.5.A and Fig 3.5.C). Most importantly, transfection of both SCLC1 and NSCLCS with the si-Dual showed an enhancement of 2-FDG induced cytotoxicity under anaerobic conditions than when either HKI or HKII alone were blocked (Fig 3.5.B and Fig 3.5.D). Collectively, these data highlight the importance of both isoforms of HK, HKI and HKII, in maintaining tumor cell viability under anaerobic conditions.
Fig 3.5 Under anaerobic conditions reducing HK levels by siRNA in cisplatin-sensitive cell lines increases 2-FDG induced cytotoxicity. (A). Immunoblot showing protein expression of HKII and HKI in the presence of siRNA targeting luciferase, HKI only, HKII only, or both HKI and HKII. β-Actin was used as a loading control. (B). SCLC1 was transfected with siRNA targeting luciferase, HKI, HKII or HKI and HKII. Cells were treated 48 hours post-transfection as indicated. 72 hours post transfection, cells were collected for immunoblot and cytotoxicity experiments. Under anaerobic conditions, 12 mM FDG treatment is effective in inducing cell
death in SCLC1 cells transfected with siRNA targeting HKI, HKII, or both HKI and HKII. Transfection with siRNA targeting luciferase alone was not cytotoxic. *, p < 0.0001. (C). Immunoblot showing protein expression of HKII and HKI in the presence of siRNA targeting luciferase, HKI only, HKII only, or both HKI and HKII. β-Actin was used as a loading control. (D). NSCLCS cells were transfected with siRNA targeting luciferase, HKI, HKII or HKI and HKII. Cells were treated 48 hours post-transfection as indicated. 72 hours post-transfection, cells were collected for immunoblot and cytotoxicity experiments. Under anaerobic conditions, 12 mM FDG treatment is effective in inducing cell death in NSCLCS cells transfected with siRNA targeting HKI, HKII, or both HKI and HKII. Transfection of siRNA targeting luciferase alone was not cytotoxic in NSCLCS. ##, p<0.0001; #, p=0.0001.
Chapter 4: Under hypoxia, cisplatin-resistant lung cancer cell lines can be targeted utilizing glycolytic inhibitors

4.1 Glycolytic inhibitors are more cytotoxic in cisplatin-resistant lung cancer cell lines under hypoxic conditions

Solid tumors in vivo contain hypoxic regions that are comprised of slowly growing tumor cells that are resistant to standard chemotherapy treatments that are aimed at killing rapidly dividing cells. Similar to treatment with oligomycin, under hypoxia cells become dependent on glycolysis for survival. In the hypoxic regions of a tumor in vivo, cells express HIF-1 to upregulate survival genes, such as HK, to adapt to this physiological stress. Our lab previously showed that HIF-1 upregulates HKII and that might be a mechanism of resistance to 2-DG under hypoxia (50). Based on these findings, the question asked was whether cisplatin-resistant cell lines were able to upregulate HK levels under hypoxic conditions (0.5% O₂) in vitro in a HIF-1 dependent manner. Both cisplatin-resistant cell lines tested increased not only HKII protein under hypoxia, but also HKI (Fig 4.1.A). However, HKII protein levels are not increased to the same level that is observed in cisplatin-sensitive parental cell lines.

Fig 4.1 Glycolytic inhibitors are more cytotoxic in cisplatin-resistant lung cancer cell lines expressing low HK under hypoxia. All cells were treated with indicated doses of 2-DG or 2-FDG and placed under hypoxic conditions, 0.05% O₂. (A.) Immunoblot showing protein expression of HIF-1α, HKII, and HKI under normoxia and hypoxia in lung cancer cell lines. β-Actin was used as a loading control. (B). Quantification of HKII protein levels normalized to β-Actin. Cisplatin-resistant cell line values are normalized to the cisplatin-sensitive parental cell line.
(C). Quantification of HKI protein levels normalized to β-Actin. Cisplatin-resistant cell line values are normalized to the cisplatin-sensitive parental cell line. D). 24 hour 2-DG treatment under hypoxia selectively targets cisplatin-resistant SR2. *, p=0.0016; **, p = 0.0020; ***, p <0.001. (E). 24 hour 2-DG treatment under hypoxia selectively targets cisplatin-resistant NSCLCSC. #, p =0.0048; ##, p <0.0001. (F). 2-FDG is more cytotoxic in cisplatin-resistant SR2 under hypoxic conditions. *, p<0.001; **, p =0.003. (G). 2-FDG is cytotoxic in cisplatin-resistant NSCLCSC under hypoxic conditions. #, p = 0.0250; ##, p<0.0001; ###, p =0.0012
To evaluate the therapeutic relevance of glycolytic inhibitors against cisplatin-resistant cell lines under hypoxia, cells were treated with glycolytic inhibitors 2-DG and 2-FDG and then placed under hypoxic conditions for 24 hours. Interestingly, both SCLC and NSCLC cisplatin-resistant cell lines were significantly sensitive to 2-DG under hypoxia despite induction of HIF-1 (Fig 4.1.B, 4.1.C). When using the potent glycolytic inhibitor 2-FDG, there was a significant difference in cell death under hypoxic conditions between cisplatin-sensitive and resistant cell lines (Fig 4.1.D and 4.1.E). Agreeing with the results with chemically-induced anaerobiosis, both SCLC and NSCLC cisplatin-resistant cell lines displayed significant sensitivity to both 2-DG and 2-FDG under hypoxia despite HIF-1 activity. These data clearly demonstrate that cisplatin-resistant lung cancer cells under hypoxic conditions are particularly vulnerable to glycolytic inhibitors.
4.2 Increased cell death in cisplatin-resistant cell lines under hypoxia correlates with decreased lactate and ATP production

