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 with their nonmalignant counterparts, we found that upregulation 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, is a powerful antagonist of lipogenesis through pyruvylation of CoA. We also provide evidence that 3-bromopyruvate promotes cell death via a necrotic mechanism that does not involve reactive oxygen species and that 3-bromopyruvate impaired the growth of endometrial cancer xenografts Cancer Res; 74(20): 5832–45. ©2014 AACR.

Introduction

Endometrial cancer is the most common gynecologic malignancy in the developed world and affects more than 287,000 women and attributes to 74,000 deaths worldwide each year (1). Endometrial tumors originate from 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 most commonly arises in postmenopausal women and is classically categorized in two clinicopathologic 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, noninvasive, 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/m2] 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 noncancer; *P* = 0.049). However, other cancer-related parameters including estrogen and insulin were not significantly elevated in obese women with 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 PI3K/Protein kinase B (Akt) pathway is altered in up to 93% of type I endometrial tumors, through loss of PTEN and mutations in PI3K family members (4, 11). Other aberrations include mutations in V-Ki-
ras2 Kirsten rat sarcoma viral oncogene homolog, overexpression of epidermal growth factor receptor, loss of liver kinase B1 (LKB1), and tuberous sclerosis 2 (TSC2; refs. 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 (Charlottesville, VA). Endometrial samples were collected at surgery (hysterectomy) from four women with and four women without type I endometrial cancer (mean age, 52.6 years; BMI, 44.5 kg/m²) and RNA was analyzed, as described (14). For protein expression analyses, nontumor and tumor endometrial tissue (matched for each patient) were obtained from an independent cohort of postmenopausal women (mean BMI, 35.3 kg/m²), with Federation Internationale des Gynaecologistes et Obstetrices (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 ATCC (HEC-1-A, AN3CA, KLE, RL95-2); Sigma Aldrich (Ishikawa); Deutsche Sammlung von Mikroorganismen und Zellkulturen (MFE-296, MFE-319); Dr. Hui Li at the University of Virginia (MAD11) and Dr. Kelle Moley at Washington University School of Medicine, St. Louis, MO (hUE-Ts). MAD11 and hUE-Ts are human telomerase reverse transcriptase (hTERT)-immortalized endometrial stromal and uterine epithelial cells, respectively, which were both derived from patients without cancer. Cancer cells have not been validated since purchase.

Immunohistochemistry
Pathologic analyses of tumor and nontumor tissue were performed by a certified gynecologic oncology pathologist in a blinded manner. Immunohistochemistry was performed at the University of Virginia Biorepository and Tissue Research Facility using a mouse monoclonal antibody against human GLUT6 (Abcam). 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) 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 nontargeting SMARTpool control (Ctrl) siRNA (Thermo-Fisher Scientific) using JetPRIME reagent (Polyplus Transfection Inc.) as the delivery vehicle.

qPCR
RNA was isolated with TRIzol reagent (Life Technologies) and reverse transcribed using a High Capacity cDNA kit (Life Technologies). qPCR was performed using iQ SYBR Green SuperMix (Bio-Rad) on an iCycler (MyiQ Optical Module).

