AWARD NUMBER: W81XWH-05-1-0036

TITLE: The Effect of Glycolytic Modulation in Prostate Cancer

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REPORT DATE: November 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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Advanced prostate cancer is only temporarily controlled by androgen ablation therapy or chemotherapy, warranting the study of novel approaches. In this regard, recent studies have demonstrated that abnormal growth factor and apoptotic pathways, required by tumor cells to resist multiple insults, can drive tumor cells to even further dependence on glycolysis, supporting a rationale for selectivity of abrogating glycolysis in tumor cells compared to normal cells. Additional recent studies have demonstrated that starvation of tumor cells may induce the process of autophagy, or cell self digestion, and that autophagy may represent a mechanism of tumor cell resistance if allowed to continue only temporarily, followed by cell death if the process of autophagy continues for a prolonged period. In this proposal, we test the hypothesis that modulation of glycolysis will improve clinical results. We therefore hypothesize that 2-deoxyglucose will be safe and active in patients, and abnormal cell pathways such as constitutive activation of Akt, abnormal regulation of autophagy, and other oncogenes may increase sensitivity to inhibition of glycolysis. To test this hypothesis we have the following specific aims: 1. To inhibit glycolysis in patients with prostate cancer in a phase I/II study of 2-deoxyglucose. 2. To determine the mechanism of inhibition of tumor cell growth through modulation of glycolysis.

15. SUBJECT TERMS
2-deoxyglucose, prostate cancer, autophagy
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INTRODUCTION:
One in six men will be diagnosed with prostate cancer in their lifetime. For patients that have tumor recurrence following local therapy, all current standard systemic therapies are inadequate due to mechanisms of tumor resistance. In this proposal, we are developing a new paradigm to target metabolism and mechanisms of resistance to agents that induce metabolic stress in prostate cancer. Prior studies demonstrated the dependence of early tumor growth and progression on anaerobic metabolism through glycolysis. In fact, the preference for tumor cells to depend on glycolysis over normal cells is the basis for the successful development of FDG-PET imaging. Despite these prior data, clinical development of agents that target glycolysis has been limited with initial concern over the lack of a therapeutic window. However, more recent studies have demonstrated that abnormal growth factor and apoptotic pathways, required by tumor cells to resist multiple insults, can drive tumor cells to even further dependence on glycolysis, supporting a rationale for selectivity of abrogating glycolysis in tumor cells compared to normal cells. For example, studies have recently demonstrated that activation of Akt kinase, which occurs commonly in tumor such as prostate cancer that are PTEN deficient, increases dependence on glycolysis.

In this proposal, we have been testing the hypothesis that modulation of glycolysis or the induction of metabolic stress will be active against prostate cancer and that the mechanisms responsible for resistance to metabolic stress can be understood. We began to test 2-deoxyglucose, an inhibitor of glycolysis in a phase I clinical trial while conducting parallel studies to understand resistance mechanisms in preclinical models. Our studies from this proposal have demonstrated that starvation of tumor cells may induce the process of autophagy, or cell self digestion, and that autophagy may represent a mechanism of tumor cell resistance if allowed to continue (1).

Our results have now allowed us to hypothesize that induction of metabolic stress in tumor, as demonstrated with the prototypical glycolytic inhibitor 2-deoxyglucose, will induce autophagy as a resistance mechanism, and future efforts to abrogate autophagy will improve clinical results. We also hypothesize that our initial study of 2-deoxyglucose will be safe and lead to the feasibility of correlative studies in patients, which can be used for future studies of autophagic modulation. The specific aims of this proposal have been:

1. To inhibit glycolysis in patients with prostate cancer in a phase I/II study of 2-deoxyglucose.
2. To determine the mechanism of inhibition of tumor cell growth through modulation of glycolysis.
BODY:

This report represents an update of the annual report submitted Oct 2008; we had recent approval on June 18, 2009 to extend the period of performance to July 31, 2010. New progress in this period includes additional clinical trial accrual, initial correlative assessment of p62 and LC3 protein as markers of autophagy, continued clinical trial follow-up, publication of a clinical abstract in ASCO 2008, and the final publication of preclinical studies proposed in aim 2. The details of new outcomes in aim 1 and 2 are discussed below:

Aim 1:
In this period we have again made progress despite clinical trial delays. In prior reports, clinical trial drug supply and the approval process delayed trial accrual and the original statement of work was modified and approved to allow more time for the clinical trial approval process. The budget period was also revised and approved to begin 7/1/06 and extend to 7/09. Within aim 1, despite these delays, we were successful in obtaining drug supply for 2-deoxyglucose, an FDA IND in August 2006, protocol revisions to include data from ongoing laboratory studies (described below for aim 2) and final approval by the U.S. Army Medical Research and Materiel Command’s Human Subjects Research Review Board (HSRRB) on 10/13/06 (work within Task 4). The trial was then approved by our IRB and activated November 2006 (the time of the prior report). Since that time, an additional delay occurred in early 2007 because of a delay on drug supply, followed by a hold on the study secondary to concern over EKG abnormalities noted in a different study with 2-deoxyglucose combined with docetaxel. To address this concern, we have made an amendment in 2007 to add additional EKG monitoring in the protocol and consent, and these changes were approved by HSRRB and our local IRB.