To determine the functional consequences of 2-DG treatment under hypoxia, lactate levels in cisplatin-sensitive and resistant lung cancer cell lines were assayed. Under hypoxic conditions, untreated cisplatin-resistant cell lines produced less lactate than their cisplatin-sensitive counterparts. These results suggest that although upregulation of HIF-1 and subsequent increased HK levels were detected, this was not sufficient to increase lactate production to levels observed in the cisplatin-sensitive parental lines (Fig 4.2.A and 4.2.B). Furthermore, 2-DG and 2-FDG were found to be more effective at inhibiting lactate production under hypoxia in cisplatin-resistant cell lines than in the cisplatin-sensitive parental cell lines. It should be noted that these experiments, as those above where cytotoxic effects are assayed, were performed within 24 hours of initiation of the hypoxic conditions. Therefore, under this short time frame the delay in HK reaching high enough levels to have dampening effects on the effectiveness of glycolytic inhibitors in either lowering lactate levels or decreasing their cytotoxicity may not have been sufficient. Nonetheless, the data clearly demonstrate that decreased HK expression in cisplatin-resistant cell lines correlates with decreased lactate production indicating that glycolytic activity is reduced in these cell lines.

Next, ATP production was measured in these four lung cancer cell lines under hypoxic conditions. As previously noted, cells were treated as indicated prior to initiation of hypoxia (0.5% O₂). A significant reduction in ATP production was observed for cisplatin-resistant cells in the presence of 2-DG and 2-FDG under hypoxia compared with the parental cisplatin-sensitive cell lines that express higher levels of HKI and HKII
Furthermore, the more potent glycolytic inhibitor, 2-FDG, was more effective than 2-DG at lowering ATP levels. These data correlate with our previously observed decrease in lactate production and increased cell death under hypoxia in these cell lines, however future siRNA experiments under hypoxia are necessary to conclusively state that the observed sensitivities are a result of the differences in HK protein expression.

Fig 4.2 Glycolytic inhibitors block lactate production more effectively in cisplatin-resistant cell lines under hypoxia. Lactate production was measured after 6 hours of treatment. (A). After 6 hours, 2-DG and 2-FDG significantly block lactate production in cisplatin-resistant SR2. *, p =0.025; **, p < 0.0001. (B). After 6 hours, 2-DG and 2-FDG significantly blocked lactate production more effectively in cisplatin-resistant NSCLCSC. #, p=0.013; ##, p=0.0029; ###, p=0.005. (C). 2-DG and 2-FDG block ATP production more effectively in cisplatin-resistant SR2 and
NSCLCSC under hypoxic conditions, *, p <0.0001; #, p=0.0012; **, p=0.0015; ##, p=0.0016.
Chapter 5: Preclinical testing of HK inhibitor GSK X in small cell lung cancer cell lines

5.1 GSK compound X is effective at targeting cisplatin-resistant cell line SR2 under anaerobic conditions

GlaxoSmithKline (GSK) synthesized and verified several compounds targeting HKI and HKII with varying efficacy and sent five compounds to our laboratory to be characterized in high HKII expressing cisplatin-sensitive and low HKII expressing resistant cell lines. The first compound used, GSK X, targets HKI (pIC50 = 6.7) more effectively than HKII (pIC50 = 5.6) in cell-free enzymatic assays as determined by GSK. These synthesized compounds are more potent glycolytic inhibitors than either 2DG or FDG, and were synthesized with the goal of potentially being clinically applicable agents. Due to proprietary considerations additional functional data regarding these novel small molecule inhibitors was not provided. The first experiment completed was to determine the efficacy of GSK X under normoxic and anaerobic conditions. While treatment with this small molecule inhibitor has no effect on HKII or HKI protein expression under normoxia (Fig 5.1.A), GSK X is significantly more cytotoxic at 24 µM in cisplatin-sensitive SCLC1 than in cisplatin-resistant SR2 (Fig 5.1.B). This finding may suggest that glucose metabolism is important under normoxia in the high HKII and HKI expressing cell line SCLC1, while SR2 is able to rely on alternate sources of metabolism.

