An Inverse Relationship Between Resistance to 2-Deoxy-D-Glucose and Glucose Starvation in Tumor Cells under Normoxia

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AN INVERSE RELATIONSHIP BETWEEN RESISTANCE TO 2-DEOXY-D-GLUCOSE AND GLUCOSE STARVATION IN TUMOR CELLS UNDER NORMOXIA

By

Katherine Beth Philips

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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A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

AN INVERSE RELATIONSHIP BETWEEN RESISTANCE TO 2-DEOXY-D-
GLUCOSE AND GLUCOSE STARVATION IN TUMOR CELLS UNDER
NORMOXIA

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A distinguishing feature of tumor cells is their upregulation of glucose metabolism. This metabolic change from oxidative phosphorylation to aerobic glycolysis creates a selective window that can be exploited for anticancer therapy. Here we investigate mechanisms by which pancreatic cancer cells respond to therapeutic (2-deoxy-D-glucose, 2-DG) and physiologic (glucose starvation, GS) forms of glucose restriction. 2-Deoxy-d-Glucose (2-DG) is a sugar analog of both glucose and mannose and thus has established activities inhibiting glycolysis and interfering with glycosylation, respectively. Due to 2-DG’s dual functions, it has classically been thought of as a glucose deprivation mimetic. Additionally, these roles render 2-DG an attractive anticancer agent with potential to kill both hypoxic and normoxic tumor cells.

In order to anticipate and understand resistance mechanisms that may arise when 2-DG is used clinically, here we investigate survival mechanisms in cell lines that are either selected for resistance or intrinsically resistant to the sugar analog. Generally, the resistant cell lines examined display muted induction of the unfolded protein response (UPR) when treated with 2-DG. Additionally, we find multiple mechanisms upstream of ER stress/UPR that
appear to contribute to resistance including: lower 2-DG uptake and reduced glycosylation interference.

Due to 2-DG’s inhibitory activities, it is thought to mimic glucose starvation (GS). Surprisingly, cell lines that were resistant to 2-DG were found to be more sensitive to GS than the 2-DG sensitive cell line, 1420. Sensitivity to GS coincided with reduced breakdown of glycogen to glucose, which correlated with lowered levels of glycogen phosphorylase (PYGB). Inhibition of PYGB prevented glycogen breakdown and restored sensitivity to GS in 1420. Overall the data demonstrate that the manner in which glucose is restricted in tumor cells, i.e. therapeutic or physiologic, leads to differential biological responses involving distinct glucose metabolic pathways. Moreover, in evolving tumors where glucose restriction occurs, the identification of PYGB as a metabolic target may have clinical application.
Dedication

This dissertation is dedicated to my family.
Acknowledgements

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Table of Contents

List of Figures........................................................................................................ix
List of Tables............................................................................................................xi
List of Abbreviations................................................................................................xii

Chapter 1: Introduction..............................................................................................1
  1.1 General cancer background..............................................................................1
  1.2 Current cancer therapies..................................................................................2
  1.3 Metabolism.......................................................................................................6
    1.3.1 Cellular metabolism....................................................................................6
    1.3.2 Tumor glucose metabolism.........................................................................8
    1.3.3 Exploiting tumor metabolism....................................................................8
    1.3.4 2-Deoxy-D-Glucose (2-DG) and inhibition of tumor metabolism.............10
    1.3.5 Exploiting tumor metabolism with 2-DG under normoxic conditions....12
  1.4 Unfolded protein response and cancer.............................................................14
  1.5 2-DG vs. GS......................................................................................................17
  1.6 Glycogen..........................................................................................................18
  1.7 Summary...........................................................................................................19

Chapter 2: Materials and Methods.........................................................................21
  2.1 Cell lines..........................................................................................................21
  2.2 Drugs and chemicals.........................................................................................22
Chapter 3: An inverse relationship between resistance to 2-DG and glucose starvation in tumor cells under normoxia

3.1 An inverse relationship exists between resistance to 2-DG and GS

3.2 Activation of UPR proteins (Grp78, eif2a, and CHOP) correlates with 2-DG induced cell death but does not correspond to GS toxicity

3.3 Low-dose resistant cells not kept in 2-DG do not maintain resistance whereas high-dose resistant cells preserve resistance
3.4 Uptake of 2-DG is found to be lower in 14DG2 and 1469 than 1420, suggestive of a resistance mechanism.

3.5 Increased respiration and reduced lactate production found in 14DG2 and 1469 suggest a less glycolytic phenotype than 1420.

Chapter 4: The UPR and resistance to 2-DG

4.1 Inhibition of Grp78 by chemical or genetic means reverses resistance to 2-DG indicating that UPR plays a role in toxicity.

4.2 Constitutive overexpression of Grp78 protects 1420 cells from 2-DG induced toxicity.

4.3 Autophagy does not appear to play a role in 2-DG resistance.

4.4 Tunicamycin, a LLO synthesis inhibitor, is toxic to 1420 and 14DG2.

4.5 Brefeldin A, an ER stressor that does not affect glycosylation, is toxic to 1420, 14DG2 and 14DG5.

4.6 Bortezomib, an inhibitor of proteasome function, is toxic to 1469.

Chapter 5: Glycosylation

5.1 LLO synthesis is enhanced in 2-DG resistant cell lines.

5.2 UPR Array and cholesterol synthesis.

5.3 Inhibition of cholesterol synthesis sensitizes resistant cells to 2-DG.

Chapter 6: Resistance to GS
6.1 2-DG resistant cell lines have low protein and mRNA levels of glycogen storage enzymes ................................................................. 60
6.2 Glycogen staining correlates with reduced ability to store and/or breakdown glycogen in 14DG2, 1469 and 14DG5 cells ........................................ 62
6.3 Inhibition of PYGB in 1420 restores sensitivity to GS ........................................ 63
6.4. PYGB expression in NM cells ......................................................................... 64
6.5 Increased ROS levels correlate with cell death in response to GS .............. 65

Chapter 7: Discussion ......................................................................................... 67
7.1 2-DG Resistance and Apoptosis ..................................................................... 68
7.2 2-DG Resistance and Metabolism ................................................................. 69
7.3 Inverse relationship between 2-DG and GS .................................................. 70
7.4 UPR and ER stress role in 2-DG response ..................................................... 72
7.5 Glycosylation and 2-DG resistance ................................................................. 73
7.6 GS sensitivity correlates with increased ROS production in sensitive cell lines ......................................................................................... 75
7.7 Glycogen and GS sensitivity ........................................................................ 76
7.8 2-DG vs. GS ............................................................................................... 77
7.9 Clinical perspective ........................................................................................ 77
7.10 Summary and Conclusions .......................................................................... 78

References .............................................................................................................. 80
List of Figures

Figure 1-3-1: Graphical representation of cell metabolism.................................7
Figure 1-3-3: Overview of tumor cell glucose metabolism.................................9
Figure 1-3-4: Two-dimensional structure of D-Glucose, 2-Deoxy-D-Glucose and
D-Mannose........................................................................................................12
Figure 1-3-5: 2-DG use in hypoxic vs. normoxic cells.....................................13
Figure 1-3-6: Lipid-linked oligosaccharide production in the ER membrane......14
Figure 1-4-1: The Unfolded Protein Response..................................................16
Figure 3-1: An inverse relationship between resistance to 2-DG and GS........34
Figure 3-2: Grp78 and CHOP activation correlate with 2-DG-induced cell death
but do not correspond with GS toxicity..............................................................36
Figure 3-3: Low-dose resistant cells not grown in the presence of 2-DG do not
maintain resistance whereas high-dose resistant cells do.............................38
Figure 3-4: 2-DG uptake is lower in 14DG2 and 1469 than in 1420, which
correlates with low hexokinase II levels in 1469 and is suggestive of a resistance
mechanism...........................................................................................................40
Figure 3-5: Decreased lactate production in concert with increased oxygen
consumption suggests a shift to mitochondrial metabolism correlating with
reduced sugar uptake found in 14DG2 and 1469.............................................42
Figure 4-1: Inhibition of Grp78 by chemical or genetic means reverses resistance
to 2-DG..............................................................................................................44
Figure 4-2: Constitutive overexpression of Grp78 protects 1420 cells from 2-DG-induced toxicity..........................................................46

Figure 4-3: Autophagy did not contribute to 2-DG resistance.................................48

Figure 4-4: 1420 and 14DG2 respond similarly to TM, while 1469 and 14DG5 both display resistance to TM.........................................................50

Figure 4-5: 1420, 14DG2 and 14DG5 all display sensitivity to BFA while 1469 demonstrates resistance.........................................................52

Figure 4-6: 1469 cells are sensitive to proteasome inhibition.................................54

Figure 5-1: 14DG2 and 14DG5 display increased basal LLO synthesis as can be seen in Fluorophore-Assisted Carbohydrate Electrophoresis (FACE) correlating with their resistance to 2-DG and lowered UPR induction...............................56

Figure 5-2: 14DG2 and 14DG5 appear to upregulate production of the cholesterol pathway to generate dolichol for LLO synthesis..........................57

Figure 5-3: Inhibition of the dolichol synthesis pathway sensitizes resistant cells to 2-DG..............................................................................58

Figure 6-1: Protein and mRNA levels of PYGB and/or GYS are decreased in 2-DG resistant cell lines.................................................................61

Figure 6-2: Glycogen staining correlates with reduced ability to store and/or breakdown glycogen in 14DG2, 1469 and 14DG5 cells............................62

Figure 6-3: Inhibition of PYGB in 1420 restores sensitivity to GS...........................63

Figure 6-4. Resistant cell lines not maintained with 2-DG display unique phenotypes.................................................................................65

Figure 6-5: Molecular mechanisms of GS-induced cell death.................................66
List of Tables

Table 1. Select anticancer drugs and their mechanism of action..................5

Table 2. Compounds that target metabolic pathways and enzymes are currently being investigated for anti-cancer activity......................................................10

Table 3. UPR-targeting compounds that are being investigated for anticancer activity...............................................................16

Table 4: Overview of UPR response and cytotoxicity in each cell line when challenged with ER stressing agents.................................................................54
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-AAG</td>
<td>17-N-allylamino-17-demethoxygeldanamycin</td>
</tr>
<tr>
<td>AKT/PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B-cell CLL/lymphoma 2</td>
</tr>
<tr>
<td>BCL-xL</td>
<td>BCL2-like 1</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>BTZ</td>
<td>Bortezomib</td>
</tr>
<tr>
<td>CAFF</td>
<td>Caffeine</td>
</tr>
<tr>
<td>CaMKKβ</td>
<td>Ca2+/calmodulin-dependent protein kinase kinase-beta</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary cells</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP-homologous protein</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per Minute</td>
</tr>
<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>17-DMAG</td>
<td>17-Dimethylaminoethylamino-17-demethoxygeldanamycin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
</tbody>
</table>
ERAD  Endoplasmic Reticulum-Associated Protein Degradation
ERK  Extracellular Signal-regulated Kinases
FACE  Fluorophore-Assisted Carbohydrate Electrophoresis
FDG  Fluorodeoxyglucose
GFP  Green Fluorescent Protein
GRP78  Glucose-regulated protein 78kDa
GS  Glucose Starvation
GYS  Glycogen Synthase 1
Her2/Neu  v-erb-b2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2
HK2  Hexokinase II
LDHA  Lactate dehydrogenase isoform A
LLO  Lipid-Linked Oligosaccharide
3-MA  3-Methyladenine
MBTPS1  Membrane-Bound Transcription Factor Peptidase site 1
MBTPS2  Membrane-Bound Transcription Factor Peptidase site 2
MYC  V-myc myelocytomatosis viral oncogene homolog
PERK  PKR-like ER Kinase
P-EIF2α  Phosphorylated Eukaryotic Initiation Factor 2 (eIF2) α subunit
PGI  Phosphoglucone Isomerase
PYGB  Glycogen Phosphorylase Brain Isoform
RAPA  Rapamycin
RAS  Kirsten rat sarcoma viral oncogene homolog
ROS  Reactive Oxygen Species
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCAP</td>
<td>SREBF Chaperone</td>
</tr>
<tr>
<td>SEER</td>
<td>Surveillance Epidemiology and End Results</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SREBF1</td>
<td>Sterol Regulatory Element Binding Transcription Factor 1</td>
</tr>
<tr>
<td>SREBF2</td>
<td>Sterol Regulatory Element Binding Transcription Factor 2</td>
</tr>
<tr>
<td>TM</td>
<td>Tunicamycin</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded Protein Response</td>
</tr>
<tr>
<td>VST</td>
<td>Versipelostatin</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 General Cancer Background

Cancer is an inclusive collection of diseases commonly defined as involving upregulated cell growth leading to malignant neoplasms. Tumor cells demonstrate uncontrolled proliferation, invasion of surrounding tissues and may spread to distant sites in the body through the blood stream or lymphatic system in a process known as metastasis [1]. The increased growth of cancer cells is caused by mutations resulting in altered oncogene and tumor suppressor functions. Mutated oncogenes allow damaged cells to survive and these damaged cells then may go on to replicate, thus initiating a tumor. On the other hand, tumor suppressor genes are normally tasked with preventing cell cycle or inducing apoptosis in damaged cells. A loss of function mutation in two alleles of a tumor suppressor gene will allow unchecked cell cycle and permits injured cells to survive. These genetic alterations that cause unregulated tumor growth can arise due to familial inheritance or certain environmental factors such as tobacco use, poor diet and exposure to pollutants and radiation.