We are now accruing to the 3rd cohort of the study, but again had a delay in 2008 due to IRB continuing review and a new required amendment, which was approved on 11/05/08. We have obtained PET scans prior to study drug and on day 2 to determine if PET is a marker for drug uptake. The hypothesis that is being tested here is that 2-deoxyglucose drug should block PET tracer uptake, if the drug is taken up by tumor tissue. This would then be predicted to turn a hot spot on a baseline PET to cold on day 2. In the clinical study, 11 patients have now been treated at doses 30, 45 and 60mg/kg/day orally. Therapy was well tolerated with no dose-limiting toxicity. We now have treated 8 patients with prostate cancer, and two patients have received more than 11 cycles and were stable by RECIST criteria. Current toxicity data is shown in Table 1 and data from PET scans in Table 2 and Figure 1.
Table 1: Most common toxicity by grade (n=11 patients)

<table>
<thead>
<tr>
<th>Toxicity</th>
<th>Max Grade</th>
<th>% of Pts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anorexia</td>
<td>1</td>
<td>9%</td>
</tr>
<tr>
<td>AV block</td>
<td>1</td>
<td>9%</td>
</tr>
<tr>
<td>Constipation</td>
<td>2</td>
<td>9%</td>
</tr>
<tr>
<td>Fatigue</td>
<td>3</td>
<td>27%</td>
</tr>
<tr>
<td>QTc Prolonged</td>
<td>3</td>
<td>36%</td>
</tr>
</tbody>
</table>

Table 2: Data on patients with PET at baseline and day 2

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Ca</th>
<th>Dose level</th>
<th>Tumor site</th>
<th>Baseline PET SUV site/Liver</th>
<th>Day 2 PET</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>PC</td>
<td>2</td>
<td>Mediast</td>
<td>9.2/2.0</td>
<td>9.3/2.0</td>
</tr>
<tr>
<td>5</td>
<td>L</td>
<td>2</td>
<td>Chest</td>
<td>9.4/1.5</td>
<td>12.8/1.1</td>
</tr>
<tr>
<td>6</td>
<td>CC</td>
<td>2</td>
<td>Mediast</td>
<td>7.3/1.7</td>
<td>4.7/2.3</td>
</tr>
<tr>
<td>7</td>
<td>PC</td>
<td>3</td>
<td>L1</td>
<td>3.8/3.2</td>
<td>2.4/2.3(Food)</td>
</tr>
<tr>
<td>8</td>
<td>PC</td>
<td>3</td>
<td>Ischium</td>
<td>5.8/2.1</td>
<td>4.0/2.0</td>
</tr>
</tbody>
</table>

Figure 1: Picture of PET scan on day 1 and 2 of a patient with mediastinal tumor demonstrating decreased SUV within one day of initiating 2-DG.
The clinical data were published in an abstract at ASCO 2008 (Attached in appendix). Based on the rationale described in aim 2, recent analysis of LC3 and p62 were completed on 6 patients. P62 decreased in 5/6 patients at 24 hours of therapy in cycle 1. Figure 2 demonstrates p62 and LC3 in the first cycle in 4 patients. Although the pattern of LC3 change is inconsistent, the decrease in p62 over 24 hours is consistent with what would be expected if DG induced autophagy clears p62 protein aggregates.

Aim 2:
Within aim 2, we have already begun to determine mechanisms of resistance, or activity, of 2-deoxyglucose in prostate cancer. Since the prior period, we have completed, revised, submitted and re-submitted a manuscript, which was accepted for publication (Prostate 68: 1743 – 1752, 2008, December 2008 - See attachment in Appendix). Additional laboratory studies have allowed us to find additional correlative markers such as p62 that could be measured in clinical trials. In fact, we found in the laboratory that in response to stress autophagy-defective tumor cells preferentially accumulate p62/SQSTM1 (p62) protein aggregates, endoplasmic reticulum (ER) chaperones, damaged mitochondria, reactive oxygen species (ROS), and genome damage.
Thus, autophagy suppresses oxidative stress and protein, organelle and DNA damage. Suppressing ROS or p62 accumulation provided protection from damage resulting from autophagy defects. Moreover, stress-mediated p62 accumulation caused by defective autophagy stimulated ROS and the DNA damage response and promoted tumorigenesis. These findings suggest that the tumor-suppressive function of autophagy is prevention of sustained p62 accumulation, oxidative damage and genome instability. Thus suppression of autophagy is a mechanism for p62 upregulation. These data are hypothesis generating and allow us to test the hypothesis that p62 will be a marker of the effect of agents that modulate autophagy in clinical trials.
The following represents our planned tasks and status of each:

**Task 1:** Creation of additional isogenic cell lines (months 1-12). We have completed this task and created these additional isogenic cell lines derived from rat prostate epithelial cells in the laboratory of Dr. E. White (Co-investigator), and created cell lines derived from mouse prostate epithelial cells to take advantage of mutant mice models. Dr. White (Co-PI) published a methods paper on generation of immortal mouse epithelial cell lines including iMPECs (Mathew R, Degenhardt K, Haramaty L, Karp CM, White E. Immortalized mouse epithelial cell models to study the role of apoptosis in cancer. Methods Enzymol. 2008;446:77-106.