Next, GSK X was tested in SCLC cells cultured under anaerobic conditions. Cells were treated with 0.1 µg/ml of oligomycin and the indicated doses of GSK X, and incubated for 24 hours. In contrast to what was seen under normoxia, GSK X was more effective at 24 µM in low HKII and HKI expressing cisplatin-resistant lung cancer cell
line SR2 (Fig 5.1.B). This shows that targeting HK in SR2 is an effective strategy under anaerobic conditions. Further experiments under anaerobic conditions should be performed in the NSCLC cell lines with this compound to expand this finding.

Fig 5.1 (A) HKI and HKII protein levels are not altered by GSK X treatment under normoxia at 3µM or 6µM. β-Actin was used as a loading control. (B) Under normoxia, GSK X is more cytotoxic in SCLC1 than SR2, * = GSK X 24 µM, p = 0.0120. Under anaerobic conditions, GSK X is more cytotoxic in cisplatin-resistant SR2, ** = GSK X 24 µM, p = 0.0033.
5.2 Under hypoxia, GSK compound X is effective at targeting cisplatin-resistant lung cancer cell line SR2

To further characterize GSK X, an experiment under hypoxic conditions was completed. Under hypoxia, HK isoforms are elevated by HIF-1 in order to help the cell survive when mitochondrial function is compromised by the lack of oxygen. When SCLC1 and SR2 cells are placed under 0.5% O$_2$ (hypoxia) and treated with GSK X, SR2 is significantly more sensitive at all three doses compared to parental SCLC1 (Fig 5.2). These data supports previously shown data utilizing 2-DG and 2-FDG, namely that inhibiting glycolysis by blocking this pathway at the level of HK correlates with cell death in these cisplatin-resistant lung cancer cell lines. This result highlights the concept that selective targeted inhibition of HK activity using a potent small molecule inhibitor may be a viable preclinical strategy with clinical implications. Again, further characterization of this compound is needed in the NSCLC cell lines, as well as in vivo, in order to determine the efficacy of this compound as a clinical agent.

Fig 5.2 Under hypoxia, GSK X is more cytotoxic in cisplatin-resistant SR2 than in cisplatin-sensitive SCLC1. * = GSK X 6 µM, p = 0.0328. ** = GSK X 12 µM, p = 0.0004, *** = GSK X 24 µM, p < 0.001.
Chapter 6: Targeting alternate metabolic pathways is toxic in cisplatin-resistant lung cancer cell lines under normoxia

6.1 Inhibition of fatty acid β-oxidation correlates with cell death in cisplatin-resistant lung cancer cell line SR2

Our previous data showed that cisplatin-resistant cell lines expressing low HK are sensitive to glycolytic inhibitors under anaerobiosis and hypoxia after 24 hours. However they are resistant to glycolytic inhibition under normoxia after 24 hours despite lowered lactate production. This observation led us to investigate whether these cells might be utilizing alternate metabolic pathways under normoxia, such as fatty acid β-oxidation or glutaminolysis, and whether perturbing one of these pathways would be effective at eliciting cell death. To begin addressing this hypothesis we identified the rate-limiting enzyme in the fatty acid β-oxidation pathway, carnitine palmitoyltransferase-1 or CPT-1, which transfers long chain fatty acids from the cytosol into the mitochondria, as a possible target for inhibition. Conveniently, there is a commercially available irreversible inhibitor of CPT-1 called etomoxir. To determine if fatty acid β-oxidation is an important metabolic pathway in cisplatin-resistant lung cancer cells, both cell pairs were treated with Etomoxir for 72 hours under normoxia. Cells were placed under normoxia, because fatty acid β-oxidation utilizes oxygen in the mitochondria to breakdown fatty acids. Of the four cell lines used in these studies only the low HK-expressing SR2 cell line was found to be sensitive to etomoxir treatment (Fig 6.1.A and 6.1.B). Taken together these data support the possibility that the low HK levels found in this cisplatin-resistant cell line may impair its ability to utilize glucose for ATP production, rendering it more
dependent on fatty acid β-oxidation for maintaining cell viability under normoxia than its parental cell line with high HK expression.