The Centers for Disease Control and Prevention (CDC) reported that in 2010 cancer was the second leading cause of death in the US, following only heart disease [2]. According to the National Cancer Institute’s Surveillance Epidemiology and End Results (SEER) Cancer Statistics Review, in 2013 approximately 1.66 million people will be diagnosed with and more than 580,000
will die of some form of cancer [3]. The overwhelming number of cancer patients and complicated web of signaling events that drive tumorigenesis, a cell’s progression from normal to malignant, make it challenging to develop safe and effective cancer therapies. Aside from rapid DNA replication and cell propagation, a widespread feature of many if not all cancers is a shift to dependence on aerobic glycolysis rather than oxidative phosphorylation for energy production, as was first described by Otto Warburg in the early 1920s [4]. In this work, we examine using this metabolic change as a target for cancer therapy.

1.2 Current Cancer Therapies

At present, cancer treatment is limited to a small number of options including removal of all or part of the diseased organ or gland by surgical resection as well as targeting the rapidly dividing DNA with radiation therapy and/or chemotherapeutic intervention. Cancers are often treated using a combination of these modalities to increase the chance of removing or killing the entire tumor and to reduce the risk of recurrence and resistance to therapy. Common anti-mitotic chemotherapeutic agents include etoposide, docetaxel and cisplatin [5]. Etoposide is an agent developed in the 1960s that causes DNA strand breaks by inhibiting topoisomerase, an enzyme that unwinds DNA in preparation for replication [6]. This agent is used to treat small cell lung cancers and can be employed as a second, third and fourth line therapy for other cancers. Docetaxel stabilizes assembly of microtubules, while preventing their
depolymerisation. This interference leads to a decrease in the free tubulin needed for microtubule formation and results in inhibition of mitotic cell division between metaphase and anaphase [7]. Docetaxel is typically used in breast, prostate and other non-small cell cancers but also has demonstrated cytotoxic effects on other forms of cancer including: colorectal, ovarian, lung and gastric cancers. Cisplatin is a broad-spectrum chemotherapeutic agent composed of platinum-containing complexes that bind to and cause crosslinking of DNA ultimately triggering apoptosis. The platin based agents are utilized for the treatment of lung cancers, head and neck as well as urothelial malignancies [8]. These examples of anti-cancer treatments target the rapid division of DNA that occurs in cancer cells to support their high rate of proliferation. However, since the selectivity of these therapies is based solely on rapid cell division, they also target non-cancerous dividing cells such as the skin, hematologic cells originating in the bone marrow, and cells lining the gut. Off-target consequences of chemotherapy and radiation therapy result in the adverse side effects that often accompany cancer treatment such as fever, vomiting and hair loss. Another concern of using chemotherapeutic agents is the tumor’s ability to develop resistance to these drugs [9]. Currently, most chemotherapy protocols involve combinations of drugs with different targets to avoid resistance; however many patients still succumb to cancer death in spite of combination therapy.

In addition to DNA damaging agents, there are many other classes of compounds that are being used and tested for cancer therapy, see Table 1. In some cases, a cancer is caused by overexpression of a specific pathway or
protein and targeted therapies can be effective. For example the estrogen receptor antagonist tamoxifen has shown great anticancer activity in estrogen receptor positive (ER+) breast cancers [10]. Herceptin is another targeted therapy that has displayed efficacy in breast cancer patients whose tumors contain a particular mutation which causes upregulation of the Her2/neu gene resulting in increased expression of epidermal growth factor receptor (EGFR) [11]. HER receptors are embedded in the cell membrane and are activated by epidermal growth factors (EGF) to stimulate cell proliferation [12]. Targeted therapies appear to be ideal since they are specific and selective; however, in general it is difficult to identify one targetable trait that can be exploited for cancer therapy in a patient. Furthermore, the therapies presented thus far, all have been shown to cause adverse side effects or are not effective enough at killing cancer cells. Hence, it is imperative to find new avenues of cancer treatment that spare benign cells, and do not have the aforementioned undesirable side effects. Therefore, the finding of enhanced glucose metabolism in tumor cells is an attractive target for anti-cancer therapy.
Table 1. Select anticancer drugs and their mechanism of action. Targeted therapies are shown in italics.

<table>
<thead>
<tr>
<th>Compounds (Generic Names)</th>
<th>Mechanism of Action</th>
<th>Used to Treat</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Docetaxel, Paclitaxel, Vinblastine, Vincristine</td>
<td>Anti-mitotic</td>
<td>Breast, Prostate, Lung, Ovarian, Non-small cell cancers, etc.</td>
<td>[13]</td>
</tr>
<tr>
<td>Carboplatin, Cisplatin</td>
<td>DNA crosslinking</td>
<td>Testicular cancer, Sarcomas, Lymphomas, Germ cell tumors, etc.</td>
<td>[14]</td>
</tr>
<tr>
<td>Busulfan, Cyclophosphamide, Melphalan</td>
<td>DNA crosslinking, alkylating agents</td>
<td>Multiple Myeloma, Ovarian cancer</td>
<td>[15-17]</td>
</tr>
<tr>
<td>Gemcitabine, 5-Flurouracil</td>
<td>Nucleoside analog</td>
<td>Lung, Pancreatic, Bladder, Breast cancer</td>
<td>[18, 19]</td>
</tr>
<tr>
<td>Bleomycin, Doxorubicin</td>
<td>DNA intercalation</td>
<td>Leukemias, Lymphomas, Bladder, Breast, Stomach cancer, etc.</td>
<td>[20, 21]</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Topoisomerase inhibition</td>
<td>Kaposi’s sarcoma, lung cancer, testicular cancer, lymphoma, glioblastoma, etc.</td>
<td>[6]</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>Estrogen receptor antagonist</td>
<td>ER+ Breast Cancer</td>
<td>[22]</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>Proteasome inhibitor</td>
<td>Multiple Myeloma</td>
<td>[23]</td>
</tr>
</tbody>
</table>
1.3 Metabolism

1.3.1 Cellular metabolism

In general, cells require four building blocks for growth: lipids to form membranes, amino acids to make proteins, nucleotides for nucleic acid synthesis, and sugars for the production of energy and macromolecules. Animals consume, produce or recycle these nutrients, which then can be distributed to cells throughout the body via the circulating blood. A healthy cell balances anabolism and catabolism through use of sensors of its energetic state and surrounding stimuli. Conversely, a defining hallmark of tumor cells, as defined by Hanahan and Weinberg, is that they become unresponsive to or independent of these signals and arbitrarily upregulate growth [24]. Therefore tumor cells must alter signaling pathways or circumvent them in order to stimulate metabolism in order to provide the extra building blocks required for proliferation.

Two signaling pathways that are integral to glucose metabolism are Protein Kinase B (Akt/PKB) and AMP-activated protein kinase (AMPK). Akt is a protein kinase that when activated increases glucose uptake in response to insulin stimulation and inhibits glycogen synthase kinase 3 (GSK3) resulting in enhanced glycogen storage [25]. AMPK is a cellular energy sensor that responds to low levels of ATP, particularly the AMP/ATP ratio [26]. High levels of this ratio signify a lack of energy and thus signal the cell to enhance glucose uptake and metabolism to provide additional ATP for the cell. Non-transformed cells balance their use and production of energy through these signaling cascades and should energy stores become depleted, AMPK and Akt will shift the cells from energy
consuming states to energy producing and retaining conditions. An overview of
the anabolic and catabolic signal transduction pathways in a normal cell can be
seen in Figure 1-3-1.

Figure 1-3-1 Graphical representation of cell metabolism. Proteins, fats and
amino acids are extracted from digested food and then circulated via the
bloodstream. Nutrients are then available to participate in either anabolic
(shown in blue) or catabolic (shown in red) processes. Adapted from
http://healthywellbeinginitiative.wordpress.com/ [27].
1.3.2 Tumor glucose metabolism

In addition to their tumor promoting activities, oncogenes such as Myc, Ras and others have now been shown to play a role in the increased glucose consumption found in most tumor cells [28, 29]. Glucose is the major ATP source for most tumors, and can also be used as a supply of carbon to generate metabolic intermediates for proliferation. Therefore, this sugar is funneled into many cellular pathways including glycolysis, glycosylation, the pentose phosphate pathway. The previously mentioned metabolic switch from oxidative phosphorylation to aerobic glycolysis that cancer cells experience, known as the Warburg effect, provides the energy and biomass required for tumor cell growth and proliferation. However, due to aberrant angiogenesis and rapid tumor growth, the availability of glucose often becomes restricted [28-31]. Thus, as tumors grow they are likely to encounter periods and locations of glucose deprivation and must employ mechanisms to survive this starvation and its consequent stress [32].

1.3.3 Exploiting tumor metabolism

As is highlighted below in Figure 1-3-3, heightened glucose metabolism appears to be essential for tumor vitality making this pathway an attractive mark for anticancer therapy. Table 2 shows a selection of compounds currently being investigated for antitumor activity that target the unique metabolism of tumor cells. One example of this type of agent is resveratrol, a compound found in red wine that has been shown to downregulate pyruvate kinase muscle isozyme 2...
(PKM2) expression via inhibition of mammalian target of rapamycin (mTOR) signaling. This downregulation was found to suppress cancer metabolism in that cells treated with resveratrol exhibit decreased glucose uptake as well as reduced lactate production, a measure of a cell’s glycolytic activity [33]. Direct inhibitors of metabolic enzymes are also being developed and explored such as bromopyruvic acid, an inhibitor of hexokinase (HK), the first enzyme of glycolysis [34].

Figure 1-3-3 Overview of tumor cell glucose metabolism. In tumor cells, glycolysis is enhanced while mitochondrial oxidative phosphorylation is downregulated. Potential therapeutic targets are shown in red. Adapted from http://jem.rupress.org/content/209/2/211.full [35].
Table 2. Compounds that target metabolic pathways and enzymes are currently being investigated for anti-cancer activity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mechanism of action</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromopyruvic Acid</td>
<td>Hexokinase Inhibitor</td>
<td>[34]</td>
</tr>
<tr>
<td>AICAR</td>
<td>AMPK Inhibitor</td>
<td>[36, 37]</td>
</tr>
<tr>
<td>Sodium Dichloroacetate</td>
<td>Pyruvate Dehydrogenase kinase Inhibitor</td>
<td>[38]</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>mTOR Inhibitor</td>
<td>[33]</td>
</tr>
<tr>
<td>Phloretin</td>
<td>GLUT1 Inhibitor</td>
<td>[39]</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>GLUT4 Inhibitor</td>
<td>[40]</td>
</tr>
<tr>
<td>Oxamate</td>
<td>Lactate Dehydrogenase A Inhibitor</td>
<td>[41]</td>
</tr>
<tr>
<td>Dichloroacetate</td>
<td>Pyruvate Dehydrogenase Kinase Inhibitor</td>
<td>[42]</td>
</tr>
<tr>
<td>Cerulenin</td>
<td>Fatty Acid Synthase Inhibitor</td>
<td>[43]</td>
</tr>
</tbody>
</table>

1.3.4 2-Deoxy-D-Glucose (2-DG) and inhibition of tumor metabolism

Another method to exploit the increased glucose usage found in most tumor cells is by use of the sugar analog 2-Deoxy-D-Glucose (2-DG). This agent has demonstrated activities both in vitro and in vivo that mimic the glucose-deprived environment of a tumor due to its inhibitory effects on sugar metabolism. Glucose and mannose are both six-carbon sugars that differ only in the position of the hydroxyl group located on the second carbon. 2-DG is
structurally identical to both glucose and mannose except that it lacks a hydroxyl group on its second carbon, shown in Figure 1-3-4. As a glucose analog, 2-DG inhibits glycolysis by blocking several enzymes including HK as well as phosphoglucone isomerase (PGI) thus reducing energy production. This blockage is particularly detrimental to cells growing under hypoxic conditions where mitochondria are unable to efficiently produce ATP through oxidative phosphorylation, rendering cells dependent on glycolysis for survival [44]. In animal models of retinoblastoma, cancer of the retina, 2-DG was effective at killing cells in hypoxic regions of the tumor while carboplatin, a conventional chemotherapeutic anti-mitotic agent similar to cisplatin, was toxic to the normoxic tumor regions demonstrating a successful combination therapy [45]. Furthermore, additional preclinical in vitro and in vivo data supporting the use of 2-DG to kill hypoxic tumor cells led to a Phase I clinical trial in which 2-DG was combined with the anti-mitotic chemotherapeutic agent docetaxel to target the faster replicating aerobic cells [46]. The safety of 2-DG use in patients was established at a dose of 63 mg/Kg delivered orally once per day.
1.3.5 Exploiting tumor metabolism with 2-DG under normoxic conditions

In contrast to data demonstrating that under hypoxic conditions all cell lines tested to date are killed with 2-DG treatment, under normoxic conditions the majority of cell lines examined display growth inhibition but not cell death in response to low-dose 2-DG challenge [47]. 2-DG’s growth inhibitory effects may be clinically useful, but even more striking is that 2-DG alone provokes a lethal response in a select few cancer cell lines under normoxia, including pancreatic adenocarcinoma cell line 1420 [48]. This toxicity was revealed not to be due to inhibition of glycolysis but rather through interference with N-linked glycosylation, a post-translational protein modification that is dependent on lipid-linked oligosaccharide (LLO) production [49]. LLO synthesis is a step-wise procedure wherein dolichol acts as a lipid anchor in the endoplasmic reticulum (ER) membrane and activated sugars are added sequentially to form a final oligosaccharide with two N-acetylglucosamines, three glucoses, and nine mannoses (G$_3$M$_9$). The completed LLO is transferred from the dolichol to an
asparagine residue on a growing polypeptide where it then directs protein folding, Figure 1-3-5. As a mimetic of mannose, 2-DG can be incorporated into an emerging LLO and prevent its completion. Obstruction of LLO production and subsequent N-linked glycosylation leads to disruption in the folding of glycoproteins, which initiates a signaling cascade, known as the unfolded protein response (UPR), to reestablish homeostasis in the ER [50].