**Task 2:** Determine the effect of various oncogenes on glycolysis modulation (Months 7-18). We completed this task. We assessed mechanisms of 2-deoxyglucose effect and discovered that 2-deoxyglucose induces autophagy in prostate tumor cell lines. We also assessed the importance of *beclin1* and *akt* on cytotoxicity and autophagy. This finding is important, given recent studies demonstrating the importance of autophagy as a cell death pathway, or resistance pathway (2). Since the prior report, these data are now published in a manuscript (Prostate 68: 1743 – 1752, 2008, December 2008-See attachment in Appendix).

**Task 3:** Determine the effect of various treatments on protein expression (Months 18-36). This was also completed as part of task 2 and is included in the manuscript in the Appendix.

**Task 4.** Complete phase I clinical trial to determine phase II dose (Months 24-36). This task is ongoing, despite multiple unavoidable delays and holds and we have accrued additional patients in the last quarter. As noted above, we have been successful in accruing patients in 3 cohorts. No SAEs have occurred. PET scanning as a marker for drug uptake has been obtained on some patients, as well as peripheral blood LC3 and p62 protein expression as a marker of autophagy. Because autophagy has been found to be regulated by beclin-1, we have also established the ability to assess patient specimens for beclin-1 by immunohistochemistry. These data are included in an abstract accepted at ASCO 2008. Although a concern that the company may have limited drug supply was expressed on our prior report, the data supporting autophagy as a resistance mechanism leads us to conclude that future studies should be testing a combination of agents that induce metabolic stress such as 2DG along with agents that inhibit autophagy. Therefore, our current study will be most helpful to determine the feasibility of markers for use in these additional combination studies, which is one of the aims of the trial and can be completed with fewer patient numbers. We now plan to continue the clinical study with closure of the trial following the full assessment of the correlative markers and clinical data over the next grant period. We have also begun to develop future studies based on these data to use agents that induce metabolic stress with hydroxychloroquine, an inhibitor of autophagy.
**Task 5.** Interim analysis to determine phase II dose (Months 30-36). As noted above, our data supports analysis over the next grant period to determine the feasibility of marker assessment for future studies of combination therapy combined with an agent that will abrogate autophagy.

**Task 6:** Although this task initially planned, to begin a phase II study in patients with hormone refractory prostate cancer (Months 30-36) if drug was available, the limited availability of drug and findings from this project, lead us to conclude that a future study (not part of this proposal) should be designed to assess combination therapy, and completion of our part of the initial study phase I study to determine the feasibility of marker assessment in patients.

**Task 7:** Final Analysis to determine clinical activity of 2-deoxyglucose as a single agent and plan future studies. As noted above, we will be assessing both activity and marker assessment in the current clinical trial over this last grant period. We have already planned and developed future studies, based on these data.
KEY RESEARCH ACCOMPLISHMENTS

1. IND for use of 2-deoxyglucose in clinical trial

2. Full approval of the phase I and II clinical trial


4. Creation of additional isogenic cell lines

5. Finding of a potential mechanism of 2-deoxyglucose cell death modulation


7. Additional enrollment and assessment of p62 and LC3 in patient PBMCs

REPORTABLE OUTCOMES (New since revised last report in bold):

1. IND for drug use (prior report)

2. IRB approved Clinical trial (prior report)


4. Abstract publication ASCO 2007 (prior report)

CONCLUSIONS:

In summary, we have continued productivity within the aims and tasks of this proposal despite delays. The discoveries from the laboratory work (identifying autophagy as an important mechanism that may modulate tumor sensitivity to drugs that induce tumor starvation) and clinic (generating an additional hypothesis regarding the importance of PET imaging) have already been significant, and allow for the foundation to design future clinical trials. We have already been successful in publishing the data from the laboratory portion of this proposal, since the last report, which supports a new paradigm in the treatment of prostate cancer (Prostate 68: 1743 – 1752, 2008, December 2008-See attachment in Appendix). Based on these data generated in this project, we conclude that future studies should be testing a combination of agents that induce metabolic stress such as 2DG along with agents that inhibit autophagy. Therefore, our current study will be most helpful to determine the feasibility of markers for use in these additional combination studies, which is one of the aims of the trial and can be completed with fewer patient numbers. Our initial assessment of correlative markers already suggests that p62 assessment in further studies is warranted. We now plan to continue the clinical study with closure of the trial following the final assessment of the correlative markers of metabolic stress and autophagy over the extended grant period. We have also begun to develop future studies based on these data to use agents that induce metabolic stress with hydroxychloroquine, an inhibitor of autophagy. In fact, these data from this project, using a prototypical metabolic inhibitor such as 2DG, is hypothesis generating that any agent that induces metabolic stress, such as anti-angiogenesis inhibitors, mTOR inhibitors, or TKIs, should be combined with an agent that inhibits autophagy, supporting a new paradigm that should be tested for the treatment of prostate cancer.