Table 1. Glycolytic–lipogenic gene alterations in gynecologic malignancies

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Total cases</th>
<th>Cases with gene alterations (%)</th>
<th>Cases with genes only downregulated</th>
<th>Survival P</th>
</tr>
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<tr>
<td>Endometrial type I</td>
<td>307</td>
<td>123 (40)</td>
<td>4</td>
<td>0.003384*</td>
</tr>
<tr>
<td>Stage I</td>
<td>231</td>
<td>88 (38)</td>
<td>4</td>
<td>0.241869</td>
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<tr>
<td>Stages II–IV</td>
<td>76</td>
<td>35 (46)</td>
<td>0</td>
<td>0.023995*</td>
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<tr>
<td>All stages lean (BMI &lt; 30)</td>
<td>109</td>
<td>51 (47)</td>
<td>2</td>
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<tr>
<td>All stages obese (BMI ≥ 30)</td>
<td>195</td>
<td>71 (36)</td>
<td>2</td>
<td>0.007452*</td>
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<tr>
<td>Endometrial type II</td>
<td>66</td>
<td>45 (68)</td>
<td>0</td>
<td>0.967244</td>
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<tr>
<td>Stage I</td>
<td>23</td>
<td>16 (70)</td>
<td>0</td>
<td>0.294052</td>
</tr>
<tr>
<td>Stages II–IV</td>
<td>43</td>
<td>29 (67)</td>
<td>0</td>
<td>0.611672</td>
</tr>
<tr>
<td>All stages lean (BMI &lt; 30)</td>
<td>33</td>
<td>23 (70)</td>
<td>0</td>
<td>0.826925</td>
</tr>
<tr>
<td>All stages obese (BMI ≥ 30)</td>
<td>33</td>
<td>22 (67)</td>
<td>0</td>
<td>0.751774</td>
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<tr>
<td>Ovarian</td>
<td>316</td>
<td>254 (80)</td>
<td>78</td>
<td>0.580492</td>
</tr>
</tbody>
</table>

NOTE: Analyses were performed on cBioPortal using uterine corpus endometrial carcinoma (TCGA; ref. 11) and ovarian serous cystadenocarcinoma (TCGA; ref. 17) datasets. Genes included in the analysis were GLUT6, HK2, PFKP, LDHA, ENO1, GAPDH, BPGM, ENO2, ALDOA, PKM2, ACLY, ACC1, ACC2, and FASN. Alterations considered were mRNA expression (up- or downregulated), gene amplification, deletion, and mutations.

*Significantly worse survival (P < 0.05) compared with those cases (within a type of cancer) without these alterations.
Bio-Rad system. GLUT mRNA expression was normalized to β2M using the Pfaffl method (19).

**Western blotting**
Protein lysates (20 μg) were resolved by PAGE and electrotransferred to nitrocellulose membrane. Protein expression was detected with rabbit antibodies such as GAPDH, PKM2, LDHA, ACLY, ACC, FASN, PTEN, AKT, PPARγ, TSC2, G6PD (Cell Signaling Technology); GLUT1 (Abcam); and mouse antibodies such as 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).

**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 14CO2 and de novo lipogenesis (DNL) from substrates was measured by hexane/isopropanol (3:2) extraction of 14C-lipids. For glycosylation measurements, D-[3-3H] glucose was separated from tritiated [3H]O2 by diffusion. Detailed methods for these assays are provided in Supplementary Material. For OCR and ECAR measurements, cells (10,000 to 20,000/well) were seeded in 24-well Seahorse tissue culture plates a day before incubation in KRP nutrient buffer (37 °C for >30 minutes). Basal OCR (pnaO2/min) and ECAR (mpH/min) were measured using the Seahorse XF-24 Flux Analyzer (Seahorse Biosciences). 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 lactate dehydrogenase (LDH) were prepared in glycine–hydradine buffer (500 mM/L glycine, 127 mM/L hydrazine sulfatate, pH 9.5). Cells were incubated in DMEM (without phenol red or FBS) for 45 minutes. Media removed from cells was incubated with 2.5 mM/L NAD+ and 25 μg/mL LDH for 1 hour at room temperature and absorbance measured at 340 nm.

**Glucose uptake**
Cells were incubated with 5 mM/L 2-deoxy-D-glucose (2-DG) in KRP with 0.2 μCi of 3H-2-DG per well for 2.5 minutes. Cells were rinsed twice in ice-cold PBS before permeabilization in 1% TritonX-100 (1 hour) before counting.

**Analyses of free thiol groups**
CoA and 3-bromopyruvate (BrPA) were prepared in PBS (pH 7.4), and N-acetyl cysteine (NAC) in water, and coincubated 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 10 μM/L ThioGlo1 (Covalent Associates Inc.). Fluorescent signal was measured 5 minutes later at 379/513 nm.