![Diagram of aerobic vs hypoxic cells](image)

**Figure 1-3-5.** 2-DG use in hypoxic vs. normoxic cells. Non-transformed aerobic cells can produce energy from alternate energy sources via their mitochondria when 2-DG blocks glycolysis. In cells under hypoxic conditions the mitochondria is unable to efficiently produce energy due to the lack of oxygen and thus 2-DG can be toxic. Adapted from Kurtoglu 2007, [51].
1.4 Unfolded Protein Response and Cancer

The UPR functions to alleviate ER stress by assisting protein folding and in the event that it is unable to relieve the strain, this pathway can initiate apoptosis, Figure 1-4-1. As a result of unfolded protein accumulation, the UPR induces production of folding chaperone Glucose-regulated Protein 78kDa (Grp78) in an attempt to alleviate ER stress [53]. In an unstressed cell, Grp78 is located in the ER membrane and is bound to transducers of the UPR: PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme (IRE1) and activating transcription factor 6 (ATF6). Grp78 will dissociate from and thereby activate these transducers when it senses a misfolded protein and relocates to aid refolding. Activated PERK attempts to reduce the folding burden by slowing
protein production through the phosphorylation of eukaryotic translation initiation factor 2 alpha (eif2α), [54]. ATF6 and IRE1 mainly function as transcription factors to produce additional folders and chaperones in order to aid in the stress response. If Grp78, PERK and the other UPR components are unable to efficiently relieve ER stress, apoptosis ensues through activation of C/EBP-homologous protein (CHOP) via downregulation of the anti-apoptotic mitochondrial protein Bcl-2 [55].

Grp78 has been shown to be upregulated in many cancers and its expression correlates with poor prognosis as well as chemo- and radio-resistance in many solid tumors [53, 56]. Furthermore, it has been reported that UPR inhibition, in particular blockage of Grp78, can reverse these forms of resistance. Therefore, compounds that interfere with UPR processes are being investigated as potential cancer therapies (Table 3).
The Unfolded Protein Response. Grp78 senses misfolded proteins and dissociates from and thus activates the UPR transducers. Grp78 bonds with the misfolded proteins to aid in their refolding while the transducers slow protein production and produce additional folders and chaperones to prevent additional stress and protect the cell from apoptosis. Adapted from Szegedi, et al., [57].

Table 3. UPR-targeting compounds that are being investigated for anticancer activity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Versipelotstatin</td>
<td>Grp78 inhibition</td>
<td>[58]</td>
</tr>
<tr>
<td>Metformin</td>
<td>AMPK inducer</td>
<td>[59, 60]</td>
</tr>
<tr>
<td>SubA</td>
<td>Grp78 Inhibition</td>
<td>[61]</td>
</tr>
<tr>
<td>PERK inhibitor</td>
<td>PERK inhibition</td>
<td>[62]</td>
</tr>
</tbody>
</table>
1.5 2-DG vs. GS

2-DG is conventionally thought of as a glucose starvation (GS) mimetic; however, there are intrinsic differences between these two conditions that affect how a cell responds. Both 2-DG and GS restrict glycolysis, but 2-DG does so by competitively inhibiting PGI and allosterically inhibiting HK, enzymes of this pathway. Blocking glycolysis with 2-DG may cause shuttling of glucose to other processes. On the other hand, GS reduces the available sugars for glycolysis and other metabolic pathways driving cells to utilize other sources of energy if they are capable of doing so. One example of these differences is that 2-DG has recently been shown to cause a decrease in reactive oxygen species (ROS) generation, while GS surprisingly increases its production [63]. This can be explained by 2-DG-6-P inhibiting PGI and forcing glucose-6-P through the PPP thus generating more antioxidants such as glutathione. Conversely, GS would prevent glucose from fueling the PPP inhibiting antioxidant production and therefore increasing production of ROS.

Another example of differing outcomes from 2-DG and GS treatment can be seen in their effects on glycosylation. As was previously mentioned, 2-DG can be incorporated into LLOs thereby preventing their full formation for glycosylation. Transfer of LLOs containing 2-DG to a protein causes misfolding and malfunctioning which in turn trigger UPR and other stress responses. On the contrary, GS simply limits the availability of sugar precursors for this process and results in hypoglycosylated proteins [64, 65]. Overall, data suggests that although
2-DG has been used to mimic GS, many differences exist in these two distinct forms of glucose deprivation.

1.6 Glycogen

In the face of limited nutrients, a cell will mobilize storage units to enhance its chances of survival. Fats can be broken down to lipids, proteins degraded to their amino acids and glycogen can be metabolized to provide glucose. Sensors alert the cell to a lack of a particular building block and in response stores can be mobilized. Glycogen is produced under the direction of glycogen synthase (GYS), which is active in its dephosphorylated state, thus when glycogen synthase kinase (GSK) is inhibited. Conversely, at times of glucose deprivation glycogen can be mobilized through the action of glycogen phosphorylase (PYGB). Although it seems counterintuitive since cancer cells divide rapidly and would likely benefit from keeping nutrients in flux, many publications have demonstrated that cancer cells do store glycogen and in many cases they store even more than normal cells do [66]. One explanation for this phenomenon is that glycogen storage and breakdown have been shown to prevent senescence in cancer cells and to sustain proliferation [67]. Furthermore, it has been hypothesized that tumor glycogen is utilized to promote survival during transient hypoxia or hypoglycemia. Overall, since glycogen storage and breakdown appear to be essential to tumor cell growth and survival, inhibition of these pathways and thus the cell’s ability to store or use glycogen may have therapeutic potential.
1.7 Summary

Our lab recently completed a Phase I clinical trial using 2-DG as an anti-cancer agent, thus in hope of future clinical use of this sugar, this work aims to understand and anticipate resistance mechanisms that could occur in patients treated with this sugar analog. We previously demonstrated that a cell line (1469) that is intrinsically resistant to 2-DG displays reduced uptake of the sugar analog, lower LLO interference and muted induction of UPR as compared with the sensitive cell line (1420) when treated with 2-DG [48]. In anticipation of the emergence of acquired resistance in the clinical application of this sugar analog, pancreatic cancer cell line 1420 was used to isolate two-fold (14DG2) and five-fold (14DG5) resistant variants by continuous exposure to increasing doses of 2-DG under normoxia. Since the biologic activities of 2-DG are conventionally recognized as mimicking GS, the resistant cell lines were used here to study mechanisms of survival under both 2-DG treatment as well as when starved of glucose (GS). Surprisingly, our results reveal an inverse relationship between survival to 2-DG and GS. Although here we identify several mechanisms that are associated with the evolution of resistance to 2-DG including reduced uptake of the sugar analog as well as decreased interference with glycosylation, we find that resistance to GS may be explained by a single mechanism of glycogen utilization due to increased levels of glycogen phosphorylase isoform PYGB in
1420 as compared to 14DG2 and 14DG5 and increased levels of glycogen synthase (GYS) in 1420 in comparison with 1469.

The paradigm of cancer treatment is shifting from use of traditional chemotherapeutic drugs and radiation to more tumor specific therapies. Here we demonstrate that the unique metabolism of tumors can be exploited in order to selectively target cancer cells while sparing adjacent benign cells. 2-DG has shown anticancer efficacy in preclinical and phase I clinical studies and warrants further exploration both as a single agent therapy as well in combination with other treatment strategies. Additionally, in this work we demonstrate a role of glycogen in cancer cells that may make it a viable target for anticancer therapy.
Chapter 2: Materials and Methods

2.1 Cell Lines

Human pancreatic tumor cell lines 1420 (MIA PaCa-2) and 1469 (Panc-1) were purchased from ATCC (Manassas, VA). From cell line 1420, a two-fold 2-DG resistant variant cell line (14DG2) was generated by continuous treatment with 2mM 2-DG for a period of months. Additionally, from 14DG2, a five-fold 2-DG resistant variant (14DG5) was selected by continuous treatment with 5mM 2-DG for a period of months. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 2 g/L glucose (Life Technologies, 11885-084). 14DG2 and 14DG5 were continually cultured with 2mM 2-DG or 5mM 2-DG, respectively. Unless otherwise specified, 24 h prior to start of experimental treatment media was replaced on all cell lines with 2-DG-free DMEM with 2 g/L glucose.

14DG2 and 14DG5 cells were grown in the absence of 2-DG for over 8 weeks to develop cell lines 14DG2NM and 14DG5NM, respectively. Media for cell passage was supplemented with penicillin/streptomycin (AMRESCO, Solon, OH, K952) and 10% FBS (Life Technologies, Grand Island, NY, 16000). For glucose starvation, no glucose DMEM (Life Technologies, 11966-025) was used with the antibiotics listed above and dialyzed FBS (Life Technologies, 26400). Cells were seeded and allowed to attach and grow. The next day, culture medium was removed, cells were washed twice with PBS and starvation medium was added to the culture.
2.2 Drugs and Chemicals

2-Deoxy-d-glucose (D6134), mannose (M6020), tunicamycin (T7765), etoposide (E1383), 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (D5193), 3-methyladenine (M9281), rapamycin (R0395), simvastatin (S6196), and caffeine (27600) were purchased from Sigma-Aldrich (St. Louis, MO).

Bortezomib was purchased from LC Laboratories (Woburn, MA, B-1408). Brefeldin A was purchased from Calbiochem (203729). Versipelostatin was a kind gift from Dr. Kazuo Shin-Ya (Biomedicinal Information Research Center (BIRC), National Institute of Advanced Industrial Science and Technology (AIST), Biological Systems Control Team, Tokyo, Japan). The following rabbit primary antibodies were obtained from Cell Signaling Technology (Danvers, MA): Grp78 (3177), phospho-eif2a (3597), GYS1 (15B1), Bcl-2 (2870), Bcl-xl (2762), and HK2 (2106). The following mouse primary antibodies were from Cell Signaling Technology (Danvers, MA) and Santa Cruz Biotechnology Inc. (Dallas, TX), respectively: CHOP (5554), PYGB (sc-81751). Mouse anti-β-actin (A5441) primary antibody was from Sigma-Aldrich. Horseradish peroxidase-conjugated anti-rabbit (W4011) and anti-mouse (W4021) secondary antibody were purchased from Promega (Madison, WI). Primary anti-glycogen antibody was a kind gift from Dr. Otto Baba (Department of Hard Tissue Engineering, Tokyo Medical and Dental University, Tokyo, Japan). Alexa Fluor 555 goat anti-mouse secondary antibody was purchased from Invitrogen (Grand Island, NY, A21424).
2.3 Cytotoxicity Assay

Cells were incubated for 24 h at 37°C in 5% CO₂, at which time drug treatments began and continued for 72 h. At this time, attached cells were trypsinized and combined with their respective culture media followed by centrifugation at 400g for 5 min. The pellets were resuspended in 1mL of Hanks Balanced Salt Solution (HBSS) (Mediatech) and analyzed with a Vi-Cell Cell Viability Analyzer (Beckman Coulter) based on trypan blue exclusion. Results were shown as the percentages of dead cells out of total cells counted. Data shown are the averages of triplicate samples +/- SD from one representative experiment out of at least three independent analyses unless otherwise indicated.

2.4 Western Blot Analysis

Cells were seeded onto six-well plates and grown overnight to reach approximately 50% confluence. Following drug exposure for 24 h, cells were harvested and lysed with buffer (100mM Tris-HCl at pH 7.4, 1% SDS, protease inhibitor cocktail and phosphatase inhibitor cocktail 2 from Sigma-Aldrich). Protein concentrations of each sample were determined using a Micro BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer’s instructions, and equal amounts of proteins were loaded onto 4-15% Tris-HCl gradient gels (Bio-Rad). After SDS-PAGE, proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore), blocked with 5% milk and probed with primary antibodies overnight (except 1 h for β-actin). The membrane
was then washed and probed with secondary antibodies for 1 h and washed again. Membrane was then incubated with SuperSignal West Pico or Femto Chemiluminescent Substrate (Thermo Scientific) and protein intensity was visualized on Blue Lite Autorad Films (ISC BioExpress). All primary antibodies were used at a dilution of 1:1000 except for β-actin (1:10000). Representative blots from at least three independent experiments were shown unless otherwise indicated. All parallel blots shown were developed on the same membranes. However, for clear presentation, irrelevant samples in some of the figures were cut out and the remaining blots presented. Quantification of blot intensity was performed using ImageJ (National Institutes of Health, Bethesda, MD).