REFERENCES:
APPENDIX:


Therapeutic Starvation and Autophagy in Prostate Cancer: A New Paradigm for Targeting Metabolism in Cancer Therapy

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BACKGROUND. Autophagy is a starvation induced cellular process of self-digestion that allows cells to degrade cytoplasmic contents. The understanding of autophagy, as either a mechanism of resistance to therapies that induce metabolic stress, or as a means to cell death, is rapidly expanding and supportive of a new paradigm of therapeutic starvation.

METHODS. To determine the effect of therapeutic starvation in prostate cancer, we studied the effect of the prototypical inhibitor of metabolism, 2-deoxy-D-glucose (2DG), in multiple cellular models including a transfected pEGFP-LC3 autophagy reporter construct in PC-3 and LNCaP cells.

RESULTS. We found that 2DG induced cytotoxicity in PC-3 and LNCaP cells in a dose dependent fashion. We also found that 2DG modulated checkpoint proteins cdk4, and cdk6. Using the transfected pEGFP-LC3 autophagy reporter construct, we found that 2DG induced LC3 membrane translocation, characteristic of autophagy. Furthermore, knockdown of beclin1, an essential regulator of autophagy, abrogated 2DG induced autophagy. Using Western analysis for LC3 protein, we also found increased LC3-II expression in 2DG treated cells, again consistent with autophagy. In an effort to develop markers that may be predictive of autophagy, for assessment in clinical trials, we stained human prostate tumors for Beclin1 by immunohistochemistry (IHC). Additionally, we used a digitized imaging algorithm to quantify Beclin1 staining assessment.

CONCLUSIONS. These data demonstrate the induction of autophagy in prostate cancer by therapeutic starvation with 2DG, and support the feasibility of assessment of markers predictive of autophagy such as Beclin1 that can be utilized in clinical trials. Prostate 68: 1743–1752, 2008.

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Received 7 April 2008; Accepted 15 July 2008

KEY WORDS: prostate cancer; deoxyglucose; beclin1; autophagy; glycolysis; metabolism
One area that has received renewed attention is the metabolic fragility of cancer cells, which preferentially utilize glycolysis to metabolize glucose rather than oxidative phosphorylation. This difference, initially termed “The Warburg effect,” remains one of the fundamental features that distinguishes normal cells from tumor cells [3,4]. Whereas aerobic metabolism can generate 36 molecules of ATP per molecule of glucose, anaerobic glycolysis can only generate two. This fragility is magnified because cancer cells must survive in hostile environments with poor blood supply, limited oxygen, reduced growth factors, limited nutrients, and high metabolic demand. Our prior studies demonstrated induction of multiple glycolytic enzymes resulting from autocrine stimulation specifically in prostate cancer cells, suggesting that inhibition of glycolysis would exploit the metabolic fragility of prostate cancer [5].

Few studies to date have been completed with agents that directly target glycolysis to induce cytotoxicity, despite diagnostic studies developing positron emission tomography (PET), which uses a trapped agents that directly target glycolysis to induce cytotoxicity. In this regard, the process of autophagy, which is induced by nutrient deprivation, has been identified as an important mechanism of cellular resistance, or alternatively cell death if allowed to continue unabated [10–12]. Autophagy is a response to starvation whereby cellular organelles and bulk cytoplasm are targeted to lysosomes for degradation to supply an alternate energy source during periods of nutrient limitation. In addition to nutrient recycling, autophagy also plays an essential role in the proteolytic degradation of damaged proteins and organelles to maintain quality control. Sustained autophagy under conditions of protracted starvation has also been proposed to lead to cell death; thus, the survival or death consequences of autophagy are condition-dependent. Autophagy is also often impaired in human prostate cancers, due to either activation of the PI-3 kinase/Akt pathway and thereby mTOR, which inhibits autophagy, or through allelic loss of the essential autophagy gene beclin1 [11]. Therefore, growth in a hostile environment, inefficient utilization of glucose and defective autophagy predict that prostate cancers may be particularly sensitive to therapies that inflict metabolic stress.

