Metabolic vulnerabilities in endometrial cancer


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Abstract

Women with metabolic disorders, including obesity and diabetes, have an increased risk of developing endometrial cancer. However, the metabolism of endometrial tumors themselves has been largely understudied. Comparing human endometrial tumors and cells to their non-malignant counterparts we found that up-regulation of the glucose transporter GLUT6 was more closely associated with the cancer phenotype than other hallmark cancer genes including hexokinase 2 and pyruvate kinase M2. Importantly, suppression of GLUT6 expression inhibited glycolysis and survival of endometrial cancer cells. Glycolysis and lipogenesis were also highly coupled with the cancer phenotype in patient samples and cells. To test whether targeting endometrial cancer metabolism could be exploited as a therapeutic strategy, we screened a panel of compounds known to target diverse metabolic pathways in endometrial cells. We identified that the glycolytic inhibitor, 3-bromopyruvate (BrPA), is a powerful antagonist of lipogenesis through pyruvylation of coenzyme A. We also provide evidence that BrPA promotes cell death via a necrotic mechanism that does not involve reactive oxygen species and that BrPA impaired the growth of endometrial cancer xenografts in vivo. Overall, this study identifies GLUT6 and glycolytic-lipogenic pathways as novel liabilities in endometrial cancer that may be exploited for therapeutic intervention.
Introduction

Endometrial cancer is the most common gynecological malignancy, affecting more than 287,000 women and attributing to 74,000 deaths worldwide each year [1]. Endometrial tumors originate from glandular epithelial cells of the uterus and may locally progress to invade the myometrium and further metastasize to lymph nodes, liver, and lungs in advanced stages [2]. Endometrial cancer is most common in post-menopausal women and is classically categorized in 2 clinicopathological subtypes; Type I (endometrioid) and Type II (predominantly clear cell and papillary serous). Type II tumors are generally more invasive, estrogen receptor and progesterone receptor (ER/PR) negative, and confer a poor prognosis; but account for less than 15% of all cases [3, 4]. In contrast, the more common Type I tumors are frequently low grade, non-invasive, ER/PR positive and survival rates are higher due to early identification and treatment with primary surgery [4].

Obesity [body mass index (BMI) > 30 kg/m$^2$] is associated with increased incidence, risk of death, and lower age of diagnosis for endometrial cancer [5-7]. Disorders associated with hyperglycemia (Type 1 and 2 diabetes) also have increased risk of endometrial cancer, indicating that poor control of blood glucose may be an important contributor to the growth of these tumors in women [8, 9]. In our previous study comparing obese women with and without Type I endometrial cancer, circulating glucose levels were higher in women with cancer (119.5 vs. 90.7 mg/dl for non-cancer; p = 0.049). However, other cancer-related parameters including estrogen and insulin were not significantly different between obese women with and without cancer [10]. These findings suggested that, independent of adiposity and its associated
hormonal changes, increased blood glucose levels may play an important role in the growth and/or development of Type I endometrial cancer.

Endometrial cancers are well-studied at the genetic level, but few studies have rigorously evaluated endometrial cancer metabolism. Many of the genetic aberrations that are thought to drive endometrial cancer initiation and progression also regulate cell metabolism. For example, the phosphatidylinositol 3-kinase (PI3K)-Protein kinase B (Akt) pathway is altered in up to 93% of Type I endometrial tumors, through loss of phosphatase and tensin homologue (PTEN) and mutations in PI3K family members [4, 11]. Other aberrations include mutations in V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, over-expression of epidermal growth factor receptor, and loss of liver kinase B1 (LKB1) and tuberous sclerosis 2 (TSC2) [4, 12, 13]. Despite this information it remains unclear whether the flux of nutrients through metabolic pathways contributes to endometrial cancer cell survival and tumor growth. Herein we show that glycolytic-lipogenic metabolism is increased in endometrial cancer cells and that they are dependent upon this metabolism for survival.

Materials and Methods

Patient sample analyses

Institutional review board approval in accordance with Federal regulations was obtained from the University of Virginia Health System. Endometrial samples were collected at surgery (hysterectomy) from four women with, and four women without Type I endometrial cancer (mean age 52.6 years and BMI 44.5 kg/m²) and RNA was analyzed, as described [14]. For protein expression analyses, non-tumor and tumor endometrial tissue (matched for each
patient) were obtained from an independent cohort of postmenopausal women (mean BMI 35.3 kg/m$^2$), with FIGO-defined low stage (1A-1C) and grade (1-2) Type I endometrial cancer. Analyses of gene alterations (shown in Table 1) from endometrial and ovarian cancers were performed using cBioPortal [15, 16] and are based solely upon data generated by The Cancer Genome Atlas (TCGA) Research Network [11, 17, 18].

**Endometrial cells**

Cells were obtained from American Type Culture Collection, Manassas, VA, USA (HEC-1-A, AN3CA, KLE, RL95-2) Sigma Aldrich, St. Louis, MO, USA (Ishikawa), Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany (MFE-296, MFE-319), Dr. Hui Li (University of Virginia, VA, USA) and Dr. Kelle Moley (Washington University School of Medicine, St. Louis, MO, USA) [human Telomerase Reverse Transcriptase (hTERT)-immortalized endometrial stromal (MAD-11) and uterine epithelial (hUE-Ts), both derived from patients without endometrial cancer]. Cancer cells have not been validated since purchase.