2.5 qPCR

Total cellular RNAs were extracted by the RNeasy Plus Mini Kit (QIAGEN, Valencia, CA, 74134), DNase treated with Turbo DNase Free (Invitrogen) and reverse-transcribed using the ImProm-II Reverse Transcription System (Promega, A3800), according to manufacturer instructions. qPCR experiments were performed on an ABI 7300 Real-Time PCR System (Life Technologies) using PerfeCTa SYBR Green FastMix, ROX (Quanta Biosciences, Gaithersburg, MD, 95073-250). Primer sequences used are as follows:

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human β-Actin Forward</td>
<td>5’-AGAGCTACGAGCTGCCTGAC-3’</td>
</tr>
<tr>
<td>Human β-Actin Reverse</td>
<td>5’-AGCCTGTGGCGTACAG-3’</td>
</tr>
</tbody>
</table>
Melting curve analysis was performed to verify specificity of products as well as water and RT controls in every run. Data were analyzed using the ΔCt method as in Mutlu et al. [68]. Target gene expression was normalized to β-actin by taking the difference between Ct values for target genes and β-actin (ΔΔCt value). These values then were calibrated to that of the control sample to give the Ct value. The fold target gene expression is given by the formula: $2^{-\Delta\Delta Ct}$. Graphs given show fold change induced in treated over untreated control cells of each cell line, unless otherwise specified.
2.6 UPR PCR Array

The RT² Profiler Human Unfolded Protein Response PCR Array (384-well plate format) was purchased from QIAGEN (previously SABiosciences, PAHS-089E-4). Total RNAs were extracted as mentioned above and the RT² First Strand Kit (QIAGEN, 330401) was used to reverse-transcribe mRNAs. Gene expression was measured via qPCR using the RT² qPCR Mastermix (QIAGEN, 330529) on an ABI 7900HT Fast Real-Time PCR System (Life Technologies). PCR array data were analyzed using the RT² Profiler PCR Array Data Analysis tools available on the manufacturer’s website.

2.7 2-DG Uptake

Cells were seeded and cultured for 24 h under normoxic conditions in low glucose (2 g/L) DMEM. The medium was then replaced with fresh “hot” low glucose DMEM (300 mmol/L cold 2-DG and 1 mCi $^3$H 2-DG), and the sample incubated for 5 min under normoxia. Next, the hot medium was removed, cells were washed 3 times with fresh, glucose- and serum-free medium, and the cells were lysed with 0.5 mL of 1N NaOH, collected to 1.5mL eppendorf tubes and gently vortexed. A 0.25-mL sample was used for protein analysis. Radioactivity from the remaining sample was counted using a Packard CA2000 liquid scintillation spectrometer. Radioactivity was measured as counts per minute (CPM) and normalized to protein level as measured by Micro BCA Protein Assay Kit (Thermo Scientific).
2.8 Fluorophore-Assisted Carbohydrate Electrophoresis (FACE)

Cells were cultured until 90% confluent, harvested in methanol, and dried under N\textsubscript{2}. LLOs were recovered in chloroform/methanol/water (10:10:3), hydrolyzed, modified with 7-amino-l,3-naphthalenedisulfonic acid (ANDS) fluorophore, and analyzed by fluorophore-assisted carbohydrate electrophoresis (FACE) as previously described by Gao, et al., [69].

2.9 siRNA

Anti-Luciferase siRNA 1 (siLuc, D-002050-01) and ON-TARGETplus SMARTpool siRNAs against PYGB (siPYGB, L-009587), Grp78 (siGrp78, L-008198-00-0005) or were obtained from Thermo Scientific (Rockford, IL). Cells were seeded into 6-well plates and cultured for 24 h to reach ~60% confluence using antibiotic-free media. Then, cells were transfected with anti-Luc (control) or siRNA that targeted either PYGB or Grp78 as specified in figure, using DharmaFECT siRNA transfection reagent #1 (Dharmacon). Twenty-four hours after transfection, cells were collected and re-seeded onto 6-well or 24-well plates and drug-treated for immunoblotting or cytotoxicity analyses, respectively. Lowest concentrations of siRNAs that could produce saturated knockdown efficiency were used.

2.10 Glycogen Staining

In a method adapted from Louzao et. al 2008 [70], cells were seeded on coverslips in 6-well plates and grown overnight then washed 3 times with PBS
and changed to glucose-free media for 24 h. Cells were washed then fixed with 4% paraformaldehyde in PBS for 15 min at RT and then permeabilized with 2% BSA-0.2% Triton X-100 PBS for 5 min on ice. After three PBS washes, non-specific binding was blocked with 2% BSA-PBS for 1 h at RT. Cells were incubated with Anti-glycogen antibody (at 1:100 dilution) in 2% BSA-PBS overnight at 4°C. The following day cells were washed and incubated with Goat Anti-mouse IgG Alex Fluor 555 in 2% BSA-PBS for 1 h at RT at a 1:500 dilution. This step was followed with PBS washes and finally coverslips were mounted on slides using ProLong Gold Antifade Reagent with dapi medium (Life Technologies, Grand Island, NY, P-36931). Slides were allowed to dry overnight in the dark and then imaged on a fluorescent microscope. Images shown are from one representative experiment out of at least three independent analyses.

2.11 Oxygen Consumption

Cells were grown in 75-cm² flasks until they were approximately 70% confluent, and then were trypsinized, collected and spun to pellet. Cells (5 × 10⁶) were resuspended in 1 mL of DMEM, without glucose or fetal bovine serum. Oxygen consumption was measured with Clark electrode (Hansatech) for 10 min as previously described [71].

2.12 Lactate Production

Lactate was measured by adding 0.025 mL of deproteinated medium from treated or untreated cultures to a reaction mixture containing 0.1 mL of lactic
dehydrogenase (1000 U/mL), 2 mL of glycine buffer (glycine, 0.6 mol/L and hydrazine, pH 9.2), and 1.66 mg/mL of NAD. Deproteinization required treating 0.5 mL of medium from sample cultures with 1 mL perchloric acid (8%, wt/vol), vortexing for 30 seconds, then exposing this mixture to 4°C for 5 minutes, and centrifuging at 1500g for 10 minutes at room temperature. The supernatant was centrifuged 3 more times, and 0.025 mL of a final clear supernatant was used for lactic acid measurements. Formation of NADH was measured with a Beckman DU r 520 UV/vis spectrophotometer at 340 nm, which directly corresponds to lactic acid levels as determined by a lactate standard curve.

2.13 Grp78 Overexpression

T1-Phage resistant E. coli from Mammalian Gene Collection vector pOTB7 that contain Grp78 (Invitrogen) were streaked on LB agar plates containing chloramphenicol and grown overnight. Colonies were picked and grown in liquid LB while shaking overnight then DNA was harvested using Mini Prep Kit (Qiagen). PCR was performed on plasmid DNA to add KpnI and Bam HI restriction sites using the following primers (restriction sites are underlined):

Grp78-Kpn1-Fw 5’-CGC CGC GGT ACC ATG AAG CTC TCC CTG GTG GCC GCG-3’
Grp78-BamHI-Rv 5’-GCG GCG GGA TCC CTA CAA CTC ATC TTT TTC TGC TGC TGT-3’

The DNA was run on a 0.8% agarose gel and gel purified with the Gel Extraction Kit (Qiagen) and cleaned using Reacting Cleanup Kit (Qiagen). Next, the plasmid DNA and the expression vector pAc-GFP1-N1 (Clontech) were
digested using Kpn1 and BamHI Fast Digest enzymes (Fermentas) overnight. Thermosensitive Alkaline Phosphatase (Fermentas) was added to the plasmid digest to prevent self-ligation. The plasmid and insert were ligated using LigaFast Rapid DNA Ligation System (Promega). PrimePlus bacteria (Open Biosystems) were transformed with the new construct, colonies were picked and authenticity of the human Grp78 cDNA sequence was confirmed by fluorescence-based double-stranded DNA sequencing. Bacteria with the construct were grown and DNA isolated using a Miniprep kit (Qiagen).

To establish stable 1420 cell lines overexpressing Grp78, cells grown to 50% confluence were transfected with 5ug of the pAc-Grp78-N1 expression plasmid using 30uL of Optifect Transfection reagent (Invitrogen), as described by the manufacturer. As a vector control, 1420 cells were transfected with pAc-GFP1-N1 under the same conditions. Stable transfectants were selected in complete medium containing 1.2mg/mL G418 (Life Technologies Inc.) for 2 weeks. G418-resistant clones were subsequently isolated and cultured in complete medium containing 1.0mg/mL G418. Overexpression of Grp78 was assessed using immunoblot and indirect immunofluorescence.

2.14 Reactive Oxygen Species (ROS) Measurement

As previously described, cells were collected and intracellular H$_2$O$_2$ was measured by incubating with 10 µmol/L of acetyl-penta-fluorobenzenesulfonyl fluorescein (APFB; EMD) at 37°C for 30 minutes in the dark [72]. Then, the cells were washed once with PBS and centrifuged to remove impermeable reagents.
Cells were resuspended in 500 µL of PBS and analyzed in a fluorometer, FLUOstar OPTIMA, BMG Labtech (excitation at 485 nm and emission at 520 nm).

2.15 ATP Measurement

Intracellular ATP levels were measured with the CellTiter- Glo Luminescent Cell Viability Assay Kit (Promega) according to the manufacturer's directions. Briefly, cells were seeded onto 96-well plates and cultured for 24 h to reach >70% confluence. After 24 h of glucose deprivation, cells were lysed in the same plate with the reagent included in the assay kit for 10 min. Then the mixtures were transferred onto opaque-walled 96-well plates and luminescence produced from ATP-mediated chemical reaction was read by FLUOstar OPTIMA microplate reader (BMG LABTECH). Results shown are the averages of duplicate samples +SD from one representative experiment out of at least three independent analyses.

2.16 Statistics

Statistical analyses were performed by two-way analysis of variance (ANOVA), followed by Bonferroni posttest, and a P value less than 0.05 was considered significant.
Chapter 3: An inverse relationship between resistance to 2-Deoxy-D-Glucose and Glucose Starvation in tumor cells under normoxia

In 2007 our lab published work demonstrating that 2-DG is deadly to certain cancer cell lines under normoxic conditions and that this toxicity is due to the drug’s activity as a mannose analog and consequent interference with N-linked glycosylation. Glycosylation is a co- or post-translational modification wherein LLOs are attached to growing polypeptides and then direct proper protein folding and function. 2-DG can masquerade as mannose and be added in its place to a growing LLO which will prevent further addition of sugars to the structure. In brief, 2-DG causes production of incomplete LLOs and since glycosylation directs protein folding, aberrant LLO synthesis results in accumulation of misfolded proteins that trigger an unfolded protein response (UPR). Activation of the UPR can either rescue a cell from ER stress or this pathway can activate apoptosis in sensitive cell types, such as 1420.

In addition to its role as a glycosylation obstructer and its toxicity under normoxic conditions, 2-DG has also been successfully used to kill cancer cells under hypoxic conditions due to its inhibition of glycolysis [51]. In this way, 2-DG is classically thought of as a glucose deprivation mimic. It is highly likely that a glucose starvation event will occur at some point during tumor pathogenesis; however, tumors are generally able to survive this challenge [73]. Thus, since most tumor cell lines previously tested were not sensitive to 2-DG treatment, we investigated whether the same mechanisms which cells use to survive physiologic GS were employed to endure 2-DG challenge.
3.1 An inverse relationship exists between resistance to 2-DG and GS

2-DG has previously been shown to result in cytotoxicity in pancreatic cancer cell line 1420 under normoxic conditions and this toxicity was found to be due to interference with N-linked glycosylation and correlated with high UPR induction [48, 74]. The 2-DG sensitive cell line (human pancreatic 1420) was used to isolate two-fold (14DG2) and five-fold (14DG5) resistant variants that were then compared with the parental cell line as well as with an intrinsically resistant pancreatic cell line (1469), which our lab has previously studied. Surprisingly, the order of sensitivity to 2-DG was reversed when these cell lines were placed under GS conditions (Fig. 3-1a and b). Thus, although 2-DG has been traditionally used to mimic GS, the inverse relationship observed here suggests fundamental differences between these two modes of glucose restriction. These findings also suggest that 2-DG resistance is not indicative of a general survival advantage which is further supported by experiments in which the anti-mitotic agent etoposide produced similar levels of toxicity in 1420, 14DG2 and 14DG5 (Fig. 3-1c). Similarly, the three aforementioned cell lines displayed toxicity when treated with another anti-mitotic agent, staurosporine (data not shown). Additionally, levels of the anti-apoptotic proteins Bcl-2 and Bcl-xl were also examined [75]. 14DG2 and 14DG5 exhibited slightly elevated levels of Bcl-2; however, this did not appear to have a functional effect since these cells were not protected from etoposide or staurosporine as compared with 1420 (Fig.
3-1d). 1469 displayed minor upregulation of Bcl-xl which may explain this cell line’s relative resistance to the anti-mitotic agents and 2-DG (Fig. 3-1d).

**Figure 3-1.** An inverse relationship between resistance to 2-DG and GS. (A.) 1420, 14DG2, 1469 and 14DG5 cell lines were treated with the indicated doses of 2-DG for 72 h in normoxia and percent dead cells was assayed by trypan blue exclusion. The bars represent the average of triplicate samples +/- SD. (B.) 1420, 14DG2, 1469 and 14DG5 cell lines were challenged with the indicated levels of glucose in media with dialyzed serum for 72 h in normoxia and percent dead cells was assayed by trypan blue exclusion. The bars represent the average of triplicate samples +/- SD. (C.) 1420, 14DG2, 1469 and 14DG5 cell lines were treated with the indicated doses of etoposide for 72 h in normoxia and percent dead cells was assayed by trypan blue exclusion. The bars represent the average of triplicate samples +/- SD. (D.) Cells were grown in normoxia then harvested and immunoblotting was performed to detect protein levels of Bcl-2 and Bcl-xl.
β-Actin was used as a loading control. (*) denotes p value < 0.05, (**) denotes p value < 0.01 and (*** ) denotes p value < 0.001.