We, therefore, hypothesize that prostate cancer is metabolically fragile because of dependence on glycolysis, increased activity of Akt, and impaired autophagy. This creates an opportunity to improve therapy through promotion of metabolic stress with agents that inhibit glycolysis. To begin to understand this novel paradigm, we studied the effect of a prototypical inhibitor of glycolysis, 2DG, a glucose analogue that inhibits glucose uptake, to determine if, in fact, 2DG induces cytotoxicity and autophagy in prostate cancer cells. To develop markers of autophagy for assessment in clinical trials, we studied Beclin1 in our cell systems and human prostate tissue from patients with prostate cancer.

**MATERIAL AND METHODS**

**Cell Culture and Viability Assay**

PC-3 (human androgen insensitive prostate cancer cell line), LNCaP (human androgen sensitive prostate cancer cell line) were obtained from ATCC. LNCaP and PC-3 cells were maintained in RPMI-1640 media with glucose concentration 2 g/L and 10% FBS. 2DG was obtained from Sigma (St. Louis, MO). Cells were plated initially in 96-well microtiter plates. After 24 hr they were treated with different concentrations of 2DG. After 72 hr of incubation in the presence or absence of drug, viability studies were performed by the MTT method as previously described [13]. Trypan Blue exclusion viability assay was performed on cells plated in 100 mm dishes. Cells were removed with trypsin after 72 hr and triplicate samples from each dish were counted on Vi-Cell (Beckman Coulter Fullerton, CA). Mean and standard error was calculated. Time lapse microscopy was performed as previously described [14].

**RNA Interference**

LNCaP and PC-3 cells were transfected with annealed, purified, and desalted double-stranded siRNA (30 μg/3 x 10⁶ cells) using the Amaxa nucleofection system (Gaithersburg, MD) (kit V, program G-16), as previously demonstrated [14]. siRNA targeted against beclin1 (5'-CAGUUUGGCACAAUCAAUUAU-3') and
LaminA/C were obtained from Dharmacon Research (Lafayette, CO).

Fluorescence Microscopy/LC3-GFP Autophagy Assay

LNCaP and PC-3 cells were co-transfected with EGFP-LC3 reporter along with LaminA/C siRNA (control) or beclin1 siRNA and plated on cover slips, treated with 2DG and cultured in maintenance media. After 72 hr cover slips were fixed in Formalde-Fresh solution (Fisher Scientific, Pittsburgh, PA). Following the washing and mounting the cover slips, the cells with GFP translocation were counted (>30 total) and photographed using fluorescence microscope (Nikon).

Immunoblot Analysis

Cells treated with 2DG were lysed in ice-cold RIPA buffer with protease inhibitors cocktail from (Sigma). Equivalent amounts of protein from each sample were electrophoresed on 12% or 15% gel SDS–PAGE and transferred to nitrocellulose. For cell cycle protein assessment, Cyclin D1, Cdk4, Cdk6 and secondary goat anti-mouse HRP conjugated antibody were used (Sigma). Beclin1 was assessed using rabbit primary antibody (Santa Cruz Biotechnology, Inc., cat # sc-11427, Santa Cruz, CA). LC3 was assessed using primary rabbit antibody from MBL International (Woburn, MA) and secondary goat anti-rabbit HRP conjugated antibody (Santa Cruz Biotechnology, Inc.). Cleaved Caspase3 antibody was obtained from Cell Signaling (Beverly, MA).

Immunohistochemistry of TMA

Tissue microarray slides with paraffin embedded prostate cores were placed into a Ventana Medical Systems Discovery automated slide stainer, heated to 75°C for 8 min. Deparaffinization (de-waxing) of tissues is accomplished using heat and Ventana de-waxing solutions for 8–10 min. Slides were washed in buffer at 37°C for 10 min. Antigen retrieval is performed for over 72 min at a pH 8 using EDTA buffer. Anti-BECN1 (H-300) (Santa Cruz Biotechnology, Inc., cat # sc-11427) was applied to the tissue sections at a dilution of 1:240 with 1% BSA/PBS with amplification and incubated overnight at room temperature. Biotinylated secondary antibody (Discovery Universal detection #2) was applied to the tissue sections and developed with Ventana Strept-Avidin Horseradish Peroxidase. Hematoxylin was used as a tissue counterstain.

Imaging

Immunostained tissue microarrays were imaged and digitized using a 40× volume scan on a high-throughput Trestle/Clariant MedMicro whole slide scanner. The resulting imaged specimens were stored in multi-tiled TIFF format on a redundant array of independent devices (RAID). Tissue Microarray analysis software automatically performs registration of the arrays, decomposes the specimen into its constituent staining maps and generates the measures for integrated staining intensity (ISA), effective staining area (ESA), and effective staining intensity (ESI) [15]. The software manages imaged tissue microarrays along with all related descriptive text and data fields into an Oracle 10 g database. The expression metrics that are generated during processing are automatically populated into the database and can be used to query and locate any given imaged specimen and correlated dataset to facilitate subsequent retrieval.