**Immunohistochemistry**

Pathological analyses of tumor and non-tumor tissue were performed by a certified gynecological oncology pathologist in a blinded manner. IHC was performed at the University of Virginia Biorepository and Tissue Research Facility using a mouse monoclonal antibody against human GLUT6 (Abcam, Cambridge, UK). 3,3'-Diaminobenzidine was used as a substrate for the peroxidase reaction and hematoxylin as the counterstain. Slides were scanned using an Aperio ScanScope XT Slide Scanner (Aperio, Vista, CA, USA) and images analyzed using the ImageScope software (Aperio).
siRNA transfections

Cells were double-transfected (24 hours apart) with 50 nmol/L ON-TARGETplus siRNA; SLC2A6 (GLUT6) SMARTpool (Pool), GLUT6 individual siRNA sequences found in the SMARTpool (Seq.1-4), or equivalent concentrations of non-targeting SMARTpool control (Ctrl) siRNA (ThermoFisher Scientific, Lafayette, CO) using JetPRIME reagent (Polyplus Transfection Inc, New York, NY, USA) as the delivery vehicle.

qPCR

RNA was isolated with TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and reverse transcribed using a High Capacity cDNA kit (Life Technologies). qPCR was performed using iQ SYBR green SuperMix (Bio-Rad, Hercules, CA, USA) on an iCycler (MyiQ Optical Module) Bio-Rad system. GLUT mRNA expression was normalized to β2M using the Pfaffl method [19].

Western blotting

Protein lysates (20µg) were resolved on polyacrylamide gels and electro-transferred to nitrocellulose membrane. Protein expression was detected with rabbit antibodies; GAPDH, PKM2, LDHA, ACLY, ACC, FASN, PTEN, AKT, PPARγ, TSC2, G6PD (Cell Signaling Technology) GLUT1 (Abcam), and mouse antibodies; pAKT (S473), P-p70S6 kinase (T389) (Cell Signaling Technology), HK2, MCT1, 14-3-3 (Santa Cruz), GLUT6 (Abcam) and β-actin (Sigma Aldrich). Primary antibodies were detected with goat anti-mouse IgG (DyLight 800 conjugate) or anti-rabbit IgG (DyLight 680 conjugate) and membranes scanned on the LI-COR ODYSSEY System (LI-COR, Lincoln, NE, USA).
**Metabolic Assays**

Cells were incubated in Krebs Ringer Phosphate (KRP) nutrient buffer containing either D-[3-3H] glucose, D-[14C(U)] glucose, L[14C(U)]-glutamic acid, [1-14C]-palmitic acid, or [2-14C] acetic acid sodium salt. Substrate oxidation was measured capturing evolved 14C-CO$_2$ and DNL from substrates was measured by hexane:isopropanol (3:2) extraction of 14C-lipids. For glycolysis measurements, D-[3-3H] glucose was separated from tritiated [3H]$_2$O by diffusion. Detailed methods for these assays are provided in supplemental material. For OCR and ECAR measurements, cells (10,000-20,000/well) were seeded in 24-well Seahorse tissue culture plates a day prior to incubation in KRP nutrient buffer (37°C for >30 minutes). Basal OCR (pmolesO$_2$/min) and ECAR (mpH/min) were measured using the Seahorse XF-24 Flux Analyzer (Seahorse Biosciences, North Billerica, MA). OCR and ECAR rates for each cell line were averaged over 3 plates and normalized to protein content per well.

**Lactate release**

Lactate standards, NAD+ and LDH were prepared in glycine-hydrazine buffer (500mM glycine, 127mM hydrazine sulfate, pH 9.5). Cells were incubated in DMEM (without phenol red or FBS) for 45 minutes. Media removed from cells was incubated with 2.5mM NAD+ and 25μg/mL LDH for 1 hour at room temperature and absorbance measured at 340nm.

**Glucose Uptake**

Cells were incubated with 5mM 2-DG in KRP with 0.2μCi of $^3$H-2-DG/well for 2.5 minutes. Cells were rinsed twice in ice-cold PBS prior to permeabilization in 1%TritonX-100 (1 hour) before counting.
**Analyses of free thiol groups**

CoA and BrPA were prepared in PBS (pH 7.4), and NAC in water, and co-incubated at room temperature (15 minutes). CoA and NAC alone were used as positive controls, and BrPA alone as a negative control. Solutions were added to black-walled 96-well plates with an equivalent volume of 10µM ThioGlo®1 (Covalent Associates Inc.). Fluorescent signal was measured 5 minutes later at 379/513nm.

**Cytotoxicity assays**

Cells were exposed to drugs for 48-72 hours and viability was detected by addition of thiazolyl blue tetrazolium bromide (MTT) reagent. Formazan crystals were solubilized and absorbance read at 590/620nm. Cell viability is displayed as a percentage of control cells i.e. cells with equivalent concentrations of the appropriate drug vehicle. Refer to supplemental material for detailed method.

**Cell viability and cycle analyses**

Cells were stained with 7-AAD (2μg/mL) and Annexin V (AV). Viable (AV-) and non-viable (AV+) cell populations determined by flow cytometry (BD FACSCanto I). For cell cycle, cells were permeabilized/stained with 0.4% Triton X-100, 2μg/mL RNase, and 2 μM TOPRO-3 in warm phosphate buffered saline (PBS). Profiles were determined by flow cytometry and sub-G1 populations gated using FlowJo software (Tree Star Inc. Ashland, OR, USA).

**Cell death assays**
LDH release (LDH-Cytotoxicity Assay Kit II, Abcam) and caspase activity (Caspase-Glo 3/7 assay reagent, Promega) were measured by luminescence, as per manufacturer’s instructions. DNA fragmentation was analyzed by agarose gel electrophoresis, as previously described [20].

**Mass spectrometry**

Cell samples (in ice-cold 6% perchloric acid) contained $^{13}$C3-malonyl-CoA (0.5µM final) as an Internal Standard. Supernatants were loaded onto pre-equilibrated solid-phase extraction columns (Oasis HLB, 1cc/30mg, Waters, Milford, MA) and dried eluates reconstituted in chromatography solvent. Analyses were performed using a triple quadrupole mass spectrometer (AB-Sciex 4000 Q-Trap) coupled to a Shimadzu LC-20AD LC system equipped with a Supelco Discovery C18 column (50 mm × 2.1 mm × 5 µm bead size).