**Cytotoxicity assays**
Cells were exposed to drugs for 48 to 72 hours, and viability was detected by addition of thiazoyl blue tetrazolium bromide (MTT) reagent. Formazan crystals were solubilized and absorbance read at 590/620 nm. Cell viability is displayed as a percentage of control cells, that is, cells with equivalent concentrations of the appropriate drug vehicle. Refer to Supplementary Material for detailed methods.

**Cell viability and cycle analyses**
Cells were stained with 7-aminoactinomycin D (7-AAD; 2 μg/mL) and Annexin V (AV). Viable (AV−) and nonviable (AV+) cell populations were determined by flow cytometry (BD FACS Canto I). For cell cycle, cells were permeabilized/stained with 0.4% Triton X-100, 2 μg/mL RNase, and 2 μg/mL propidium iodide (PI)TOPRO-3 in warm PBS. Profiles were determined by flow cytometry and sub-G1 populations gated using FlowJo software (Tree Star Inc.).

**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 13C3-malonyl-CoA (0.5 μM/L final) as an internal standard. Supernatants were loaded onto pre-equilibrated solid-phase extraction columns (Oasis HLB, 1 cc/30 mg, Waters) 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)-Foxn1nu, Charles River Laboratories Inc.) were subcutaneously inoculated with 3 × 106 296 cells. Mice with palpable tumors (~2 mm diameter) were administered 2.5 mg/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 (CO2 asphyxiation) when the largest tumor neared 1 cm³.

Tumor volumes 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.

**Statistical analysis**
Unpaired 2-tailed Student 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) were calculated.
and linear regression graphs were derived from the GraphPad Prism program.

Results

**Glucose transporters, and glycolytic and lipogenic enzymes, are upregulated 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/m²; ref. 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; Fig. 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; Fig. 1A). We also performed bioinformatics analyses on genes that were upregulated by more than 6-fold (371 genes) using the Database for Annotation, Visualization and Integrated Discovery (DAVID; refs. 21, 22) and matched these hits to the Panther biologic processes gene lists. These data identified that glycolytic and lipogenic metabolic pathways were enriched in malignant compared with nonmalignant endometrium (Fig. 1B). In an independent cohort of six 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 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 tumors versus adjacent nonmalignant tissue (Supplementary Fig. S1A).

Aberrations in the PTEN/PK3/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 (Supplementary Fig. S1B). As shown in Fig. 2A, GLUT6 expression and glycolytic–lipogenic metabolism are upregulated in endometrial cancer cells.

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 six of seven endometrial cancer cells compared with noncancerous endometrial cells (Fig. 2C and D), and glycolysis significantly correlated with ECAR (r = 0.858, P = 0.005; Fig. 2E). In contrast, glucose oxidation was markedly decreased in cancerous glandular epithelial cells, particularly those closest to blood vessels in the surrounding stroma (Fig. 1G and Supplementary Fig. S2). Blood vessels also stained positive for GLUT6. Glandular cells from the normal human endometrium had little or no expression of GLUT6 (Fig. 1G and Supplementary Fig. S2). In comparison, the ubiquitously expressed glucose transporter, GLUT1, was elevated by 4.3-fold at the gene level in the malignant endometrium (Fig. 1E) and in three of six tumors compared with nontumor endometrium (Fig. 1F and Supplementary Fig. S1A).