RESULTS

Effect of 2DG in Cancer Cell Lines

To determine if 2DG inhibits prostate cancer cell viability, we treated LNCaP and PC-3 cells with increasing concentrations of 2DG. As shown in Figure 1, both LNCaP cells and PC-3 cells are inhibited by 2DG in a dose dependent fashion. The cytotoxic effect was demonstrated by cell counts using a trypan blue assay (Fig. 1A) and an MTT cell viability assay (Fig. 1B). To determine the effect of 2DG over time, we observed morphological changes by time-lapse microscopy over 5 days (100×). As shown in Figure 1C, proliferation was decreased more by therapeutic starvation with exposure of cells to 2DG (bottom two rows) compared to control (top row) or compared to cells in which glucose was absent from the media (although present in added serum). To determine the effect of 2DG on cell cycle checkpoint proteins, we assessed the effect of 2DG on expression of Cyclin-D1, cdk4, and cdk6. As shown in Figure 2, 2DG decreased expression of Cyclin-D1, cdk4, and cdk6 in both LNCaP and PC-3 cells. Thus, 2DG arrests cell growth and promotes cell death of prostate cancer cell lines in a dose-dependent fashion.

Autophagy and Beclin-1 in Tumor Cell Lines

To determine the effect of 2DG on autophagy, we expressed the fluorescent autophagy marker GFP-LC3 in LNCaP and PC-3 cells, and modulated the expression of the autophagy regulator Beclin1 with siRNA. As shown in Figure 3A, siRNA for Beclin1 efficiently decreased expression in PC-3 cells compared to control siRNA. Treatment of 2DG induced autophagy as demonstrated by redistribution of the autophagosome marker GFP-LC3 from a diffuse cytoplasmic pattern to
form punctate localization indicative of autophagosome formation (Fig. 3B shows a photograph of representative cells and C quantification of percentage of cells with punctate redistribution). In Figure 3B, the arrows identify punctate localization in cells treated with 2-DG, which do not develop with Beclin1 siRNA treatment (second row of Fig. 3B). As shown in Figure 3C, over 40% of cells contain such punctate GFP-LC3 localization with 5 mM 2DG, which is decreased to less than 20% of cells with the addition of Beclin1 siRNA. To determine if the effect of 2DG to induce Beclin1 dependent autophagy was limited to PC-3 cells, we also assessed the effect of 2DG in LNCaP cells. As shown in Figure 4, treatment of LNCaP cells
with 2DG resulted in similar decreased Beclin1 with siRNA (A) and increased autophagy, as demonstrated by punctate distribution of LC-3 (B,C). As was the case with PC-3 cells, 2DG induced autophagy in LNCaP cells was also dependent on Beclin1 expression.

**Effect of 2DG on LC3 and Caspase Activation**

To further assess the effect of 2DG on autophagy, we also assessed the expression of LC3 protein by Immunoblot. As shown in Figure 5A, LC3-II protein increased relative to LC3-I protein, as would be expected with induction of autophagy over 72 hr of treatment with 5 or 25 mM 2DG. To begin to determine the effect of Beclin1 on apoptotic proteins such as caspase-3, we assessed the effect of 2DG on the cleaved (activated fragment of caspase-3). As shown in Figure 5B, caspase-3 is cleaved to the active fragment in PC3 cells treated with 2DG. Of note, treatment of Beclin1 siRNA, which was shown to abrogate autophagy (Figs. 3 and 4), allowed increased activation of caspase-3 at lower 2DG concentration, suggesting that Beclin1 and autophagy were associated with resistance to apoptosis with these specific experimental conditions, and at concentrations more relevant to what can be achieved in patients. The effect of autophagy on 2DG induced cytotoxicity was further assessed by a cytotoxicity assay. LNCaP and PC-3 cells were treated with various concentrations of 2DG over 72 hr, with and without beclin1 siRNA or Lamin control, and assessed by cell counts with trypan blue (Fig. 6). Both LNCaP cells and PC-3 cells were inhibited by 2DG in a dose dependent fashion, and cytotoxicity increased with Beclin1 siRNA, consistent with the hypothesis that autophagy was a mechanism of resistance of 2DG induced cytotoxicity.

**Beclin-1 Expression in Human Tissue**

Because of the dependence of therapeutic starvation-induced autophagy on Beclin1, it would be important to develop this as a translational marker of clinical trials that develop agents that induce metabolic starvation. To determine the feasibility of measuring Beclin1 expression in human prostate tissue, we stained a human prostate tissue microarray by immunohistochemistry (IHC). As shown in Figure 7, the characteristic staining of beclin1 was in epithelial cells in normal (Fig. 7A) and cancer (Fig. 7B,C). As shown in Figure 7D, Beclin1 staining intensity (scored by a single pathologist, M.M., from 0 to 3) was increased in tumor tissue compared to normal tissue. In an effort to develop a standardized methodology for quantifying Beclin1, for use in clinical trial correlates, we performed quantitative digitized image analysis of the tissue microarray. As shown in Figure 8, a color decomposition analysis of tissue staining generated the corresponding measures for integrated staining intensity, effective staining area and effective staining intensity. Thus, Beclin1 can be assessed effectively with IHC in human tissue and quantified by automated digitized imaging, providing a complete assessment methodology to test in clinical studies.