3.2 Activation of UPR proteins (Grp78, eif2α and CHOP) correlates with 2-DG-induced cell death but does not correspond to GS toxicity

As mentioned previously, through its activity as an analog of glucose, 2-DG blocks glycolysis while as a mannose mimetic, it obstructs glycosylation thereby inducing ER stress, and subsequently stimulating the UPR. Activation of this pathway, as measured by Grp78 induction, was previously found to be greater in the 2-DG sensitive cell line (1420) than in the intrinsically resistant cell line (1469), [48]. Similarly, here we find that when treated with 2-DG, 1420 displayed robust induction of UPR markers, Grp78, p-eif2α and CHOP, while in 1469 and 14DG2, activation of these proteins was blunted (Fig. 3-2a). Moreover, in the most resistant cell line examined, 14DG5, little to no UPR induction could be seen at 1mM of 2-DG (a dose where UPR was induced in the other cell lines). These observations were further corroborated via qPCR in that 2-DG-induced Grp78 and CHOP mRNA was also found to be highest in the sensitive cell line (Fig. 3-2b). Thus, reduced UPR activation, which is indicative of lower ER stress, correlates with less cell death in response to 2-DG.

Like 2-DG, GS is also known to cause ER stress but does so by limiting the availability of glycosylation precursor sugars. However, no significant differences were observed in the induction of Grp78, p-eif2α and CHOP protein or mRNA when these cell lines were placed under conditions of GS (Fig. 3-2c and d). These latter results suggest that unlike treatment with 2-DG, differences
in ER stress and/or UPR cannot account for differential sensitivity of these cell lines to GS. These data further highlight and support our hypothesis of fundamental differences in tumor cell response to therapeutic (2-DG) and physiologic (GS) forms of glucose restriction.

Figure 3-2. Grp78 and CHOP activation correlate with 2-DG-induced cell death but do not correspond with GS toxicity. (A.) Cells were treated with the indicated doses of 2-DG for 24 h under normoxia then harvested and immunoblotting was performed to detect protein levels of Grp78 and CHOP. β-Actin was used as a loading control. (B.) mRNA levels of Grp78 and CHOP were determined by qPCR in cells treated for 24 h with 1mM 2-DG, normalized to actin mRNA levels. mRNA levels are shown as fold induction of treated/untreated control cells. The bars represent the average of duplicate samples. (C.) Cells were treated with GS for 24 h in normoxia then harvested and immunoblotting was performed to detect protein levels of Grp78 and CHOP. β-Actin was used as a loading control. (D.) mRNA
levels of Grp78 and CHOP were determined by qPCR in cells treated for 24 h with GS, normalized to actin mRNA levels and graphed as fold induction of treated/untreated control cells. The bars represent the average of duplicate samples.

3.3 Low-dose resistant cells not kept in 2-DG do not maintain resistance whereas high-dose resistant cells preserve resistance

To ensure that 2-DG and GS responses were not due to the presence of 2-DG remaining from culture medium during experiments, 14DG2 and 14DG5 were grown in 2-DG-free DMEM for >8 weeks and thus cell lines 14DG2NM and 14DG5NM, respectively, were developed. 14DG2NM reverted to a 2-DG sensitive phenotype similar to the parental cell line, 1420 (Fig. 3-3a). Conversely, 14DG5NM maintained a level of resistance to 2-DG comparable to that found in 14DG5 (Fig. 3-3a). We postulate that permanent genetic or epigenetic changes are responsible for resistance in 14DG5 while 14DG2’s resistance may be caused by transient modifications that require 2-DG maintenance. In addition, 14DG2NM lost much of 14DG2’s sensitivity to GS, again reminiscent of 1420’s response (Fig. 3-3b). However, 14DG5NM showed only a partial reversion in sensitivity to GS as compared with 14DG5 (Fig. 3-3b). These changes will be explored further in forthcoming sections.
3.4 Uptake of 2-DG is found to be lower in 14DG2 and 1469 than 1420, suggestive of a resistance mechanism

Previously, our lab reported that intrinsic resistance to 2-DG in pancreatic cancer cell line 1469 correlates with lower 2-DG uptake and decreased UPR activation when compared to sensitive cell line 1420 [76]. To confirm these uptake differences, mass spectrometry studies were performed in collaboration with Metabolon Inc. (New York, NY). Indeed, these studies showed increased levels of 2-DG in 1420 as compared with 1469, supporting the prior findings of differential uptake between these cell lines (Fig. 3-4c).

Based on this observation and the data presented in figures 3-1 and 3-2, 2-DG uptake was evaluated in the cell lines selected for two-fold and five-fold
resistance using a tritiated analog of 2-DG. Similar to earlier results in 1469, reduced uptake was found in 14DG2 as compared with 1420, correlating with its resistance to 2-DG (Fig. 3-4a). However, 14DG5, selected for resistance to high dose 2-DG, surprisingly showed greater uptake than the parental cell line, 1420. Thus, although reduced uptake correlates with 2-DG resistance in 14DG2 and 1469 it cannot explain resistance to 2-DG in 14DG5. Overall, these data indicate that reduced uptake is not a generalized mechanism of resistance to 2-DG.

There are many factors that contribute to cellular uptake of hexoses including glucose transporters, glycolytic enzymes and insulin receptors. Hexokinase is an enzyme that catalyzes the first step of glycolysis: the conversion of glucose to glucose-6-phosphate, which then cannot be removed from a cell. There are four isoforms of hexokinase: Hexokinase I, Hexokinase II, Hexokinase II and glucokinase. Hexokinase II (HK2) is the principal form in most cell types and its expression is found to be increased in many cancers [77]. Protein levels of HK2 were found to correlate with uptake in that 1469 displayed the lowest uptake and also exhibited the lowest protein expression of this enzyme (Fig. 3-4b). Moreover, mass spectrometry studies demonstrated that in addition to higher levels of 2-DG, 1420 cells also display greater accumulation of 2-DG-6-P, the product of hexokinase as compared with 1469 (Fig. 3-4c).
Figure 3-4. 2-DG uptake is lower in 14DG2 and 1469 than in 1420, which correlates with low hexokinase II levels in 1469 and is suggestive of a resistance mechanism. (A.) Untreated cells were exposed to tritiated 2-DG for 5 min. Radioactivity was measured and normalized to protein levels. The bars represent the average of duplicate samples +/- SD. (B.) Untreated cells were harvested and immunoblotting was performed to detect protein levels of HK2. β-Actin was used as a loading control. Quantification of blot can be seen to the right. (C.) Mass spectrometry analysis of metabolites 2-DG and 2-DG-6-P in 1420 and 1469 cells under control conditions and after 24 h treatment with 1mM 2-DG or 1mM fluorodeoxyglucose (FDG). (*) denotes p value< 0.05.
3.5 Increased respiration and reduced lactate production found in 14DG2 and 1469 suggest a less glycolytic phenotype than 1420

Oxygen consumption is a measure of cellular respiration, and as such is an indicator of mitochondrial oxidative phosphorylation in a cell [78]. The 2-DG resistant cell lines displayed greater oxygen consumption as compared with 1420. In particular 1469 and 14DG5 respired more than 1420 and 14DG2 also exhibited increased respiration but not to the degree found in the other two resistant cell lines (Fig. 3-5a). Furthermore, 1469 and 14DG2 both demonstrated reduced lactate production, a measure of glycolysis (Fig. 3-5b). Lactate is produced from pyruvate in the end stages of glycolysis and in aerobic conditions lactate will go on to fuel the mitochondrial TCA cycle [79]. These data, taken together with the increased respiration, suggest that these cell types are relying on mitochondrial respiration more than aerobic glycolysis. A less glycolytic phenotype, such as what can be seen in 1469 and 14DG2, may be contributing to 2-DG resistance. On the other hand, 14DG5 was shown to have greater respiration and uptake than the other three cell lines. 14DG5’s high metabolism, both in lactate production and respiration, is intriguing as it appears to have the slowest doubling time of the four cell lines tested here (data not shown). Thus, reduced uptake and glycolysis cannot explain 2-DG resistance in this cell line.
Figure 3-5. Decreased lactate production in concert with increased oxygen consumption suggests a shift to mitochondrial metabolism correlating with reduced sugar uptake found in 14DG2 and 1469. (A.) Lactate production was measured in untreated cells. (B.) Live cells were counted and placed in an oxygen chamber. The amount of oxygen consumed per cell line in 10 min was measured.
Chapter 4: The UPR and Resistance to 2-DG

Although reduced uptake of 2-DG correlated with resistance and the metabolic profiles of 14DG2 and 1469, a correlation was not found for 14DG5. However, since an association was established between sensitivity to 2-DG and high UPR induction (Fig. 3-1 and 3-2), we further interrogated this pathway. The UPR is a dual-pronged response in that it has the ability to rescue a cell by reducing protein folding issues, but this pathway can also signal apoptosis if it deems the cell too severely damaged [80]. The UPR has been extensively studied in both cancer and other diseases and high expression of UPR genes has been found to correlate with poor prognosis. Due to this correlation, inhibition of UPR has been shown to result in cancer cell death or sensitization to therapy [81]. Therefore, as mentioned previously, inhibitors of UPR proteins are currently being developed for cancer therapy (Table 3).

4.1. Inhibition of Grp78 by chemical or genetic means reverses resistance to 2-DG indicating that UPR plays a role in toxicity

Due to the differential Grp78 induction observed between the sensitive and resistant cell lines when treated with 2-DG, versipelostatin (VST), a chemical inhibitor of the protein chaperone was employed [58]. The mechanism by which VST inhibits Grp78 has yet to be fully resolved, however it has been suggested that it blocks the activity of Grp78’s promoter and hence its upregulation in response to ER stress [58]. We observed an additive toxic effect when VST and 2-DG were combined in the resistant cell lines (14DG2, 1469 and 14DG5)
whereas no significant additive effect was found when this agent and 2-DG were combined in 1420 cells (Fig. 4-1a). Western blot analysis confirmed decreased induction of Grp78 by 2-DG when combined with VST; however, CHOP levels were found to increase in response to the combination of agents suggesting that UPRmediate cell death is occurring (Fig. 4-1b).

Furthermore, similar findings to the VST results were observed in the resistant cell lines when Grp78 was blocked with siRNA and the cells were subsequently treated with 2-DG (Figure 4-1c and d). We hypothesize that VST and siGrp78 reduce free Grp78 but not those bound to UPR transducers in the ER and therefore the ER’s ability to fold proteins is diminished leading to a stronger activation of the UPR transducers that consequently activate apoptosis.

Enhanced CHOP induction in cells treated with the combination of VST and 2-DG supports this hypothesis. It is of note that this effect was not recapitulated with inhibitors of Grp94 and the hsp90 family such as the geldanamycin derivatives, 17-DMAG (Fig. 4-1e) or 17-AAG (data not shown). These data suggest that UPR but not general cellular stress or heat shock responses play a role in 2-DG resistance.
Figure 4-1. Inhibition of Grp78 by chemical or genetic means reverses resistance to 2-DG. (A.) Cells were treated with either 2mM 2-DG, 5uM VST or the combination for 72 h in normoxia and percent dead cells was assayed by trypan blue exclusion. The bars represent the average of triplicate samples +/- SD. (B.) Cells were treated with 2-DG, VST or the combination for 24 h in normoxia then harvested and immunoblotting was performed to detect protein levels of Grp78 and CHOP. β-Actin was used as a loading control. (C.) siRNA directed at Grp78 or a scrambled control sequence was employed in each of the four cell lines and combined with 72 h 2-DG treatment in normoxia. (D.) Cells were transfected with siRNA for 24 h then treated with 2-DG for an additional 24 h then harvested and immunoblotting was performed to detect protein levels of Grp78. β-Actin was used as a loading control. (E.) Cells were treated for 72 h with the indicated doses of 17-DMAG, 2-DG or the combination in normoxia and percent dead cells was assayed by trypan blue exclusion. The bars
represent the average of triplicate samples +/- SD. (*) denotes p value < 0.05, (**) denotes p value < 0.01 and (***) denotes p value < 0.001.

4.2. Constitutive overexpression of Grp78 protects 1420 cells from 2-DG-induced toxicity

Next, to further examine the role of UPR in 2-DG resistance, 1420 cells were stably transfected with a plasmid designed to constitutively overexpress Grp78. The 1420 “oxGrp78” cells were compared with 1420 cells transfected with a plasmid containing green fluorescent protein (GFP) as a control, “oxGFP” cells. The oxGrp78 cells displayed reduced sensitivity to 2-DG as compared with the oxGFP cells suggesting that enhanced Grp78 present prior to 2-DG treatment has a protective role due to improved protein folding and decreased UPR transducer activation (Fig. 4-2a and b). This may explain 1469’s 2-DG resistance since it displays a higher basal level of Grp78 than the sensitive cell line, 1420.

Figure 4-2. Constitutive overexpression of Grp78 protects 1420 cells from 2-DG-induced toxicity. (A.) 1420 cells were stably transfected with a plasmid to overexpress either Grp78 or GFP and then were treated with 2-DG for 72 h in normoxia and percent dead cells was assayed by trypan blue exclusion. The bars represent the average of triplicate samples +/- SD. P-values shown compared oxGrp78 with oxGFP. (B.) 1420, oxGrp78 and
oxGFP cell lines were treated with 2-DG for 24 h and then harvested and immunoblotting was performed to detect protein levels of Grp78 and PERK. β-Actin was used as a loading control. (*) denotes p value < 0.05.