**Animal studies**

Six week-old female athymic nude mice (Crl:NU(NCr)-Foxn1<sup>nu</sup>, Charles River Laboratories Inc.) were subcutaneously inoculated with $3 \times 10^5$ 296 cells. Mice with palpable tumors (~2mm diameter) were administered 2.5mg/kg freshly-prepared BrPA (pH 7.5) or equivalent volumes of PBS (vehicle) by intraperitoneal injection 4 days a week, for 3 weeks. Mice were killed (CO<sub>2</sub> asphyxiation) when the largest tumor neared 1cm<sup>3</sup>. Tumor volumes (length x width<sup>2</sup>/2) were calculated each week during treatment and at time of harvest. All animal experiments were approved by the University of Virginia Animal Care and Use Committee.

**Statistics**
Unpaired two-tailed Student’s *t* tests were used to determine the statistical differences between experimental and control groups where appropriate with *p*<0.05 considered statistically significant. Pearson correlation coefficients (*r* and *p* values) and linear regression graphs were derived from the GraphPad Prism program.

**Results**

*Glucose transporters, and glycolytic and lipogenic enzymes, are up-regulated in the malignant endometrium*

We previously reported gene expression data from women with Type I endometrial cancer who were matched for age (mean 52.6 years) and BMI (mean 44.5 kg/m$^2$) [14]. Mining the microarray data using gene set enrichment analysis identified the glycolysis and gluconeogenesis gene set as highly enriched in tumor-derived endometrium (NES 1.87, *q*=0.02, *p*<0.001, Figure 1A). Among the most elevated genes in malignant tissue were the glycolytic enzymes hexokinase 2 (*HK2*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), pyruvate kinase M2 (*PKM2*), and lactate dehydrogenase isoform A (*LDHA*) (Figure 1A). We also performed bioinformatics analyses on genes that were up-regulated by more than 6-fold (371 genes) using the Database for Annotation, Visualization and Integrated Discovery (DAVID) [21, 22] and matched these hits to the Panther biological processes gene lists. These data identified that glycolytic and lipogenic metabolic pathways were enriched in malignant compared with non-malignant endometrium (Figure 1B). In an independent cohort of 6 patients with Type I endometrial cancer, we validated the gene expression data at the protein level. Figure 1C shows the expression of glycolytic (*HK2*, *PKM2*, *GAPDH* and *LDHA*) and *de novo* lipogenesis (DNL) enzymes, including ATP citrate lyase (ACLY), acetyl-CoA
carboxylases (ACC1 and ACC2), and fatty acid synthase (FASN). All of these enzymes except GAPDH were elevated in the majority of tumor vs. adjacent non-malignant tissue (Figure S1A).

Aberrations in the PTEN-PI3K-Akt pathway are common in Type I endometrial cancer. In our samples, PTEN expression was reduced in one tumor sample, but activation of Akt (phosphorylation at Ser473) was evident in most tumor samples (Figure 1D and S1A). In contrast, the expression of peroxisome proliferator-activated receptor-gamma (PPARγ), glucose-6-phosphate dehydrogenase (G6PD), TSC2, and phosphorylation of p70 S6 ribosomal kinase (pS6K), were not dramatically altered between tumor and non-tumor endometrial tissue (Figure S1B). Although alterations in LKB1 expression were observed in some tumors (Fig S1B), these changes were inconsistent between patient samples i.e. LKB1 expression was higher in one tumor and lower in other tumors, compared with adjacent non-tumor tissue. Overall, these data indicate that Akt activation is associated with increased expression of glycolytic and lipogenic enzymes in endometrial tumor tissue (Figure S1A).

Glucose is a primary carbon source for glycolysis and lipogenesis and enters cells through glucose transporters. We manually ranked the GLUT family of transporters and identified GLUT6 (SLC2A6) as the 11th most up-regulated gene overall and the most significantly elevated GLUT in the malignant endometrium (36.9-fold increase) (Figure 1E). Elevated GLUT6 protein expression was confirmed in tumor tissue compared with matched non-tumor endometrium in 5 of the 6 patients (Figure 1F and S1A). GLUT6 is highly expressed in cancerous glandular epithelial cells, particularly those closest to blood vessels in the surrounding stroma (Figures 1G and S2). Blood vessels also stained positive for GLUT6.
Glandular cells from the normal human endometrium had little or no expression of GLUT6 (Figure 1G and S2). In comparison, the ubiquitously expressed glucose transporter, GLUT1, was elevated by 4.3-fold at the gene level in the malignant endometrium (Figure 1E), and in 3 of 6 tumors compared to non-tumor endometrium (Figure 1F and S1A).

Analyses of TCGA data sets found that 40% of Type I and 68% of Type II endometrial cancers harbor alterations in the glycolytic-lipogenic gene set found in our cohort of patient samples (Table 1). The percentage of cases with alterations did not vary dramatically between women with stage I vs more advanced stages, or lean vs obese, within Type I and within Type II cancers (Table 1). However, Type I cancers (all cases) containing alterations in these genes had significantly poorer survival than those without gene alterations. Further examination of specific groups of Type I cancers revealed poorer survival among obese women or those with stage II-IV cancers if they had the gene alterations (Table 1). Alterations in this gene set were also found in 80% of ovarian cancers. However, these genes were down-regulated in a higher percentage of ovarian cancer cases (78/254, 30.7%) compared to Type I (4/123, 3.25%) and Type II (0/45, 0%) endometrial cancers and were not associated with worse survival (Table 1).

**GLUT6 expression and glycolytic-lipogenic metabolism are up-regulated in endometrial cancer cells**

To further investigate endometrial cancer metabolism, we metabolically profiled 7 human endometrial cancer cells including HEC-1-A (HEC), Ishikawa (ISH), MFE-296 (296), MFE-319 (319), AN3CA (AN3), RL95-2 (RL) and KLE, and 2 immortalized cell lines derived from non-cancerous endometrial tissue, MAD-11 (MAD) stromal and hUE-Ts (hUE) uterine epithelial
cells. As shown in Figure 2A, GLUT6 expression was up-regulated in all 7 endometrial cancer cells compared with non-cancerous endometrial cells, while the expression of GLUT1 and all other glycolytic and DNL enzymes varied between cancer and non-cancer cells (Figure 2A-B). Akt was activated (phosphorylated) in the majority of cancerous endometrial cells and PTEN expression was lost in 3 of the 7 cancer cell lines (Figure 2B).