**DISCUSSION**

Targeting metabolism is an attractive new paradigm for investigation because of increased metabolic fragility of cancer. The understanding and development of clinically available therapies capable of modulating metabolism is critically important. We found that 2DG, a prototypical inhibitor of glycolysis, was cytotoxic in prostate cancer cells. Additionally, we found that 2DG induced the state of autophagy, now known to modulate the effectiveness of targeting metabolism in tumor cells. Autophagy is thought to be a resistance mechanism to cellular stress, or, alternatively, if left to completion, a cause of cell death. Additionally, we demonstrated the importance of Beclin1 as a regulator of autophagy and established methodology to quantitatively assess Beclin1 in human tissue. These data, therefore, support future translational efforts by providing a rationale to assess therapeutics that target metabolism, by demonstrating that autophagy may be a mechanism that modulates cell death, by providing support for the importance of Beclin1 as a regulator of autophagy, and by establishing methodology for the assessment of Beclin1 in patient material in future clinical trials.

The finding that 2DG induced autophagy is important because this may represent either a mechanism of cell death or survival that warrants further study with agents developed for therapeutic starvation such as
2DG. Autophagy is conserved, genetically controlled catabolic response to starvation whereby cells self-digest intracellular organelles by targeting them for degradation in lysosomes to generate energy. This may serve to regulate normal turnover of organelles and to remove those with compromised function to maintain homeostasis. Autophagy can also be a survival mechanism during periods of starvation where self-

**Fig. 3.** Induction of Beclin1-dependent autophagy by 2DG in human PC3 cells. **A**: Western blot for Beclin1 and the actin control of PC3 cells treated with Beclin1 siRNA (+) or lamin control (–) siRNA. Beclin1 protein levels were reduced specifically by Beclin1 siRNA. **B**: Representative examples of predominantly diffuse EGFP-LC3 localization without 2DG (0 mM 2DG) and membrane translocation (red arrows) upon 2DG treatment (5 and 25 mM) in the upper row are shown. This punctate pattern represents the localization of the marker GFP-LC3 in autophagosome formation. The localization of GFP-LC3 is abrogated by Beclin1 siRNA (all three lower panels in 3B). **C**: Quantitation of the percentage of counted cells that contained EGFP-LC3 localization indicative of autophagy after treatment with 2DG. A decrease in the percentage of cells with punctate localization of EGFP-LC3 was noted with treatment of Beclin1 siRNA. Each bar represents the percentage of cells with the translocation ± SEM.
digestion provides an alternative energy source and facilitates the disposal of unfolded proteins under stress conditions. It has recently become clear that normal and tumor cells require the catabolic process of autophagy to survive nutrient deprivation [11]. We found that autophagy was dependent on Beclin1 (Figs. 3 and 4) and functioned as a survival mechanism under our experimental conditions (Figs. 5 and 6), which is consistent with prior studies [16]. We also found that Beclin1 can be detected in human tumor by IHC, establishing the feasibility of measuring Beclin1 in prostate tissue (Figs. 7 and 8), and realize that additional studies would be needed to determine if intensity of expression is associated with the propensity of tumor to undergo autophagy. The implication of measuring

Fig. 4. Induction of Beclin1-dependent autophagy by 2DG in human LNCaP cells. A: Western blot for Beclin1 and the actin control of LNCaP cells treated with lamin control siRNA or Beclin1 siRNA. B: Representative examples of predominantly diffuse EGFP-LC3 localization without 2DG (0 mM 2-DG) and membrane translocation (red arrows) upon 2DG treatment (25 mM) in the upper row are shown. This punctate pattern represents the localization of the marker GFP-LC3 in autophagosome formation. The localization of GFP-LC3 is abrogated by Beclin1 siRNA (lower panels in 4B). C: Quantitation of the EGFP-LC3 localization showing induction of autophagy with 2-DG that is inhibited by siRNA for Beclin1. Each bar represents the percentage of cells with the translocation ± SEM.

Fig. 5. Effect of 2DG on LC3 protein (A) and caspase3 (B). LC3-I and LC3-II protein is shown by immunoblot after treatment with 5 and 25 mM of 2DG at 24, 48, and 72 hr showing the characteristic increase in proportion of LC3-II/LC3-I expected with induction of autophagy (A). To determine the importance of Beclin1 in 2DG induced apoptosis, we assessed the activated cleavage product of caspase3 in PC3 cells by immunoblot after treatment with 2DG (B). Cells were treated with Lamin siRNA (control) or Beclin1 siRNA demonstrating activation and cleavage of caspase3 after treatment with 10 mM 2DG only in the setting of Beclin1 siRNA treatment, which decreased Beclin1 expression (Figs. 3A and 4A) and decreases autophagy (Figs. 3B and 4B). After treatment with 25 mM 2DG, caspase3 is activated and cleaved regardless of beclin1 expression. Actin was used as a control for equal protein loading.