Fig 6.1 (A) Etomoxir treatment under normoxic conditions effectively induces cell death in cisplatin-resistant SR2 after 72 hours. *, p=0.0002. (B) 72 hour Etomoxir treatment under normoxia does not induce cell death in NSCLC lines. (C) CPT-1a protein expression is upregulated in SR2 compared to SCLC1. No difference in CPT-1a expression was observed in NSCLC cell pair. β-Actin was used as a loading control.

To further understand the mechanism of action by etomoxir in SR2, we probed for CPT-1a in these lung cancer cell lines to determine if any changes in CPT-1a protein
expression were induced as a result of acquired cisplatin resistance. We found that in the cisplatin-resistant cell line SR2 (which demonstrated sensitivity to etomoxir) that CPT-1a levels were elevated relative to the parental SCLC1 (Fig 6.1.C). However, this trend was not duplicated in the NSCLC cell pair. It is plausible that upregulation of CPT-1a is but one of many possible metabolic changes that can occur as a result of acquiring cisplatin resistance, however only the use of additional resistant cell line pairs generated by multiple independent investigators will determine if this is a generalized phenomenon. Follow up experiments for this project may include probing for the other isoforms of CPT-1, including CPT-1c, which has been shown to increase fatty acid oxidation and promote tumor cell survival under metabolic stress (63).

6.2 Deprivation of glutamine leads to cell death in cisplatin-resistant non-small cell lung cancer cell line NSCLCSC

Since the other cisplatin-resistant cell line with lowered HK (NSCLCSC) was found to be insensitive to blockage of fatty acid oxidation, this implied that there is not a single default metabolic pathway adopted as a consequence of acquiring resistance to cisplatin. Therefore in order to determine if NSCLCSC is dependent on another metabolic substrate to maintain viability under normoxia, such as glutamine, all four cell lines were cultured in glutamine-deficient media for 72 hours. Indeed NSCLCSC was found to be the only cell line sensitive to glutamine deprivation under normoxia (Fig 6.2.A and 6.2.B). This finding of a glutamine requirement for NSCLCSC’s survival under normoxia together with those of fatty acid dependency in SR2 suggests that cisplatin-resistant cell lines with lowered HK can alter their metabolism to use energy sources other than glucose.
To investigate the mechanism behind NSCLCSC’s sensitivity to glutamine deprivation, we probed for glutaminase (GLS) and glutaminase 2 (GLS2) protein levels in our lung cancer cell lines. These glutaminase isoforms catalyze the breakdown of glutamine into glutamate with the release of ammonia, and is an important step in glutamine metabolism. GLS and GLS2 have been shown to be upregulated by the oncogene myc and tumor suppressor p53, respectively (64, 65). Once glutamine is metabolized to glutamate, it can either be exported from the cell or further broken down to α-ketoglutarate, which can feed into the tricarboxylic acid (TCA) cycle within the mitochondria. GLS is the brain/kidney isoform, while GLS2 is the liver isoform, however since it is known that cancer cells can aberrantly express gene products not native to the parental tissue, both isoforms were investigated. Unfortunately, we did not observe an increase in protein for either isoform of glutaminase enzymes in NSCLCSC compared with NSCLCS, however this experiment only assesses immunolike reactivity for the glutaminase proteins and does not measure the level of enzymatic activity of glutaminase in these cells (Fig 6.2.C). Similar to the results obtained using etomoxir in investigating CPT-1a inhibition, these results require expansion to many different cisplatin-resistant cell pairs to elucidate common patterns.
Fig 6.2 (A) 72 hour glutamine deprivation is not cytotoxic in SCLC lines. (B) 72 hour glutamine deprivation effectively targets cisplatin-resistant NSCLCSC under normoxia. #, p=0.0004. (C) GLS and GLS2 expression is downregulated in cisplatin-resistant NSCLCSC compared to NSCLCS. β-Actin was used as a loading control.
Chapter 7: Altered HK expression in ovarian cancer cells can be exploited with glycolytic inhibitors under anaerobic conditions

7.1 Lower HKII expression in cisplatin-resistant ovarian cancer cell line A2780cis correlates with increased sensitivity to 2-DG and 2-FDG under anaerobic conditions