To determine whether metabolic protein expression correlated with metabolism in endometrial cells, we measured oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and macronutrient flux using radiolabeled glucose, glutamine, palmitate and acetate tracers. Glycolysis, as measured by the conversion of tritiated glucose to tritiated water, was elevated in 6 of 7 endometrial cancer cells compared with non-cancerous endometrial cells (Figure 2C-D), and glycolysis significantly correlated with ECAR (r=0.858, p=0.003) (Figure 2E). In contrast, glucose oxidation was markedly decreased in all 7 endometrial cancer cells compared to the 2 non-cancerous cells (Figure 2F). The oxidation of glutamine (Figure 2G) and palmitate (Figure 2H), and OCR (Figure 2I), were not associated with a cancer phenotype. Finally, DNL from glucose, glutamine and acetate precursors were generally increased in most cancer-derived cells compared with non-cancerous endometrial cells (Figures 2J-L). Correlation analyses of these data revealed that GLUT6 protein expression significantly correlated with Warburg-type metabolism (low glucose oxidation/glycolysis ratio) (r=-0.837, p=0.005, Figure S3A) and the phosphorylation of Akt significantly correlated with glycolysis (r value=0.849, p value=0.004, Figure S3B) and glucose-derived lipogenesis in endometrial cells (r value=0.777, p value=0.014, Figure S3C).
GLUT6 promotes glycolysis and survival of endometrial cancer cells

Since GLUT6 has not been functionally characterized in cancer cells, we evaluated the role of GLUT6 using siRNA to knockdown this protein in 296 (Figures 3A-I and S4A-C) and RL cells (Figure S4D-H). Both of these cells were derived from Type I endometrial tumors from postmenopausal Caucasian women, with the RL cells derived from an obese woman but the BMI is unknown for the source of the 296 cells [23, 24]. Smartpool (Pool) siRNA reduced GLUT6 protein expression in 296 cells by 65%, while siRNA sequence 4 (Seq.4) (a single sequence from the Smartpool) reduced GLUT6 protein expression by 90%, compared with controls (Figures 3B and S4A). Importantly, GLUT6 knockdown did not alter the expression of GLUT1, GLUT4 and GLUT8 (GLUT8 has the most sequence homology to GLUT6) [25] in 296 cells (Figure S4B).

Functional analyses revealed that GLUT6 knockdown with the Pool siRNA reduced 296 and RL cell numbers by 62% and 59%, respectively, and with Seq.4 siRNA by 91% and 83%, respectively (Figures 3C and S4E, p<0.05). Of note, GLUT6 knockdown with other individual siRNA sequences (shown in Figure S4A) also significantly reduced endometrial cancer cell numbers (data not shown). Cell loss induced by GLUT6 knockdown was due to cell death, as evidenced by morphological alterations (Figure S4C), a marked increase in sub-G1 populations (Figures 3D and S4F), number of Annexin V positive (AV+) cells (Figures 3E and S4G), and release of lactate dehydrogenase (LDH) from permeabilized cells (Figures 3F and S4H).
To determine the role of GLUT6 in glucose metabolism, we measured glucose uptake and glycolysis in GLUT6 knockdown cells. Since complete knockdown of GLUT6 with Seq.4 siRNA induced significant cell death, the metabolism of 296 cells was investigated using the siRNA pool. Partial knockdown of GLUT6 expression significantly inhibited glucose uptake by 34% (Figure 3G, p=0.001), glycolysis by 33% (Figure 3H, p=0.004) and lactate release by 55% (Figure 3I, p<0.001).

**Targeting metabolism in endometrial cancer cells**

Glycolytic-lipogenic metabolism is increased in endometrial cancer. To test whether these metabolic phenotypes could be exploited in endometrial cancer cells, we screened a panel of compounds known to target each metabolic pathway. Selectivity and toxicity of these compounds were compared to chemotherapeutics used to treat advanced-stage endometrial cancer. Endometrial cancer and non-cancer cells displayed varying sensitivity to the DNA-damaging agent carboplatin (Figure 4A). Notably, the non-cancerous hUE cells were one of the most sensitive to carboplatin, compared with endometrial cancer and non-cancerous MAD cells (Figure 4A). In contrast, paclitaxel (a microtubule-stabilizing agent) showed greater selective toxicity towards endometrial cancer cells compared with non-cancer cells (Figure 4B). This sensitivity correlated with cell population doubling times thereby validating the role microtubules play in cell division (Figure S5A-B, r=0.796, p=0.01).

Endometrial cells were then exposed to the glycolytic inhibitors 2-deoxyglucose (2-DG) and 3-bromopyruvate (BrPA) (Figure 4C-D), the lipogenesis inhibitor 5-(Tetradecyloxy)-2-furoic Acid (TOFA) (Figure 4E), the glucose oxidation agonist dichloroacetate (DCA) (Figure 4F), the
fatty acid oxidation inhibitor etomoxir (Figure 4G), the glucose and fatty acid uptake inhibitor, phloretin (Figure 4H), the glutaminolysis inhibitor, 6-Diazo-5-oxo-L-norleucine (DON) (Figure 4I), and the pleiotropic metabolic inhibitor, metformin (Figure 4J). These compounds displayed a range of cytotoxic effects on the panel of endometrial cells including non-selective toxicity to all cells (e.g. 2-DG, metformin and etomoxir), toxicity only at supraphysiological concentrations (e.g. 2-DG and metformin), poor toxicity (e.g. DCA), or toxicity that plateaued with partial inhibition of cell viability (DON). However, as predicted by our metabolic flux data, TOFA was toxic to the cell lines with the highest rates of DNL and demonstrated mostly cancer cell-specific toxicity (Figure 4E). Curiously, of all metabolic inhibitors, BrPA was the most disparately toxic between cell lines, with a 37-fold difference in sensitivity between the most sensitive (KLE, IC$_{50}$=34µM) and the least sensitive cells (HEC, IC$_{50}$=1267µM) (Figures 4D). This suggested that sensitivity to BrPA was determined by specific cellular targets.