### 4.3. Autophagy does not appear to play a role in 2-DG resistance

Autophagy is a mechanism by which a cell degrades unnecessary or damaged components via lysosomal activity [82]. Through the process of macroautophagy, cellular constituents including organelles can be recycled and as such this pathway acts as a rescue mechanism in times of stress due to starvation [83]. Macroautophagy involves the formation of a double membrane around the damaged organelle known as an autophagosome [84]. This structure then fuses with a lysosome and the contents are degraded by the organelle’s enzymes and finally can be recycled. The following proteins work in concert to induce autophagosome development: class 3 phosphoinositide-3-kinase (PI3K), the autophagy-related genes (Atg), Beclin-1, and ubiquitin-like conjugation reactions [85]. Tumor cells have been shown to activate autophagy in response to stress as well as to increased metabolic demands due to rapid proliferation. Autophagy can enable survival by maintaining energy levels and this can result in tumor growth and therapeutic resistance. Moreover, inhibition of autophagy has been shown to enhance radio and chemo-sensitivity and increase tumor cell death [86, 87].

Agents that either induce or inhibit autophagy were used to assess if this pathway contributes to 2-DG resistance. Rapamycin, a compound that stimulates autophagy through blockage of mTOR, a cellular inhibitor of autophagy, showed
slight protection of sensitive cells, 1420, from 2-DG toxicity (Fig. 4-3a), [88]. However, 3-Methyadenine (3-MA), an inhibitor of autophagic sequestration, did not reverse resistance to 2-DG in 1469 or 14DG5 cells (Fig. 4-3b), [89]. Interestingly, 3-MA did increase the toxicity of 2-DG in 1420 and 14DG2. Thus we conclude that autophagy does not significantly contribute to 2-DG resistance in the cell lines examined here but may play a role in protecting 1420 cells from even more 2-DG damage. This hypothesis will require further study.

Figure 4-3. Autophagy did not contribute to 2-DG resistance. (A.) Cells were treated with 0.1μg/ml of the autophagy activator Rapamycin, 4mM 2-DG, or the combination for 72 h in normoxia and percent dead cells was assayed by trypan blue exclusion. The bars represent the average of triplicate samples +/- SD. (B.) Cells were treated with 10mM of the autophagy inhibitor 3-MA, 4mM 2-DG, or the combination for 72 h in normoxia and percent dead cells was assayed by trypan blue exclusion. The bars represent the average of triplicate samples +/- SD.

4.4. Tunicamycin, a LLO synthesis inhibitor, is toxic to 1420 and 14DG2

Thus far, we have established that 2-DG causes UPR induction and in a sensitive cell line high UPR activation correlates with cell death. Additionally, we demonstrated that modulation of the UPR affects 2-DG sensitivity, thus we
examined pathways upstream of UPR, such as uptake, to address whether differences in UPR were due to its transducers or if activation differences were due to pathways that converge at the UPR. In order to assess 2DG’s mechanism of interfering with glycosylation we used other ER stress-inducing agents such as tunicamycin (TM), a classic ER stress-inducing agent that directly inhibits glycosylation by blocking the first step in LLO synthesis. Both 1420 and 14DG2 exhibited similar levels of cytotoxicity and strong induction of Grp78, p-eif2α and CHOP protein and mRNA in response to TM treatment (Fig. 4-4a, b and c), [90]. Conversely, 1469 and 14DG5 were found to be comparatively resistant to TM and did not induce the UPR markers as highly as 1420 and 14DG2 (Fig. 4-4a, b, and c). Since 14DG2’s TM response is similar to that of 1420, its reduced uptake of 2-DG may best explain its resistance to the sugar analog. However, 1469 and 14DG5 are resistant to both 2-DG and TM suggesting that these cell lines are able to manage glycosylation interference by yet unknown mechanisms.
Figure 4-4. 1420 and 14DG2 respond similarly to TM, while 1469 and 14DG5 both display resistance to TM. (A.) 1420, 14DG2, 1469 and 14DG5 cell lines were treated with the indicated doses of TM for 72 h in normoxia and percent dead cells was assayed by trypan blue exclusion. The bars represent the average of triplicate samples +/- SD. (B.) mRNA levels of Grp78 and CHOP were determined by qPCR in cells treated for 24 h with 0.1ug/ml TM and normalized to actin levels and then to untreated control cells. The bars represent the average of duplicate samples. (C.) Cells were treated with the indicated doses of TM for 24 h in normoxia then harvested and immunoblotting was performed to detect protein levels of Grp78 and CHOP. β-Actin was used as a loading control. Quantification can be seen to the right of blot. (*) denotes p value < 0.05 and (**) denotes p value < 0.01.
4.5. Brefeldin A, an ER stressor that does not affect glycosylation, is toxic to 1420, 14DG2 and 14DG5

The TM findings in 1469 and 14DG5 led us to test whether these cell lines have acquired the ability to resist ER stress in general. To address this question Brefeldin A (BFA), an agent that induces ER stress by inhibiting the transport of proteins from the ER to the Golgi, was used (Nebenfuhr 2002), [91]. Little difference in sensitivity and induction of the UPR markers Grp78, p-eif2α and CHOP protein and mRNA was found between 1420, 14DG2 and 14DG5 when using BFA which differs from both 2-DG and TM in not interfering with glycosylation and yet inducing ER stress (Fig. 4-5a, b, and c). Data with BFA indicate that 14DG2 and 14DG5’s resistance to 2-DG is specific to 2-DG’s effects on glycosylation and imply that these cells are not resistant to other mechanisms by which ER stress is generated. However, 1469 was resistant to BFA as well as to 2-DG and TM suggesting that this cell line has a general advantage that enables it to manage ER stress and or apoptosis.
Figure 4-5. 1420, 14DG2 and 14DG5 all display sensitivity to BFA while 1469 demonstrates resistance. (A.) 1420, 14DG2, 1469 and 14DG5 cell lines were treated with the indicated doses of Brefeldin A (BFA) for 72 h in normoxia and percent dead cells was assayed by trypan blue exclusion. The bars represent the average of triplicate samples +/- SD. (B.) mRNA levels of Grp78 and CHOP were determined by qPCR in cells treated for 24 h with 100nM BFA and normalized to actin levels and then to untreated control cells. The bars represent the average of duplicate samples. (C.) Cells were treated with BFA for 24 h in normoxia then harvested and immunoblotting was performed to detect protein levels of Grp78 and CHOP. β-Actin was used as a loading control. Quantification can be seen to the right. (**) denotes p value< 0.01.
4.6. Bortezomib, an inhibitor of proteasome function, is toxic to 1469

In addition to its role in aiding protein folding, the UPR can also ubiquitin tag misfolded proteins designating them to be broken down in the proteasome, a process known as endoplasmic reticulum-associated protein degradation (ERAD) [92]. The proteasome breaks down aberrantly folded proteins by proteolysis and can recycle their amino acids as well as any post-translational modifiers such as oligosaccharides and phosphate groups. Bortezomib is an inhibitor of the 26S proteasome and has had success as an anticancer agent in multiple myeloma (MM), [93]. MM tumors produce large quantities of antibodies and thus are reliant on proper protein production including glycosylation [94]. Bortezomib is highly toxic to these tumors since they require proteasomal functioning. Interestingly, 1469 was uniquely sensitive to low-dose Bortezomib treatment while the other three cell lines were relatively resistant (Fig. 4-6). This suggests that 1469 is using proteasomal degradation to manage ER stress issues which could explain why this cell line is resistant to the other ER stressing agents tested: 2-DG, TM and BFA.

Overall, our results with ER stress-inducing agents, summarized in Table 4, suggest that there are multiple mechanisms of resistance to 2-DG and that 1469, 14DG2 and 14DG5 employ different mechanisms or combinations of mechanisms of resistance in response to 2-DG. Since 14DG2 displays similar levels of sensitivity as its parental cell line to TM and BFA, lower uptake of 2-DG coincides best with its resistance (Fig. 3a). In contrast, the greater accumulation of 2-DG in 14DG5 as compared to its parental cell line indicates that
mechanisms other than uptake must account for its resistance. 1469 was found to be resistant to all three ER stress inducing agents, indicating that this cell line can cope with issues in this pathway, perhaps by enhanced proteasomal degradation since this cell line is sensitive to Bortezomib. Additionally, these data, together with the findings of blunted responses to agents that interfere with glycosylation suggest that the mechanism(s) of resistance to 2-DG in 14DG5 lie upstream of ER stress/UPR.

Figure 4-6. 1469 cells are sensitive to proteasome inhibition. Cells were treated with the indicated doses of Bortezomib for 72 h in normoxia and percent dead cells was assayed by trypan blue exclusion. The bars represent the average of triplicate samples +/- SD. (*) denotes p value < 0.05.

Table 4: Overview of UPR response and cytotoxicity in each cell line when challenged with ER stressing agents

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>2-DG Response</th>
<th>TM Response</th>
<th>BFA Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1420</td>
<td>Response</td>
<td>Sensitive</td>
<td>Sensitive</td>
</tr>
<tr>
<td></td>
<td>UPR Induction</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>14DG2</td>
<td>Response</td>
<td>Resistant</td>
<td>Sensitive</td>
</tr>
<tr>
<td></td>
<td>UPR Induction</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>1469</td>
<td>Response</td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td></td>
<td>UPR Induction</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14DG5</td>
<td>Response</td>
<td>Resistant</td>
<td>Sensitive</td>
</tr>
<tr>
<td></td>
<td>UPR Induction</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Chapter 5: Glycosylation

Previously, our lab demonstrated that 2-DG toxicity in 1420 cells was due to its obstruction of N-linked glycosylation and this interference caused UPR-mediated cell death [48, 51]. Prior to glycosylation, LLOs are formed in the ER membrane and then transferred from dolichol, a lipid anchor, to an asparagine residue on a growing polypeptide [95]. Glycosylation directs a protein’s folding and hence its function. Although unglycosylated proteins have been shown to fold properly in some conditions, they are quickly degraded suggesting that glycosylation is also important for protein stability [96].

It was previously reported that Chinese Hamster Ovary (CHO) cell lines that were selected for resistance to tunicamycin showed increased expression of the first enzyme of LLO synthesis, GlcNAc-1-P-transferase (GPT), [97]. Thus, resistance to 2-DG could involve a similar amplification of genes and/or components related to LLO production.

5.1 LLO synthesis is enhanced in 2-DG resistant cell lines

In prior reports, our lab showed that when human pancreatic tumor cell lines 1469 and 1420 were treated with 2-DG, intrinsic resistance to this sugar analog in 1469 correlated with decreased reduction of fully formed LLOs as compared to the high repression found in 2-DG sensitive 1420 cells. Here we find that 2-DG at 2mM decreased levels of G_{3}M_{9}, the fully formed LLO, in 14DG2 but not to the degree observed previously in 2-DG treated 1420 cells where the dose of 2-DG was markedly lower (0.5mM), [48]. In the more resistant cell line 14DG5,
LLO levels remained unaffected even at a higher dose of 2-DG (5mM), (Fig. 5-1). Additionally, the two 2-DG resistant variants display basal upregulation of fully formed LLOs (G₃M₉) as compared to the parental cell line (Fig. 5-1).

![Image](image.png)

Figure 5-1. 14DG2 and 14DG5 display increased basal LLO synthesis as can be seen in Fluorophore-Assisted Carbohydrate Electrophoresis (FACE) correlating with their resistance to 2-DG and lowered UPR induction. Lane1: 1420 untreated; 2: 1420+ 2mM 2-DG; 3: 14DG2 untreated; 4: 14DG2+ 2mM 2-DG 5: 1469 untreated; 6: 1469+ 5mM 2-DG; 7: 14DG5 untreated; 8: 14DG5+ 5mM 2-DG.

5.2 UPR Array and Cholesterol synthesis

In general, 2-DG sensitivity appears to correlate with increased activation of the UPR by western blot and qPCR (Fig. 3-2). An analysis of genes related to the UPR was performed using a PCR Array technique. Each cell line displayed a unique genetic profile of UPR-related genes. Surprisingly, amplified mRNA levels of genes related to cholesterol synthesis were found in the resistant variants but not in the parental line (Fig. 5-2). This pathway is responsible for the generation of dolichol, the lipid anchor onto which LLOs are synthesized prior to their
transfer to polypeptides. The following genes were found to be amplified in resistant cell lines 14DG2 and 14DG5 as compared to 1420: sterol regulatory element binding transcription factor 1 (SREBF1), sterol regulatory element binding transcription factor 2 (SREBF2), insulin induced gene 1 (INSIG1), insulin induced gene 2 (INSIG2), SREBF chaperone (SCAP), membrane-bound transcription factor peptidase site 1 (MBTPS1), and membrane-bound transcription factor peptidase site 2 (MBTPS2). Taken together, our data indicate that there are multiple alterations in biologic processes related to ER stress between sensitive and resistant cells. Higher intrinsic levels of LLOs and their insensitivity to 2-DG at 5mM in 14DG5 correlate well with the degree of resistance to 2-DG in this cell line while in 14DG2 lower LLOs when treated with 2mM 2-DG reflect its lower level of resistance.

Figure 5-2. 14DG2 and 14DG5 appear to upregulate production of the cholesterol pathway to generate dolichol for LLO synthesis. A PCR Array of genes related to the cholesterol synthesis pathway is shown here. Fold change is normalized to a panel of housekeeping genes including GAPDH and tubulin. 1420 mRNA levels are set at 1.0.
5.3 Inhibition of cholesterol synthesis sensitizes resistant cells to 2-DG

Amplified cholesterol synthesis gene levels in the resistant cell lines correlate with the enhanced basal LLOs found in these cell types. Increased LLO production could provide these cells with a large pool of glycosylation precursors that may compete with LLOs that have 2-DG incorporated, thus protecting the cells from ER stress and UPR induction. To test this hypothesis, we utilized statins, a well-known class of drugs that inhibit HMG CoA-Reductase, the first enzyme of cholesterol synthesis [98]. Simvastatin sensitized 14DG2 and 14DG5 to 2-DG treatment but had little effect on 1420 and 1469 (Fig. 5-3a). This correlates with the findings of amplified cholesterol synthesis genes and increased basal LLO levels in 14DG2 and 14DG5. However, the combination treatment did not induce UPR, according to Grp78 and CHOP western blots, thus the mechanism of death will need to be explored further (Fig. 5-3b).