With our findings of lowered HK in cisplatin-resistant cell lines originating from the lung, we investigated whether this phenomenon was similar in cisplatin-sensitive (A2780) and cisplatin-resistant (A2780cis) tumor cell lines derived from another tissue: the ovary. An additional advantage of using these cell lines is that they were obtained from a commercial vendor and derived independently of the lung lines used in these studies, thus if similar trends in HK expression were detected this could assist in deflecting the argument that the observed results were an artifact of cell line preparation. Furthermore, an adriamycin-resistant (A2780adr) cell line was also available from the same vendor, allowing us to further expand these experiments into adriamycin-resistant ovarian cancer. We observed lowered HKII protein levels in the cisplatin-resistant ovarian cancer cell line A2780cis compared to its parental cisplatin-sensitive cell line A2780 (Fig 7.1.A). Lower HKII protein in A2780cis correlated with increased sensitivity to 2-DG under anaerobic conditions (Fig 7.1.B). In contrast to what we observed in lung cancer cisplatin-resistant cell lines, HKI was upregulated in A2780cis; possibly indicating that alterations in HK isozyme protein expression may vary depending the tissue of cancer cell origin (Fig 7.1.A). Additionally, treatment with oligomycin in the absence of any other intervention did not affect HK levels in ovarian cancer cell lines, as was observed in the lung cancer cell lines (Fig 7.1.A).

In contrast to what was observed in cisplatin-resistant A2780cis, the adriamycin-resistant ovarian cancer cell line A2780adr expressed elevated protein levels of both HKI
and HKII (Fig 7.1.A). Moreover, when these cell lines were treated with 2-DG under anaerobic conditions, A2780adr showed decreased sensitivity to 2-DG compared to the parental cell line A2780 (Fig 7.1.B). This result indicates that lower HKII protein and hypersensitivity to glycolytic inhibition under anaerobic conditions potentially may be specific to cisplatin resistance. Again, multiple cell lines of sensitive and resistant pairs will be required to validate this phenomenon, however the use of a adriamycin-resistant cell line serves to illustrate the point that HK protein level reductions are not a universal outcome of chemotherapeutic resistance.

Focusing solely on the parental A2780 and cisplatin-resistant A2780cis, we further investigated differences in sensitivity to glycolytic inhibitors under anaerobic conditions. Oligomycin-treated ovarian cancer cells were treated with 2-FDG for 24 hours and cell viability was determined. Here, as was seen with 2-DG treatment, 2-FDG was more effective in cisplatin-resistant ovarian cancer cell line A2780cis. As expected, 2-FDG was a more potent inducer of cell death than 2-DG. These data together with the previously presented data in lung cancer cell pairs (Chapter 3) demonstrate that utilizing glycolytic inhibitors is effective at targeting cisplatin-resistant cell lines under anaerobic conditions. Additional experiments in a broader range of cancer cell pairs are necessary to determine how effective this strategy is at targeting cisplatin-resistant cancer cell lines.
**Fig 7.1** (A) Immunoblot probing for HKII and HKI levels under normoxia in A2780, cisplatin-resistant A2780cis, and adriamycin resistant A2780adr. β-Actin was used as a loading control. (B) 24 hour 2-DG treatment under anaerobic conditions correlates to significant cell death in A2780cis. *, p=0.0013. High HKII and HKII expression in A2780adr correlates with significantly reduced cell death compared with A2780. #, p=0.030. (C). Under anaerobic conditions, 24 hour 2-FDG treatment more effectively targets cisplatin-resistant A2780cis. ***, p<0.0001; *, p=0.0002.
7.2 2-DG and 2-FDG reduce ATP production more efficiently in cisplatin-resistant A2780cis under anaerobic conditions

To probe the functional consequences of lower HK expression in cisplatin-resistant ovarian cancer cells, ATP production was measured under normoxic and anaerobic conditions. Under normoxia, 2-DG and 2-FDG did not significantly affect ATP production in either cisplatin-sensitive A2780 or cisplatin-resistant A2780cis (Fig 7.2.A). However, we observed significantly decreased ATP production in A2780cis cells treated with 2-DG and 2-FDG under anaerobic conditions compared with the parental cisplatin-sensitive cell line (7.2.B). Furthermore, the more potent glycolytic inhibitor, 2-FDG, was more effective than 2-DG in lowering ATP levels. These data correlate with our previously observed increase in glycolytic inhibitor mediated cell death under anaerobic conditions in cisplatin-resistant A2780cis. Taken together with the ATP data presented earlier in the cisplatin-resistant lung cancer cell lines, 2-DG and 2-FDG mediated ATP depletion may be a significant factor leading to cell death under anaerobic conditions. Further experiments are necessitated to determine if this is indeed the case.