**BrPA inhibits DNL by pyruvylation of CoA**

Birsoy et al. recently utilized BrPA in a global loss-of-function genetic screen to identify its selective cytotoxicity to glycolytic tumors. Their study demonstrated that BrPA requires monocarboxylate transporter 1 (MCT1) for transport into cells [26]. Other studies have also shown that BrPA inhibits glycolysis by pyruvylation of GAPDH and HK2 [27]. In evaluating the correlation between BrPA sensitivity and known cellular targets, we found that the expression of MCT1 (Figure S5C), HK2 and GAPDH (Figure 2A), individually, did not correlate with sensitivity to BrPA (Figure S5D).
Glycolysis and lipogenesis were the two metabolic pathways most up-regulated in endometrial tumors. We therefore investigated the effects of BrPA on these pathways in endometrial cancer cells. At a toxic dose (100µM), BrPA treatment inhibited glycolysis in 296 cells by 46% (Figure 5A, p<0.0001) but had a greater inhibitory effect on DNL from 3 different lipogenic precursors; glucose (79% decrease, p=0.002, Figure 5B), glutamine (99% decrease, p<0.001, Figure 5C) and acetate (86% decrease, p=0.0002, Figure 5D). DNL from these precursors requires the production of acetyl-coenzyme A (acetyl-CoA). As BrPA inhibited DNL from acetate, which enters the lipogenic pathway as acetyl-CoA, we hypothesized that BrPA inhibited DNL at, or downstream of, acetyl-CoA production. Indeed, acetyl-CoA was depleted by 99% in BrPA-treated cells (Figure 5E, p<0.002). The formation of acetyl-CoA requires coenzyme A (CoA). Since BrPA can pyruvylate free thiols (R-SH) [28], we examined whether BrPA reacted with the thiol on CoA using a dye that fluoresces once bound to free thiol groups (Figure 5F). Co-incubation of BrPA with CoA at a 1:1 micromolar ratio inhibited fluorescent signal by 44% (p<0.001), and a 10:1 micromolar ratio of BrPA to CoA completely inhibited fluorescent signal (Figure 5F). These data indicated that BrPA was directly interfering with CoA at its free thiol group. Using a previously published ionization pattern for CoA [29], we demonstrated by mass spectrometry that pyruvylation occurs at the sulfhydryl group of CoA (Figure S6).

**BrPA-mediated cytotoxicity is independent of ROS generation**

Previous studies have indicated that the anti-oxidant, N-acetylcysteine (NAC), protects against BrPA-mediated cell death by inhibiting reactive oxygen species (ROS) [30]. Since NAC also harbors a thiol group, we postulated that NAC protects against BrPA-mediated cell death
by binding to BrPA. Indeed, we found that co-incubation of BrPA with NAC at a 1:1 micromolar ratio inhibited fluorescent signal by 52% (p<0.001), and a 10:1 micromolar ratio of BrPA to NAC completely inhibited fluorescent signal (Figure 5G). These data suggested that BrPA-induced cell death may not be mediated by ROS. We therefore determined whether other antioxidants (without free thiols) could protect against BrPA-induced cell death. Indeed, although a toxic dose of BrPA increased ROS (Figure S7A-B), pre-treatment of endometrial cancer cells with apocynin (Figure S7C), allopurinol (Figure S7D), N omega-nitro-L-arginine methyl ester (L-NAME, Figure S7E), manganese [III] tetrakis (4-benzoic acid) porphyrin (MnTBAP, Figure S7F), Mito-tempo (Figure S7G), resveratrol (Figure S7H), or ascorbic acid (Figure S7I), could not protect against BrPA-mediated cell death.

Since other antioxidants could not protect against BrPA-induced cell death, we proposed that excess CoA and NAC could act as ‘sponges’ to bind BrPA and prevent cytotoxicity. Indeed, pre-treatment of 296 cells with CoA (Figure 5H) and NAC (Figure 5I) protected from BrPA-induced death. Similar results were reproduced in RL cells (data not shown).

**BrPA induces necrosis in vitro and inhibits endometrial tumor growth in vivo**

BrPA inhibits glycolytic-lipogenic metabolism and depletes intracellular ATP (60% decrease at 1.5 hours, p<0.0001) (Figure S8A). These alterations can induce cell death by multiple mechanisms. Using UV irradiation as a positive control, we evaluated the effects of BrPA on classic features of apoptosis. Treatment of 296 cells with BrPA did not induce dynamic membrane blebbing (BrPA-treated cells are shown in Figure 6A, and UV-irradiated cells in Figure S8B), DNA fragmentation (Figure 6B), an increase in apoptotic cell populations
(Annexin V positive/7-AAD negative) (Figure 6C), or activation of caspases (Figure 6D). Furthermore, BrPA-mediated cell death was associated with an increase in LDH release and this was not prevented by the pan-caspase inhibitor, Q-VD-OPH (QVD) (Figures 6E and S8C). From these observations, we conclude that BrPA-mediated cell death was due to necrosis. Since BrPA kills endometrial cancer cells in vitro, we tested the efficacy of this agent against endometrial tumors in vivo. BrPA (2.5mg/kg) or vehicle (PBS) was administered to nude mice with palpable 296 tumors (Figure 6F). As a single agent, BrPA dramatically inhibited tumor growth compared with vehicle controls (Figure 6G-H).