Figure 5-3. Inhibition of the dolichol synthesis pathway sensitizes resistant cells to 2-DG. (A.) Cells were treated with 4mM 2-DG, 5uM simvastatin or the combination for 72 h in normoxia and percent dead cells was assayed by trypan blue exclusion. The bars represent the average of triplicate
samples +/- SD. (B.) Cells were treated with 2-DG, simvastatin or the combination for 24 h in normoxia then harvested and immunoblotting was performed to detect protein levels of Grp78, CHOP, ATF6(s) and phospho-eif2α. β-Actin was used as a loading control. (*** denotes p value < 0.001.
Chapter 6: Resistance to GS

Glycogen storage and utilization has long been recognized as a means for supplying glucose under environmental conditions of nutrient limitation. When ample supplies are present, glucose will be stored as glycogen in granules found throughout the cytoplasm of a cell. The starch can then be broken down to provide glucose in times of limited supply. Many cellular pathways manage glycogen storage including: Akt, AMPK, glycogen synthase kinase beta (GSK3β), insulin signaling and more. All of these pathways recognize sugar shortage and activate compensatory mechanisms. Tumor cells’ glycogen stores have been found to be similar to or greater than amounts found in untransformed healthy cells. This is surprising as tumor cells are thought to put most of their resources toward rapid proliferation and therefore would be unlikely to store nutrients. The ability of cancer cells to store glycogen has yet to be explored as a means for therapeutic intervention. However, since this storage occurs at relatively high rates, it may provide a selective window for anticancer agents. The finding of differential responses to GS in cell lines that are sensitive and resistant to 2-DG and previous reports of enhanced glycogen storage in cancers, lead us to investigate glycogen metabolism in these cell lines.

6.1 2-DG resistant cell lines have low protein and mRNA levels of glycogen storage enzymes

According to western blot analysis the protein level of the enzyme required for synthesis of glycogen, glycogen synthase (GYS), was found to be
slightly reduced in 14DG2 and 14DG5 as compared with 1420 (Fig. 6-1a).

However, these three cell lines displayed similar levels of glycogen staining under basal conditions (Fig. 6-1c). On the other hand, 1469 exhibited decreased protein and mRNA levels of GYS than the other cell lines, which correlated with a finding of lower basal glycogen staining in this cell line (Fig. 6-1c). Furthermore, strikingly reduced protein and mRNA levels of the enzyme required for the breakdown of glycogen for its utilization, glycogen phosphorylase (PYGB), were found in 14DG2 and 14DG5 as compared with their parental cell line 1420 (Fig. 6-1a and b).

Figure 6-1. Protein and mRNA levels of PYGB and/or GYS are decreased in 2-DG resistant cell lines. (A.) Western blotting was performed to detect basal protein levels of GYS and PYGB in the four cell lines. β-Actin was
used as a loading control. (B.) mRNA levels of GYS and PYGB were
determined by qPCR in untreated cells. The bars represent the average of
duplicate samples +/- SD. (C.) Cells were grown on coverslips overnight in
normal media then incubated with anti-glycogen monoclonal antibody
(pink) and mounted with dapi (blue). Slides are imaged at 20X.

6.2 Glycogen staining correlates with reduced ability to store and/or
breakdown glycogen in 14DG2, 1469 and 14DG5 cells

In further support of the interpretation that reduced PYGB plays a role in
sensitivity to GS, we find that after 24 h in this condition 14DG2 and 14DG5
maintained near control levels of glycogen suggesting that these cell lines cannot
utilize glycogen when deprived of glucose (Fig. 6-2). On the contrary, 1420
showed reduced glycogen staining after 24 h GS, indicating its ability to
breakdown glycogen, which correlates with higher PYGB levels in this cell line.
Additionally, 1469 which exhibited lower basal glycogen then the other cell lines
also appears able to breakdown the storage molecule as it displayed reduced
staining after GS (Fig. 6-2).

![Image of glycogen staining](image)

Figure 6-2. Glycogen staining correlates with reduced ability to store and/or
breakdown glycogen in 14DG2, 1469 and 14DG5 cells. Cells were grown on
coverslips in glucose free media overnight then incubated with anti-
glycogen monoclonal antibody (pink) and mounted with dapi (blue). Slides
are imaged at 20 and 60X.
6.3 Inhibition of PYGB in 1420 restores sensitivity to GS

To test the hypothesis that PYGB is required for glycogen breakdown and protects a cell from GS, caffeine, a PYGB inhibitor was employed. Caffeine was found to increase 1420’s GS toxicity to levels similar to those found in GS challenged 14DG2 and 14DG5 cells demonstrating a survival role for glycogen breakdown (Fig. 6-3a), [99]. Further supporting the caffeine data, siRNA directed at PYGB also significantly increased the sensitivity of 1420 cells to GS (Fig. 6-3b and c).

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

Figure 6-3. Inhibition of PYGB in 1420 restores sensitivity to GS. (A.) 1420
and 14DG2 cell lines were treated with 5mM caffeine, GS or the combination for 72 h in normoxic conditions and the percent dead cells was assayed by trypan blue exclusion. The bars represent the average of triplicate samples +/- SD. (B.) 1420 and 14DG2 cell lines were transfected with siRNA directed at PYGB or luciferase as a control for 24 h. Cells were then treated with GS for 72 h in normoxic conditions and the percent dead cells was assayed by trypan blue exclusion. The bars represent the average of duplicate samples +/- SD. (C.) Western blot analysis was used to determine protein inhibition by siPYGB in 1420 and 14DG2 cell lines. β-Actin was used as a loading control. (**) denotes p value < 0.01.

6.4. PYGB expression in NM cells

Additionally, to assess the effect of 2-DG on PYGB expression 14DG2 and 14DG5 cells were maintained in the absence of 2-DG for extended periods (>8 weeks) to create cell lines 14DG2NM and 14DG5NM, respectively. 14DG2NM and 14DG5NM displayed nearly complete and partial reversals, respectively, in their sensitivity to GS as compared with 14DG2 and 14DG5 (Fig. 3-3b). This correlates with an increase of PYGB protein and mRNA levels in the NM cell lines as compared to the levels found in 14DG2 and 14DG5 suggesting that maintenance of the resistant cell lines in 2-DG is responsible (at least in part) for the downregulation of PYGB (Fig. 6-4a and b).

Additionally, 14DG2NM reverts to a nearly parental phenotype in that it regains 2-DG sensitivity; however 14DG5NM maintains a similar level of resistance to 2-DG as 14DG5 (Fig. 3-3a). These data again support our previous assertion that there are fundamental differences between 2-DG treatment and GS. Overall, our data indicate that impairment in glycogen breakdown, in the form of downregulation of PYGB in 2-DG resistant cells renders them sensitive to GS. Moreover, in cells with sufficient PYGB, blockage of this enzyme leading to GS sensitivity identifies it as a possible target for therapy.
Figure 6-4. Resistant cell lines not maintained with 2-DG display unique phenotypes. (A.) Cell lines treated with 1mM 2-DG for 24 h under normoxic conditions were harvested and immunoblotted to detect PYGB levels. β-Actin was used as a loading control. (B.) mRNA levels of PYGB were determined by qPCR in untreated cells and normalized to actin levels and then to untreated control cells. The bars represent the average of duplicate samples.

6.5 Increased ROS levels correlate with cell death in response to GS

Glycogen provides a cell with glucose, which can then go on to supply many metabolic pathways. As was previously mentioned, glucose is essential for glycolysis and subsequent ATP production while it also provides intermediates for glycosylation and other protein modifications. In addition, glucose can be funneled from glycolysis to the pentose phosphate pathway (PPP) where it is used to produce antioxidants that protect a cell from reactive oxygen species (ROS). Therefore, glucose starvation has many effects on a cell, which can activate survival mechanisms or lead to cell death.

Cell death due to glucose limitation is typically thought of as being due to energy deprivation. However, we find that all cell lines tested here demonstrate similar large drops in ATP due to GS (Fig. 6-5a). Thus, we do not find evidence of a correlation between ATP reduction and GS cell death. To this end, basal
ROS levels were found to be greater in 14DG5 and 14DG2 than in 1420 (Fig. 6-5b). Furthermore, GS caused greater increases in ROS in 14DG2 and 14DG5 than in 1420 suggesting a potential cause of GS-induced cell death (data not shown). Further exploration will be required to understand why ROS levels increase even more in 14DG2 and 14DG5 when starved of glucose and whether or not this can be attributed to their GS sensitivity.

**Figure 6-5.** Molecular mechanisms of GS-induced cell death. (A.) Intracellular ATP levels were measured in control conditions and after 24 h glucose starvation. (B.) Cell lines selected for 2-DG resistance display increased basal reactive oxygen species as compared with the parental cell line. Intracellular ROS levels were measured by flow cytometry using the ROS sensor CM-H$_2$DCFDA. 1420 is shown in black, 14DG2 in red and 14DG5 in blue.
Chapter 7: Discussion

At present, the paradigm of cancer treatment is shifting from the traditional use of anti-mitotic chemotherapeutic agents and radiation therapy to tumor specific therapies. New first line treatment modalities include immune modulation, use of antibodies which target specific upregulated pathways of a tumor, hormone ablation, and more [100, 101]. However, a need still exists for selective, safe therapies that are effective at killing a variety of tumor types. Thus, in this work we examine exploitation of the enhanced glucose metabolism found in most if not all tumors by using the glycolysis and glycosylation inhibitor, 2-DG. Due to its dual inhibitory functions, 2-DG has the ability to target multiple pathways in a tumor while sparing nearby healthy cells since they do not demonstrate the enhanced sugar uptake found in cancer cells. Previously, 2-DG was shown to have anti-cancer activity in \textit{in vitro} and \textit{in vivo} tumor models under hypoxia due to its inhibition of ATP production. In addition, 2-DG has demonstrated efficacy in killing some cancer cell lines under normoxic conditions due to its glycosylation interference and subsequent ER stress stimulation. In this work we address 2-DG’s function in cancer cells under normoxic conditions and the possibility of the emergence of resistance to 2-DG in these circumstances.

2-DG’s blockage of glycolysis through inhibition of two major glycolytic enzymes, HK and PGI, renders a cell unable to fully process glucose and thus mimics a glucose-deprived state. Cancer cells are known to naturally encounter periods of starvation due to rapid use of nutrients and aberrant angiogenesis resulting in malfunctioning blood vessels unable to deliver nutrients efficiently;
however, this does not typically result in tumor cell death. Thus, we investigated whether cells adapt to 2-DG challenge using the same mechanisms they employ to survive natural glucose deprivation. Overall, since 2-DG is thought of as a glucose starvation mimetic and physiologic glucose restriction is a common occurrence in tumor cell progression, we also examined this condition in the same cell lines.

7.1 2-DG Resistance and Apoptosis

To investigate 2-DG resistance we compared a cell line that is sensitive to 2-DG (1420) with cell lines selected for resistance (14DG2 and 14DG5) and with a cell line that displays intrinsic resistance (1469) to the sugar analog. There are many potential mechanisms of resistance that can occur at various stages of drug processing in a cell. Assuming the drug’s target remains unchanged, resistance can be due to either exclusion or metabolism. Resistance by exclusion involves either reduced uptake or increased export of the agent while resistance by metabolism is modification of the drug to a non-toxic form. Additionally, alterations in drug target can confer resistance. Examples of this include: elimination of target (e.g. induction of an alternative pathway), alteration of the target’s affinity for the drug and increased production of the target (e.g. gene amplification). With these mechanisms in mind we assessed 2-DG’s affects in the four pancreatic cancer cell lines.

2-DG cell death was accompanied by increased CHOP expression suggesting that sensitive cells are succumbing to ER-mediated apoptosis.
Therefore, anti-apoptotic pathways were analyzed to determine whether they contribute to resistance. The intrinsically resistant cell line, 1469, displayed greater Bcl-xl than the other cell lines, while 14DG2 and 14DG5 displayed slightly elevated Bcl-2 (Fig. 3-1d). The increased anti-apoptotic proteins found in 14DG2, 1469 and 14DG5 correlate with their 2-DG resistance. However, 14DG2 and 14DG5 were not found to be resistant to agents such as the anti-mitotic drug etoposide that induce death through pathways other than metabolism (Fig. 3-1c). Thus, the increased anti-apoptotic proteins in these cell types do not appear to account for their 2-DG resistance. On the contrary, 1469 was found to be relatively resistant to etoposide correlating with its enhanced expression of Bcl-xl. Thus, in this cell line, anti-apoptotic mechanisms may confer resistance to 2-DG as well as to other cytotoxic agents.