Fig 7.2 A) Under normoxia, 2-DG and 2-FDG do not significantly lower ATP production in A2780cis. (B). 2-DG and 2-FDG block ATP production more effectively in cisplatin-resistant A2780cis under anaerobic conditions, *, p = 0.0009; **, p = 0.0011.
Chapter 8: Elevated HIF and HK expression under hypoxia correlates with resistance to 2-DG in ovarian cancer cell lines

8.1 Under hypoxia, restored HK levels in cisplatin-resistant A2780cis correlates with resistance to 2-DG

To further characterize the cisplatin-resistant A2780cis, we probed for HIF-1 and HK levels under hypoxic conditions. Interestingly, under hypoxia HKII protein levels were relatively unchanged between A2780 and A2780cis, while HKI was still expressed primarily in A2780cis (8.1.A). We hypothesized that this was a result of HIF-1 activation, and also immunoblotted for HIF-1 under both normoxia and hypoxia. Ovarian cancer cells lines have previously be shown to express HIF-1 under normoxia as well as hypoxia (66). Here, HIF-1 was expressed in A2780 and under normoxia and hypoxia, while in A2780cis it was only expressed under hypoxia. Furthermore, A2780adr also expressed HIF-1a under both normoxic and hypoxic conditions, and upregulated HKI and HKII expression compared with the parental adriamycin-sensitive A2780.

We next sought to determine whether upregulation of HKII and HKI under hypoxia could protect A2780cis from 2-DG mediated cell death. We previously reported that HIF-1 could confer resistance to 2-DG under hypoxia through HKII upregulation (50). Interestingly, the cisplatin-resistant variant A2780cis was not sensitive to glycolytic inhibition with 2-DG even when the experiment was extended up to 48 and 72 hours (Fig 8.1.B, 8.1.C, 8.1.D). Here, it appears that HIF-1 may be a mechanism of 2-DG resistance in ovarian cisplatin-resistant cancer cell line A2780cis under hypoxia. Further experiments utilizing transfection of siRNA that targets HIF-1 are necessary to test the validity of this hypothesis. Furthermore, A2780adr was also resistant to 2-DG under hypoxia, which may be a result of increased HKI and HKII expression.
Fig 8.1 (A) Under hypoxia, HK expression is elevated in all three cell lines compared to normoxia. HKII expression in A2780cis is identical to HKII expression in A2780. HIF-1α is expression in all three cell lines under hypoxia, but only in A2780 and A2780adr under normoxia. β-Actin was used as a loading control. (B). After 24 hours, 2-DG is not cytotoxic in A2780, A2780cis or A2780adr under hypoxia. (B) After 48 hours, 2-DG is not cytotoxic in A2780 or A2780cis. (D.) After 72 hours, 2-DG is not cytotoxic in A2780 or A2780cis.
8.2 Under hypoxia, 2-DG and 2-FDG do not significantly decrease ATP production in ovarian cancer cell lines

Next, ATP production was measured in A2780 and A2780cis under hypoxic conditions. As previously noted, cells were treated as indicated prior to initiation of hypoxia (0.5% O₂). Interestingly, under hypoxia there is not significant reduction in ATP production observed in either cisplatin-sensitive or cisplatin-resistant cell line when treated with sub-lethal doses of 2-DG and 2-FDG (8.2). These data correlate with resistance to 2-DG mediated cell death under hypoxic conditions in A2780 and A2780cis. Again, upregulation of HIF-1 may be protecting A2780cis from the effects of 2-DG treatment under hypoxia. These experiments further show that under anaerobic and hypoxic conditions, measurements of ATP production correlate well with cytotoxicity data.

![Graph showing ATP production comparison between A2780 and A2780cis](image)

**Fig 8.2** 2-DG and 2-FDG do not significantly reduce ATP levels in A2780cis when compared to A2780.
Chapter 9: Targeting alternate metabolic pathways under normoxia is ineffective in cisplatin-resistant ovarian cancer cell line A2780cis

9.1 Inhibition of fatty acid β-oxidation is not cytotoxic in cisplatin-resistant ovarian cancer cell lines

Despite HKI elevation, HKII protein expression is low under normoxia in A2780cis compared with the parental A2780. The question of whether A2780cis would be sensitive to alternate metabolic inhibition as a result of lower HKII protein was posed. Similarly to the lung cancer cell line experiments, both ovarian cancer cell lines were treated with fatty acid β-oxidation inhibitor, etomoxir, for 72 hours and cell viability was determined using trypan blue exclusion. Again, etomoxir treatment inhibits fatty acid β-oxidation by irreversibly blocking the enzyme carnitine palmitoyltransferase-1 (CPT-1). CPT-1 shuttles carnitine-conjugated fatty acids into the mitochondria for oxidation. Neither A2780 nor A2780cis were sensitive to etomoxir treatment after 72 hours under normoxia (Fig 9.1). This indicates that fatty acid β-oxidation may not be an important source of metabolism these cell lines.