**Discussion**

Increased glucose metabolism (glycolysis) and flux through lipogenesis is a hallmark feature of many cancers, providing structural molecules and aiding intracellular signaling and transport that is required for cancer progression [31]. Analyses of TCGA data sets found that 40-68% of endometrial cancers (Type I and II) harbor alterations in the glycolytic-lipogenic genes that we observed in our cohort of patient samples. Interestingly, only women with Type I tumors that had these alterations (including those with stages II-IV and obese patients) had worse overall survival than those without. In contrast, in ovarian cancer (a malignancy not typically associated with obesity) alterations in these genes were more common (80% of cases) but were more frequently down-regulated and not associated with survival. While it is clear that alterations in genes controlling glycolytic-lipogenic processes are not unique to endometrial cancer, this metabolic pathway appears to impact the outcome of treatment and/or disease progression in women with Type I endometrial cancer. Therefore, therapeutic targeting of this pathway may improve survival rates for these subsets of patients.
Facilitative glucose uptake feeds glycolytic-lipogenic metabolism and is regulated by the GLUT family (GLUTs 1-14). Many of these are expressed in the uterus and some, including the ubiquitously expressed GLUT1, are elevated in endometrial cancer [25, 32-35]. GLUT1 is thought to contribute to basal glucose uptake in normal tissues and up-regulation of this transporter has been reported in many malignancies [36]. In this study, GLUT1 expression was only increased in some of our patient tumor samples and it was not specific to cancerous endometrial cells. In contrast, GLUT6 was the most elevated glucose transporter in malignant endometrial tissue and it was the only protein specifically up-regulated in endometrial cancer cells. Furthermore, we demonstrated that GLUT6 promotes glycolysis and survival of endometrial cancer cells, despite the expression of other glucose transporters. These data suggest that either endometrial cancer cells become dependent on glucose uptake via GLUT6, or GLUT6 may have other roles in cancer biology that remain to be discovered.

Metabolic profiling of endometrial cancer cells showed higher rates of glycolysis and lower glucose oxidation compared to their non-malignant counterparts. However, oxidative metabolism in general was not defective in endometrial cancer cells. Our results suggest that glucose oxidation is specifically decreased in endometrial cancer cells by a mechanism that is independent of a dysfunction in mitochondrial TCA cycle or oxidative phosphorylation machinery. Furthermore, our data support the concept that glucose-derived carbons are preferentially utilized by cancer cells for macromolecule synthesis to promote cell proliferation [37, 38]. Constitutive activation of the PI3K-Akt pathway in cancer cells is thought to contribute to this metabolic phenotype by stimulating glucose transport and metabolism. In our study, activation of Akt was associated with increased glycolytic-lipogenic metabolism in endometrial
cancer cells. Activation of Akt also influences protein synthesis through mammalian target of rapamycin (mTOR) and alterations in this pathway have been reported in endometrial cancer [13, 39]. However, in our cohort of patients we found that TSC2 expression (negative regulator of mTOR) and the phosphorylation of S6K1 (an mTOR target that regulates protein synthesis) were similar in endometrial tumor vs. adjacent non-tumor tissue. Together, our data provides support for Akt activation as a central regulator of glycolytic-lipogenic metabolism in cancer cells.

In recent years a number of studies have reported the anti-cancer activity of BrPA [26, 27, 40-46], and a modified formula of BrPA was effective at reducing the growth of advanced hepatocellular carcinoma in a human patient [28]. However, the metabolic consequences, molecular targets, and mechanism of action for this agent remain unclear. In endometrial cells, it appears that sensitivity to BrPA is not solely mediated by the expression of an individual protein. We also found that BrPA is a potent inhibitor of lipogenesis, by depleting the lipid precursor acetyl-CoA. Other studies have reported that BrPA lowers acetyl-CoA levels in non-cancer cells [47, 48] although our study is the first to report that the mechanism for acetyl-CoA depletion is due to pyruvlation of CoA. CoA is an important cofactor for intermediary nutrient metabolism and is thought to be involved in up to 4% of all cellular enzymatic reactions [49]. Therefore BrPA has the ability to alter numerous metabolic reactions by pyruvylating thiols, including that of the intracellular reducing agent glutathione (GSH), as proposed by [40, 50]. GSH is important for protecting cells against ROS and it has been suggested that BrPA-mediated cell death is due to oxidative stress [30]. However, in this study, BrPA-induced cell death was not protected by any antioxidant except the thiol-containing NAC. We therefore
propose that BrPA-induced cell death is not due to increased ROS generation, at least in endometrial cancer cells.

Overall, this study supports an important role for glucose metabolism in endometrial tumor growth. More specifically that endometrial cancer cells may rely on GLUT6-mediated glucose transport and glycolytic-lipogenic metabolism for survival, and these features represent vulnerabilities that are amenable to therapeutic intervention. BrPA may prove useful to treat endometrial cancer since it is a dual glycolytic-lipogenic inhibitor. However, the pleiotropic effects of this agent may limit its administration for anti-cancer therapy. As such, a modified formula or targeted delivery of BrPA to tumors may be required to reduce off-target toxicity.

From a clinical standpoint, our data suggest that maintenance of blood glucose levels by regulation of dietary intake and/or administration of hypoglycemic agents may be helpful at reducing the growth of endometrial tumors. Future studies are required to delineate the role of glucose availability and metabolism in the etiology of endometrial cancer.