Fig. 6. Effect of autophagy on 2DG induced cytotoxicity. LNCaP (A) and PC-3 (B) cells were treated with various concentrations of 2DG over 72 hr, with and without beclin1 siRNA or Lamin control, and assessed by cell counts with trypan blue. Both LNCaP cells and PC-3 cells were inhibited by 2DG in a dose dependent fashion, and cytotoxicity increased with Beclin1 siRNA. Experiments were performed in triplicate ± SEM.
beclin1 in human tumor is currently unclear, and the current assessment was focused to demonstrate the feasibility of assessing beclin1 by IHC in a small group of patient samples, and to quantify the intensity for use in larger clinical studies. This may be particularly important in prostate cancer because multiple studies have demonstrated that the beclin1 (atg6, vps30) gene is critical for autophagy to occur and allelic loss occurs with high frequency in prostate cancers [17]. Establishing the role of autophagy in prostate cancer is, therefore, an important step toward understanding the disease process and for the development of new treatments that modulate metabolism [18]. Furthermore, prior studies have demonstrated that activation of the PI-3K/Pten/Akt pathway also promotes glycolysis (in part through up-regulation of glycolytic enzymes and glucose transporters), and stimulates protein synthesis while inhibiting autophagy [4]. Thus, one of the most important events in prostate cancer profoundly alters the cellular metabolic state by increasing energy demand (stimulation of protein synthesis) while promoting inefficient energy production (dependence on glycolysis) and inhibiting catabolism (autophagy).

Therefore, it is our hypothesis that the oncogenic switch from aerobic to glycolytic metabolism, known for decades as the "Warburg effect," causes tumor cells to be predisposed to metabolic catastrophe where constitutive growth signals and inefficient energy production impair their ability to adapt to metabolic

![Image](image_url)
stress [10]. This fundamental difference between normal and tumor cells has yet to be exploited effectively in the clinic. Our data supports the importance of autophagy in the study of such approaches and gives direction toward the clinical translation of this important paradigm.

CONFLICT OF INTEREST

Views and opinions of, and endorsements by, the authors do not necessarily reflect those of the Department of Defense.

REFERENCES


Warburg science goes to the bedside: A phase I trial of 2-deoxyglucose in patients with prostate cancer and advanced malignancies.

Sub-category:
Prostate Cancer

Category:
Genitourinary Cancer

Meeting:
2008 ASCO Annual Meeting

Abstract No:
16087

Citation:
J Clin Oncol 26: 2008 (May 20 suppl; abstr 16087)

Author(s):

Abstract:

Background: A profound, but therapeutically unexploited, difference between cancer and normal tissues is the preferential utilization of glycolysis (the 'Warburg effect') for energy by cancer cells. Additionally, similar to mechanisms of chemotherapy resistance, potential mechanisms of cancer cell resistance to starvation have recently emerged. One pathway by which cells survive periods of metabolic stress is thought to be autophagy, which is a catabolic process of organelle digestion that creates ATP during periods of nutrient limitation and is regulated by the protein Beclin1. Methods: We developed this novel paradigm in pre-clinical models and a phase I clinical trial. Preclinically, we used immortalized mouse epithelial prostate cells, as well as PC-3 and LNCaP cell lines, and a transfected pEGFP-LC3 autophagy marker construct to assess cytotoxicity and autophagy induction by 2-deoxyglucose (DG). In the clinic, eligible patients receive DG orally on days 1-14 of a 21 day cycle in cohorts of 3 in a dose escalating manner. Planned correlative assessments in patients included PET scans at baseline and day 2, as a potential marker of DG uptake, Beclin1 in initial tumor blocks, and LC3 protein in peripheral blood mononuclear cells as a potential marker of autophagy. Results: In preclinical models, we demonstrated cytotoxicity and induction of autophagy, which was dependent on Beclin1 expression. To establish methods for the clinical trial, we stained a human prostate TMA (>35 patients) for Beclin1 by IHC. In the clinical study, 6 patients have been treated at doses 30 and 45 mg/kg/day orally and a 3rd cohort is accruing currently at 60 mg/kg. Therapy was well tolerated with no dose-limiting toxicity. Of three patients with prostate cancer, one patient has received more than 11 cycles with a stable PSA for over 6 cycles. Of three patients in which PET was performed at baseline and follow-up, one patient had marked decrease in tumor site SUV and a second patient a minor decrease. Accrual is ongoing. Additional PET and assessment of LC3 and Beclin1 correlatives are ongoing. Conclusions: These initial data support the safety of DG and translational advancement of the rapidly developing paradigm of targeting the metabolic fragility of cancer.
Abstract Disclosures

Abstracts that were granted an exception in accordance with ASCO's Conflict of Interest Policy and are designated with a caret symbol (^) here and in the print version.

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