Fig 9.1 Inhibition of fatty acid β-oxidation is not cytotoxic in cisplatin-resistant ovarian cancer cell line A2780cis.
Next, we wanted to determine if glutamine was an important metabolite for the viability of the A2780cis cell line. In order to address this hypothesis, a 72 hour glutamine deprivation experiment was completed using media (RPMI + 10% serum) supplemented with either 0 mg/ml or 4 mg/ml of glutamine. Again, neither A2780 nor A2780cis were sensitive to glutamine deprivation (Fig 9.2). Since neither blocking fatty acid oxidation nor depleting glutamine was effective in targeting cisplatin-resistant A2780cis under normoxia, another metabolite must be important for survival.
Fig 9.2 Under normoxia, glutamine deprivation is not cytotoxic in A2780 or A2780cis after 72 hours.

9.3 Transfection of A2780cis with siRNA targeting HKI yields increased cell death after 72 hours

Since A2780cis uniquely raises HKI protein expression, it is plausible that glucose metabolism is still a major source of ATP in these cells. To determine the validity of this hypothesis, preliminary experiments were completed using siRNA. A2780cis cells were transfected with either an siRNA control sequence targeting luciferase or an siRNA SMARTpool targeting HKI. Cells were allowed to grow for either 72 post-transfection, and were then assayed for cell death. A significant, albeit modest, increase in cell death was observed in cell treated with siRNA targeting HKI
(12.6%) compared to cells treated with siRNA targeting luciferase (18.53%). This preliminary result suggests that glucose may be an important metabolic substrate in this cell line, and additional experiments are required to further support this hypothesis.

Fig 9.3 Knockdown of HKI under normoxia leads to increased cell death 72 hours post transfection. *, p = 0.0031.
Chapter 10 Discussion

With the advent of molecular biology, Otto Warburg’s seminal discovery that tumor cells upregulate aerobic glycolysis can now be explained as a result of oncogene activation. It appears that almost every major oncogene that drives oncogenesis also initiates increased glucose metabolism. While in normal tissue glucose is broken down to pyruvate and funneled into the mitochondria for OxPhos, most tumor types engage in aerobic glycolysis despite the presence of oxygen (31). Therapies to target malignant cells relying on glycolysis for survival are being developed at the pre-clinical and clinical levels, including the glucose mimetic 2-DG. This sugar analogue has been shown to be selectively cytotoxic to a number of tumor cell lines when grown under anaerobic and/or hypoxic conditions in vitro (60, 61). Furthermore, 2-DG reduced resistance to cisplatin in an in vivo xenograft model of lung cancer [personal communication, Threshold Pharmaceuticals, South San Francisco, California]. More recently a Phase I trial in which 2-DG was used in combination with taxotere was completed (67). This trial reported that 2-DG in combination with taxotere was well tolerated and supported previously published in vivo data showing 2-DG increased the effectiveness of adriamycin and taxotere (68). These results highlight the potential of 2-DG as an adjuvant to the classical chemotherapeutic compounds used clinically, such as cisplatin, and necessitate extensive follow-up in vivo studies.

Using a chemical model of anaerobiosis in which cells were co-treated with the Complex V/ATP synthase inhibitor oligomycin, cisplatin-resistant cell pairs were used to probe the effect of lower HK expression on sensitivity to glycolytic inhibition. In Chapter 3, increased sensitivity to glycolytic inhibition under anaerobic conditions was found and
correlated with decreased lactate production in SR2 and NSCLCSC cell lines treated with 2-DG. These results were further validated by our observation that lowering either HKI or HKII protein levels in cisplatin-sensitive lung cancer cell lines using siRNA renders them sensitive to 2-FDG treatment under anaerobic conditions (Chapter 3). Moreover, utilizing a novel siRNA sequence capable of simultaneously downregulating both HKI and HKII expression, 2-FDG-mediated cell death under anaerobic conditions was found to be enhanced and greater than what is found when inhibiting either isoform of HK alone (Chapter 3). Overall, these data indicate that cisplatin-resistant lung cancer cell lines with lowered HK levels can be selectively targeted under anaerobic conditions utilizing inhibitors such as 2-DG and 2-FDG that block glycolysis at the level of these enzymes.