Acknowledgements

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References


Table 1. Glycolytic-lipogenic gene alterations in gynecological malignancies

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Total cases</th>
<th>Cases with gene alterations (%)</th>
<th>Cases with genes only down regulated</th>
<th>Survival p value</th>
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<tr>
<td><strong>Endometrial Type I</strong></td>
<td></td>
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<tr>
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<td>123 (40)</td>
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<td>0.003384*</td>
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<td>195</td>
<td>71 (36)</td>
<td>2</td>
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<tr>
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<td><strong>Ovarian</strong></td>
<td>316</td>
<td>254 (80)</td>
<td>78</td>
<td>0.580492</td>
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</table>

* significantly worse survival (p value <0.05) compared with those cases (within a type of cancer) without these alterations.
Figure and Table Legends

Figure 1. Glucose metabolism and de novo lipogenesis pathways are enriched in endometrial tumor tissue. (A) Enrichment plot of genes regulating glycolysis and gluconeogenesis in endometrial tumors compared with non-tumor tissue. Glycolytic enzymes (ranked gene expression) are listed on the left. (B) Gene expression pathways enriched in the malignant endometrium compared with non-tumor endometrium. Groups of genes regulating glycolysis, fatty acid biosynthesis and lipid metabolism are depicted with yellow circles. Larger circle diameters indicate greater numbers of genes involved in pathway. (C, D, F) The expression of glycolytic/DNL enzymes, Akt signaling pathway proteins, and glucose transporters in tumor (T) and normal adjacent (N) endometrial tissue (6 matched patient samples). 14-3-3 serves as a loading control for all blots. (E) Fold increase in gene expression of GLUT transporter family members in endometrial tumor tissue compared with non-tumor tissue. (G) GLUT6 expression in tumor and normal endometrium. Endothelial (arrow heads), stromal (S), glandular epithelial (E), and luminal (L) cells of endometrium.

Table 1. Alterations in glycolytic-lipogenic genes in gynecological malignancies.
Analyses were performed on cBioPortal using uterine corpus endometrial carcinoma (TCGA, Nature 2013) and ovarian serous cystadenocarcinoma (TCGA, Nature 2011) data sets. Genes included in analysis were GLUT6, HK2, PFKP, LDHA, ENO1, GAPDH, BPGM, ENO2, ALDOA, PKM2, ACLY, ACC1, ACC2, and FASN. Alterations considered were mRNA expression (up or down regulated), gene amplification, deletion, and mutations.
**Figure 2.** Cancerous endometrial cells display Warburg-like metabolism and elevated lipogenesis. Protein expression and metabolism of 7 human endometrial cancer (HEC, ISH, 296, 319, AN3, KLE, RL), and non-cancer stromal (MAD) and epithelial cells (hUE). (A-B) Glucose transporters, glycolytic/DNL enzymes, and Akt signaling pathway proteins in endometrial cells. 14-3-3 serves as a loading control. (C) Boxes representing each cell line depicted in the following graphs. Glycolysis (D), ECAR (E), glucose oxidation (F), glutamine oxidation (G), palmitate oxidation (H), OCR (I), DNL from glucose (J), DNL from glutamine (K) and DNL from acetate (L) were measured for all cells. Metabolic assays represent the mean (±SEM) of 3-5 technical replicates (experiments were performed at least twice).

**Figure 3.** GLUT6 regulates glycolysis in endometrial cancer cells. (A) GLUT6 mRNA expression was inhibited by 69% (Pool siRNA) and 78% (Seq.4 siRNA) in 296 cells, compared with controls (Ctrl siRNA) at 72 hr. (B) GLUT6 protein expression was partially reduced by the siRNA Pool, and inhibited by 90% with Seq.4 siRNA compared with controls at 96 hr. β-actin (loading control). (C) GLUT6 knockdown with the siRNA Pool and Seq.4 significantly reduced 296 cell counts at 96 hr, compared with controls. (D) Cell cycle analysis of siRNA-transfected 296 cells at 80 hr. Sub-G1 populations (black bars) and population counts (% of total) are shown within histograms. (E) Annexin V positive (+) and negative (-) 296 cells following GLUT6 siRNA-transfection at 80 hr; data are presented as % of total cells (positive and negative). (F) LDH release in GLUT6 siRNA-transfected 296 cells (fold-increase of control) at 96 hr. (G) Glucose uptake (3H-2-DG) in siRNA-transfected 296 cells at 96 hr. (H) Glycolysis in siRNA-transfected 296 cells at 96 hr. (I) Lactate release into media from siRNA-transfected 296 cells
at 72 hr (normalized to protein content per well). All data show the mean of at least three independent experiments ± SEM (*p<0.05). Time (hr)= time post-siRNA transfection.

**Figure 4. Sensitivity of endometrial cells to chemotherapeutics and compounds that target metabolism.** Percentage viability of endometrial cancer (HEC, ISH, 296, 319, AN3, KLE, RL) and non-cancer cells (MAD, hUE) following exposure to (A) carboplatin, (B) paclitaxel, (C) 2-DG, (D) BrPA, (E) TOFA, (F) DCA, (G) etomoxir, (H) phloretin, (I) DON, and (J) metformin. All dose response curves represent the mean of 3 independent experiments (error bars are ± SEM).

**Figure 5. BrPA inhibits lipogenesis in endometrial cancer cells.** (A-D) Glycolysis and substrate (glucose, glutamine, acetate) conversion to lipids were measured in 296 cells untreated or treated with 100 µM BrPA (90 min). (E) Acetyl-CoA levels (corrected peak area) in 296 cells untreated or treated with 100 µM BrPA for 90 min. (F) Activity of free thiol on CoA (1µM final reaction) was measured in presence of the indicated concentrations of BrPA (+CoA). (G) Activity of free thiol on NAC (1µM final reaction) was measured in presence of the indicated concentrations of BrPA (+NAC). BrPA without CoA or NAC are background controls (-CoA, -NAC). (H-I) BrPA-mediated 296 cell death is rescued by pretreatment with CoA or NAC for 30 min. All metabolic assays and dose response curves represent the mean (±SEM) of 3 independent experiments (*p<0.05).