### 7.2 2-DG Resistance and Metabolism

Next, resistance by exclusion was found to be a potential mechanism in 14DG2 and 1469 since 2-DG uptake was reduced as compared with 1420 (Fig. 3-4a). 1469's low levels of HK2 protein could explain its low uptake since this correlates with 1469's lower level of 2-DG-6-P than 1420 according to mass spectrometry studies (Fig. 3-4b and c). Unfortunately, further investigation of hexokinase and glycolysis's contribution to 2-DG resistance was difficult since glucose requires the same enzymes to enter a cell and be processed. However, studies of lactate and respiration taken together suggest that 14DG2 and 1469 are less glycolytic than 1420 (Fig. 3-5a and b). Since these cell lines tend to rely less on glycolysis, they display reduced hexose uptake, hence the reduced 2-DG
uptake which is thus a mechanism of resistance. This explanation does not hold for 14DG5, which displays greater 2-DG uptake than 1420 as well as higher levels of lactate production and respiration. Overall, 14DG5 is a highly metabolic cell type although it has a slower doubling time than the other three cell lines (data not shown) and thus it may be able to compensate for 2-DG treatment since it is also producing energy through other mechanisms and is likely also taking up more glucose and mannose than the other cell lines.

7.3 Inverse relationship between 2-DG and GS

2-DG’s dual activities, inhibiting both glycolysis and glycosylation, underlie its traditional use as a glucose deprivation mimetic. However, the findings presented here of an inverse relationship between cells that have acquired resistance or are intrinsically resistant to 2-DG treatment and their increased sensitivity to GS indicate that there are inherent differences between these two types of glucose restriction (Fig. 3-1a and b). Moreover, 2-DG is found to differentially induce UPR in sensitive vs. resistant cells whereas GS does not (Fig. 3-2). These variances in response to therapeutic vs. physiologic forms of glucose restriction may be better understood by considering how each condition affects glucose metabolism. It has been shown that 2-DG can act as a mannose mimic and thus be incorporated into LLOs thereby interfering with their normal synthesis and subsequently causing aberrant protein folding resulting in ER stress and UPR activation [48, 51, 102, 103]. Although GS also induces ER stress, its mechanism differs from that of 2-DG in that 2-DG is fraudulently incorporated into LLOs, while GS reduces the availability of LLO sugar
precursors thereby producing proteins which contain either a reduced number of N-glycans and/or smaller LLO species [65]. Furthermore, our findings that under GS, 2-DG sensitive and resistant cell lines undergo similar UPR activation but differential sensitivity to this type of glucose restriction suggest that mechanisms other than ER stress/UPR may be responsible for cell death. This is in contrast to 2-DG treated cells where increased induction of UPR (indicating increased ER stress) correlates with cell death (Figs. 3-1 and 3-2).

When studying resistance it is important to determine whether mechanisms arise due to presence of the agent or to changes forced by previously encountering the agent. Thus, the cell lines selected for resistance were grown without 2-DG supplementation for greater than 8 weeks. Thus, we developed cell line 14DG2NM from 14DG2 and 14DG5NM from 14DG5. When we compared these cell types with their parental cell line as well as 1420, we found that 14DG2NM’s 2-DG resistance and GS sensitivity were reversed (Fig 3-3). Thus, we conclude that 14DG2’s responses are at least in part due to the continued presence of 2-DG in experimental samples. On the other hand, 14DG5NM retained 14DG5’s 2-DG resistance and saw only a partial reversal in GS sensitivity. Furthermore, the reversals in sensitivity to GS in both 14DG2NM and 14DG5NM cell lines were accompanied by increases in PYGB protein levels over those found in 14DG2 and 14DG5 (Fig. 6-4a and b).
7.4 UPR and ER stress role in 2-DG response

A clear correlation was established between sensitivity to 2-DG and high induction of UPR both at the protein and mRNA levels. Thus, manipulation of this pathway was investigated to confirm its role in 2-DG response. VST, an inhibitor of Grp78 induction, was found to synergize with 2-DG and cause cell death in the 2-DG resistant cell lines (Fig. 4-1a and b). This finding was supported with similar experiments using siRNA to genetically knock down Grp78 expression (Fig 4-1c and d). Thus, although Grp78 induction correlated with cell death in response to 2-DG in 1420, inhibition of this chaperone sensitized the resistant cell lines to 2-DG. It is of note that inhibition of hsp90 by use of geldanamycin derivatives such as 17-DMAG did not enhance 2-DG toxicity (Fig. 4-1e). This supports our assertion that 2-DG causes ER specific cellular stress and not a general stress response as that found with heat shock protein induction.

Furthermore, overexpression of Grp78 was found to protect 1420 cells from 2-DG cell death (Fig. 4-2). Therefore, we hypothesize that there are multiple cellular compartments of Grp78 and they function differently in response to protein misfolding. Free Grp78 in the ER directly bind to misfolded proteins and help correct their structures. Additionally, Grp78 can exist along the ER membrane bound to the UPR transducers. Thus, if the free Grp78 can handle the accumulation of misfolded proteins, activation of the transducers can be avoided and their downstream effects including apoptosis evaded. When we inhibit Grp78 by VST or siRNA, free Grp78 may be reduced and ER stress would then cause greater activation of the UPR transducers and lead to cell death. Contrarily,
overexpression of Grp78 may enhance the free Grp78 and thus circumvent UPR transducer activation to thereby protect 1420 cells.

Autophagy, a process of cell part recycling, was also assessed as a potential resistance mechanism. Rapamycin and 3-MA were employed to induce or inhibit, respectively, autophagy [104, 105]. Here, we find that rapamycin slightly decreased 2-DG’s toxicity in 1420 but did not affect the 2-DG responses of the resistant cell lines (Fig. 4-3a). Likewise, 3-MA did not enhance the toxicity of 2-DG in the resistant cell lines but did slightly increase 2-DG’s toxicity in 1420 (Fig. 4-3b). Overall, we conclude that autophagy is not a mechanism of resistance in 14DG2, 1469 or 14DG5, but it does appear to play a role in 1420’s 2-DG response.

7.5 Glycosylation and 2-DG resistance

The UPR is generally considered to be a cell protective mechanism; however, it is known that with increased and prolonged activation, cells are signaled to undergo UPR-mediated apoptosis [106]. Indeed here we show that excessively high UPR induction observed in the sensitive cell line, 1420, when treated with 2-DG represents a shift from the protective to the apoptotic arm of the response (Fig. 3-2a and b). By contrast, 2-DG resistant cell lines showed little to no increase in UPR when challenged with 2-DG concentrations that induced UPR in 1420 cells. This latter result suggests that pathways upstream of ER stress may be responsible for resistance to 2-DG and in the case of 14DG2 decreased 2-DG uptake accounts (at least in part) for its level of resistance.
Moreover, 14DG2 is found not to be resistant to the other ER stressing agents examined and shows robust UPR induction in response to these compounds (TM and BFA, Fig. 4-4 and 4-5), supporting our contention that reduced 2-DG uptake in this cell line appears to be a major component contributing to its resistance (Fig. 3-4). On the other hand, greater 2-DG uptake is found in 14DG5 as compared to the parental cell line. Additionally, 14DG5 is also resistant to TM, an agent that induces ER stress by interfering with LLO synthesis, but this cell line is not resistant to BFA which blocks egress of fully formed glycoproteins from the ER (Fig. 4-6). Collectively, the results from sections 3 and 4 suggest that resistance to 2-DG in 14DG5 is due to mechanisms downstream of uptake but upstream of ER stress/UPR and that are likely related to the processes of LLO synthesis and N-linked glycosylation. This contention is supported by our findings where 2-DG more strongly inhibited LLO production in the sensitive cell line than it did in the resistant variants (Fig. 5-1). The increased basal fully formed LLOs (G₃M₉) in 14DG2 and 14DG5 as compared with 1420 also support this idea. Furthermore, genes related to cholesterol synthesis were found to be upregulated in 14DG2 and 14DG5 as compared with the parental cell line (Fig. 5-2). This pathway is critical for the production of dolichol, the lipid anchor of and thus integral precursor of N-linked glycosylation [107]. The upregulation in cholesterol gene expression and increased LLO synthesis found in resistant cell lines is suggestive of a compensatory mechanism to deal with 2-DG’s glycosylation interference. However, since 14DG2 cells are not resistant to TM in spite of their increased LLO levels, this mechanism may not play a substantial
role in this cell line’s 2-DG resistance. These correlative data may be better explained by further exploration of the relationship between the cholesterol synthesis pathway, LLO generation, UPR, and 2-DG resistance. Overall, the results presented here suggest that there are multiple mechanisms that alone or in concert cause resistance to 2-DG and manipulation of these pathways may be useful in combination with 2-DG treatment in a clinical setting.

7.6 GS sensitivity correlates with increased ROS production in sensitive cell lines

Similar to data presented here, differences in the induction of autophagy were recently reported when tumor cell lines (including 1420) were treated with 2-DG or GS [63]. Although both forms of glucose deprivation were found to induce ER stress leading to autophagy, 2-DG did so by phosphorylating AMPK via CaMKKβ, whereas GS induced autophagy by phosphorylating AMPK through the canonical pathway of ATP depletion as well as by increasing ROS leading to ERK stimulation [108]. Conversely, 2-DG was found to decrease ROS generation when tested in the same cell line. In accordance with this report, preliminary data suggest that the lower PYGB levels found in 14DG2 and 14DG5 correlate with increased basal ROS levels as compared to 1420 (data not shown). It remains to be determined if the increased ROS detected in 14DG2 and 14DG5 leads to sensitivity under GS conditions. However it follows that if ROS is involved with inducing cell death under GS, the breakdown of glycogen in 1420 cells may, at least in part, be responsible for its survival under this condition. A mechanism
that could explain this possibility would be that the glucose provided by glycogen could act as a substrate for the pentose phosphate pathway resulting in production of antioxidants (glutathione) thereby reducing ROS levels and adding to the cell’s ability to survive GS [109].

7.7 Glycogen and GS sensitivity

Although the mechanism by which PYGB is reduced in 14DG2 and 14DG5 cells leading to decreased survival under GS remains unknown it appears to be related to the selection process for 2-DG resistance. The two mechanisms may be dependent on each other or it may be a case of collateral sensitivity to GS due to 2-DG resistance selection. This point will be better understood by assessing PYGB protein and mRNA levels in other cell lines selected for 2-DG resistance but is suggestive of an intriguing relationship between resistance to 2-DG and sensitivity to glucose deprivation which may have widely applicable therapeutic potential. During the process of tumorigenesis, there will likely be locations and periods of time in which cancer cells outstrip their supply of glucose, therefore survival may become dependent on the capacity to utilize glycogen stores where the activity of glycogen phosphorylase becomes limiting. The ability to reverse GS resistance in 1420 by inhibition of PYGB is suggestive of a potential therapeutic target (Fig. 6-3). Moreover, based on an analysis of 59 NCI tumor cell lines, it has been shown that PYGB is the predominant glycogen phosphorylase isoenzyme found in cancer lines [110]. Adding to this possibility, it has been reported that treatment
with PYGB inhibitors under normal glucose conditions results in growth inhibition in most tumor cell lines tested [110]. These findings imply that anticancer treatment may benefit from the addition of glycogen phosphorylase inhibitors.

7.8 2-DG vs. GS

Throughout this work, we have questioned how selection for 2-DG resistance resulted in cell lines that downregulated PYGB and thus were sensitive to GS. Although at present we do not have clear evidence to explain this, we hypothesize that by having 2-DG present for long periods of time during selection for resistance glucokinase is stimulated. Glucokinase is a high Km isozyme of hexokinase that shuttles glucose directly into glycogen and its activation has been shown to cause cells to produce insulin [111]. Insulin in turn activates glycogen storage while inhibiting glycogen mobilization and thus may influence PYGB levels and activity. This is one possible explanation for how 14DG2 and 14DG5 show PYGB downregulation as compared with their parental cell line.

7.9 Clinical perspective

2-DG has previously shown promise in the clinical setting particularly in cancers where a high percentage of the tumor is anaerobic such as retinoblastoma [45, 112]. Traditional chemotherapy is used to treat the fast growing aerobic portion of the tumor while 2-DG cuts off the energy supply to the hypoxic regions by inhibiting glycolysis. Similarly, 2-DG has shown to sensitize
certain tumors to radiation therapy by inducing oxidative stress via inhibition of glycolysis [113]. Leveraging 2-DG’s use as a glycolytic inhibitor has been successful thus far in sensitizing some tumors to chemo and radiation therapy. In this work, we demonstrate that 2-DG’s inhibition of glycosylation can also be used for anti-cancer therapy. We also show that 2-DG’s effectiveness in this role can be augmented by combining it with drugs such as versipelostatin, targeting the ER stress response, as well as statins, to block cholesterol synthesis and thus inhibit LLO synthesis. Statins have been used for many years to lower cholesterol and have been proven safe for many patients. Thus, further studies of 2-DG and statin combination therapy for cancer are warranted.

7.10 Summary and Conclusions

The prevailing hypothesis that cancer cells upregulate glucose usage but also encounter glucose deprivation makes this an attractive pathway to further consider for cancer therapy [114, 115]. Tumors have been shown to evoke various metabolic pathways including Akt, AMPK and glycogen mobilization to resist death due to GS. Modulation of these pathways as well as inhibition of glucose metabolism may have potential for anti-cancer therapy. 2-DG is a safe yet potent glycolysis and glycosylation inhibitor that is known to preferentially accumulate in cancer cells [116]. Extrapolating from our in vitro findings, clinical use of 2-DG is expected to be hindered by resistance. However, as in data shown here, these 2-DG resistant cells are likely to be sensitive to glucose deprivation, an environmental condition that the tumor will encounter at some
point. Thus, clinical use of 2-DG would be augmented by specific dietary
considerations. Overall, our results in distinguishing between these two different
forms of glucose deprivation highlight the potential of exploiting glucose
restriction for improving the efficacy of cancer therapy.
References


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