Similar to the data above in lung cancer, lowered HKII protein was found to correlate with increased sensitivity to 2-DG and 2-FDG under anaerobic conditions in A2780cis, a cisplatin-resistant tumor cell line derived from the ovary (Chapter 7). However, even though HKI was elevated in this cell line, it does not appear to be sufficient to protect A2780cis from 2-DG induced cell death. It appears therefore that the common phenotype amongst cisplatin-resistant lung and ovarian cell lines (that correlates with sensitivity to glycolytic inhibition under anaerobic conditions) is lower HKII. In contrast, Wintzell et al. showed that HKII was elevated in a single cisplatin-resistant cell line that they derived from the ovarian cancer cell line SKOV-3 (69). This finding indicates that decreased HKII expression does not occur in every cisplatin-resistant cancer cell line. Further studies utilizing a larger cohort of cisplatin-sensitive and
resistant cell pairs are necessary to fully determine the role of HKI and HKII in cisplatin-resistant cell lines.

Cells in hypoxic regions invariably found within most solid tumors upon reaching a certain volume survive by using glycolysis as the primary source of ATP and are typically chemotherapy resistant. Under hypoxia, 2-DG and 2-FDG were found to inhibit lactate production more efficiently and induce greater cell death in SR2 and NSCLCSC as compared to their respective parental cells similar to what was observed under anaerobic conditions (Chapter 4). Moreover, in a transgenic in vivo model of retinoblastoma using the hypoxia-indicating compound pimonidazole, the proof of principle that 2-DG, as well as 2-FDG, actually kill hypoxic tumor cells in vivo was reported (70, 71). These results highlight the value of using glucose analogues to target the slow growing population found in most solid tumors that are traditionally resistant to chemotherapy in this tissue type.

In contrast to what was observed in the cisplatin-resistant lung cancer cell lines under hypoxia, A2780cis HKII expression was elevated to the same level as in the parental cisplatin-sensitive cell line A2780 (Chapter 8). Similar expression of HKII in both cisplatin-sensitive and cisplatin-resistant cell lines correlated with resistance to 2-DG under hypoxia. Furthermore, the adriamycin resistant A2780adr, with elevated HKII and HKI, was also resistant to 2-DG under hypoxia. These data highlight an important concept: that cisplatin-resistant cancer cells of different tissue of origin may react differently to continued exposure of this drug.

Our findings of lower HK protein expression in cisplatin-resistant lung cancer cell lines led us to question whether these results could be reflective of a shift away from
reliance on glucose metabolism and toward metabolism of fats and amino acids. In support of this possibility, our results show that cisplatin-resistant lung cancer cell lines demonstrate increased sensitivity to metabolic inhibitors of either fatty acid β-oxidation or glutamine deprivation (Chapter 6). Interestingly, cisplatin-resistant ovarian cancer cell line A2780cis was not sensitive to either fatty acid β-oxidation or glutamine deprivation, but did elevate HKI and was sensitive to treatment with an siRNA SMARTpool targeting HKI (Chapter 9). These data suggest a means of potentially targeting cisplatin-resistant lung cancer cells under normoxic conditions utilizing metabolic inhibitors. Further investigation is required to determine if glucose is an important metabolic source under normoxia for A2780cis. Additional studies in a broader range of cisplatin-resistant cell line pairs is necessary to determine if inhibitors of either fatty acid or glutamine metabolism, or other metabolites, could be an effective strategy.

How prolonged cisplatin treatment causes alteration in HK protein expression and the shift towards alternate metabolic pathways is unknown. However, the importance of HK’s binding partner, the voltage dependent anion channel 1 (VDAC1), in cisplatin treatment and resistance has been previously established (25, 26). Head and neck squamous cell carcinoma cell lines treated with cisplatin were shown to have the highest amount of cisplatin binding to the VDAC and mitochondrial proteins (25). Furthermore, VDAC1 depleted cells were found to be resistant to cisplatin induced apoptosis (26). Since HKI and HKII localize at the VDAC on the mitochondrial membrane and appear to play a significant role in prevention of apoptosis, the lowered levels of these enzymes found in this study may be a reflection of alterations in VDAC acting as selection force
leading to cell survival during exposure to cisplatin. Additional studies are required to determine how cisplatin treatment may affect HK expression in vitro.

Resistance to chemotherapeutics such as cisplatin remains a problem in the clinic, and identifying new targets to potentially exploit for treatment remains a priority. The results described here highlight the utility of investigating alterations in tumor cell metabolism induced as a direct or indirect consequence of acquired resistance to chemotherapy. Overall, our results demonstrate the potential validity of targeting metabolic pathways using glycolytic inhibitors, such as 2-FDG or 2-DG, or inhibitors of alternate metabolic pathways to kill cisplatin-resistant lung cancer cell lines under anaerobic/hypoxic and normoxic conditions respectively and provide strong rationale for pursuing these functional effects using in vivo models of cancer.
Works Cited


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