**Figure 6. BrPA induces necrosis and impairs endometrial tumor growth in vivo.** (A) Phase contrast images of 296 cells, untreated or treated with 3-bromopyruvate (BrPA)
(100µM). (B) DNA fragmentation in untreated, BrPA-treated, or UV-irradiated (150,000 µjoules/cm²) 296 cells. (C) Annexin V/7-AAD staining of 296 cells following BrPA treatment or UV-irradiation (24hr BrPA treatment, 5hr post UV-irradiation). (D) Caspase 3/7 activity in 296 cells following BrPA treatment or UV-irradiation. (E) LDH release in 296 cells treated with BrPA (+/-QVD) or UV-irradiated (+/-QVD) (18hr post treatment). LDH release is calculated as a % increase of untreated cells. All in vitro data represent the mean of at least 3 independent experiments ± SEM. (F) Schematic of BrPA dosing in mice. (G) Growth of 296 tumors in mice treated with vehicle or BrPA (2.5mg/kg) (n=3). Data points represent the mean tumor volume ± SEM. (H) 296 tumors from mice treated with vehicle (PBS) or BrPA (*p<0.05).
Figure 1

A

Genes (Rank)
HK2 (34)
PFKP (66)
LDHA (128)
ENO1 (209)
GAPDH (330)
BPGM (388)
ENO2 (921)
ALDOA (1025)
PKM2 (1286)
FBP2 (1305)
GPI (1392)
ALDOB (1596)

B

Fatty acid biosynthesis
Glycolysis
Phospholipid metabolism
Oncogenesis
Lipid, fatty acid and steroid metabolism

C

Glycolysis
HK2
GAPDH
PKM2
LDHA

D

Akt pathway
PTEN
pAKT
AKT
14-3-3

E

SLC2A6
SLC2A1
SLC2A5
SLC2A2
SLC2A12
SLC2A4
SLC2A9
SLC2A8
SLC2A3
SLC2A11
SLC2A10
SLC2A13

F

Glucose uptake
GLUT6
GLUT1
14-3-3

G

Normal Endometrium
Endometroid Tumor

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Figure 2

A. Glucose uptake and Glycolysis from Stromal, Cancer, and Epithelial tissues.

B. DNL and Akt pathway from Stromal, Cancer, and Epithelial tissues.

C. Oxidative Metabolism and De novo lipogenesis from Stromal, Cancer, and Epithelial tissues.

D. Glycolysis from Glucose to H₂O.

E. ECAR from Glucose.

F. Glucose to CO₂.

G. Glutamine to CO₂.

H. Palmitate to CO₂.

I. OCR from Acetate.

J. Glucose to Lipid.

K. Glutamine to Lipid.

L. Acetate to Lipid.
Figure 3

A

![Bar graph showing the ratio of target gene/β2M expression levels for Ctrl, Pool, and Seq.4 with asterisks indicating significant differences.]

B

![Western blot images showing GLUT6 and β-actin protein levels for Ctrl, Pool, and Seq.4.]

C

![Column graph showing the number of cells (x10^4/well) for Ctrl, Pool, and Seq.4 with asterisks indicating significant differences.]

D

![DNA content histograms for Ctrl, Pool, and Seq.4 with counts ranging from 0 to 1200 and DNA content from 50K to 250K.]

E

![Bar graph showing the percentage of total DNA content for AV- and AV+ cells with Ctrl, Pool, and Seq.4.]

F

![Bar graph showing LDH release (fold increase of ctrl) with Ctrl, Pool, and Seq.4.]

G

![Bar graph showing glucose uptake (pmol 3H-2-DG uptake/min/mg protein) for Ctrl, Pool, and Seq.4 with asterisks indicating significant differences.]

H

![Bar graph showing glucose to H2O conversion (pmol 3H-glucose to 3H2O/min/mg protein) for Ctrl, Pool, and Seq.4 with asterisks indicating significant differences.]

I

![Bar graph showing lactate release (μM Lactate/μg protein) for Ctrl, Pool, and Seq.4 with asterisks indicating significant differences.]
Figure 4

Chemotherapeutics

A  Carboplatin

B  Paclitaxel

Glycolytic Inhibitors

2-DG

BrPA

Lipogenesis Inhibitor

TOFA

Glucose Oxidation Agonist

DCA

Fat Oxidation Inhibitor

Etomoxir

Nutrient Uptake Inhibitor

Phloretin

Glutaminolysis Inhibitor

DON

Complex I Inhibitor

Metformin
Figure 5

A. Glucose \(\rightarrow\) H\(_2\)O

- Untreated
- BrPA

B. Glucose \(\rightarrow\) Lipid

C. Glutamine \(\rightarrow\) Lipid

D. Acetate \(\rightarrow\) Lipid

E. Acetyl-CoA

F. Fluorescence (379/513 nm) vs. \(\mu\)mol/L BrPA

G. Fluorescence (379/513 nm) vs. \(\mu\)mol/L BrPA + NAC

H. % viable cells vs. \(\mu\)mol/L BrPA + CoA (250\(\mu\)M)

I. % viable cells vs. \(\mu\)mol/L BrPA + NAC (250\(\mu\)M)
Figure 6

A. Images showing untreated cells and BrPA-treated cells at 0hr, 6hr, and 18hr, with a scale bar indicating 20μm.

B. Gel electrophoresis images comparing untreated and BrPA-treated samples at 6hr and 18hr.

C. Flow cytometry plots showing Annexin V and 7-AAD staining for untreated, BrPA-treated, and UV-treated samples.

D. Bar graph showing LDH release as a percentage increase of untreated controls at 6hr and 18hr.

E. Bar graph comparing LDH release for untreated, BrPA-treated, and UV-treated samples with or without QVD.

F. Diagram illustrating the timeline of s.c. injection of 296 cells, followed by 4 days of Trx treatment for 20 days, and the subsequent killing of palpable tumors (~2 mm) over 3 weeks.

G. Line graph showing tumor volume (mm³) over 3 weeks for Vehicle (PBS) and BrPA treatments, with significance levels indicated.

H. Images of tumors from Vehicle (PBS) and BrPA treatments, with a scale bar indicating 1cm.
Metabolic vulnerabilities in endometrial cancer

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