Analyses of TCGA datasets 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 versus more advanced stages or lean versus obese within type I and within type II cancers (Table 1). However, patients with 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 downregulated in a higher percentage of ovarian cancer cases (78 of 254, 30.7%) compared with type I (4 of 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 upregulated in endometrial cancer cells**

To further investigate endometrial cancer metabolism, we metabolically profiled seven 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 two immortalized cell lines derived from noncancerous endometrial tissue, MAD-11 (MAD) stromal and hUE-Ts (hUE) uterine epithelial cells. As shown in Fig. 2A, GLUT6 expression was upregulated in all seven endometrial cancer cells compared with noncancerous endometrial cells, whereas the expression of GLUT1 and all other glycolytic and DNL enzymes varied between cancer and noncancer cells (Fig. 2A and B). Akt was activated (phosphorylated) in the majority of cancerous endometrial cells, and PTEN expression was lost in three of the seven cancer cell lines (Fig. 2B).

Targeting Glucose Metabolism in Endometrial Cancer
HK2 (34)
PFKP (66)
LDHA (128)
ENO1 (209)
GAPDH (330)
BPGM (388)
ENO2 (921)
ALDOA (1025)
PKM2 (1286)
FBP2 (1305)
GPI (1392)
ALDOB (1596)

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

GLUT6
14-3-3
1
NT
NT
NT
NT
NT
2 3 4 5 6

Akt pathway
14-3-3
GAPDH
14-3-3
ACC2
ACC1
FASN
1
NT
NT
NT
NT
NT
2 3 4 5 6

GLUT1
14-3-3
GLUT6

Fold enrichment (T/N)
–log10 (P-value)

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decreased in all seven endometrial cancer cells compared with the two noncancerous cells (Fig. 2F). The oxidation of glutamine (Fig. 2G) and palmitate (Fig. 2H) and OCR (Fig. 2J) was not associated with a cancer phenotype. Finally, DNL from glucose, glutamine, and acetate precursors was generally increased in most cancer-derived cells compared with noncancerous endometrial cells (Figs. 2J–L). Correlation analyses of these data revealed that GLUT6 protein expression significantly correlated with Warburg-type metabolism (low glucose oxidation/glycolysis ratio; \( r = -0.837, P = 0.005 \); Supplementary Fig. S3A), and the phosphorylation of Akt significantly correlated with glycolysis (\( r = 0.849, P = 0.004 \); Supplementary Fig. S3B) and glucose-derived lipogenesis in endometrial cells (\( r = 0.777, P = 0.014 \); Supplementary Fig. S3C).

**GLUT6 promotes glycolysis and survival of endometrial cancer cells**

Because GLUT6 has not been functionally characterized in cancer cells, we evaluated the role of GLUT6 using siRNA to knockdown this protein in 296 (Fig. 3A–I and Supplementary Fig. S4A–S4C) and RL cells (Supplementary Fig. S4D–S4H). Both of these cells were derived from type I endometrial tumors from premenopausal 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%, whereas siRNA sequence 4 (Seq-4; a single sequence from the SMARTpool) reduced GLUT6 protein expression by 90%, compared with controls (Fig. 3B and Supplementary Fig. S4A). Importantly, GLUT6 knockdown did not alter the expression of GLUT1, GLUT4, and GLUT8 (GLUT8 has the most sequence homology to GLUT6; ref. 25) in 296 cells (Supplementary Fig. 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 (Fig. 3C and Supplementary Fig. S4E, \( P < 0.05 \)). Of note, GLUT6 knockdown with other individual siRNA sequences (shown in Supplementary Fig. 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 morphologic alterations (Supplementary Fig. S4C), a marked increase in sub-G1 populations (Fig. 3D and Supplementary Fig. S4F), number of AV+ cells (Fig. 3E and Supplementary Fig. S4G), and release of LDH from permeabilized cells (Fig. 3F and Supplementary Fig. S4H).

To determine the role of GLUT6 in glucose metabolism, we measured glucose uptake and glycolysis in GLUT6 knock-down cells. Because 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% (Fig. 3G, \( P = 0.001 \)), glycolysis by 33% (Fig. 3H, \( P = 0.004 \)), and lactate release by 55% (Fig. 3I, \( P < 0.001 \)).

**Targeting metabolism in endometrial cancer cells**

To test whether metabolic phenotypes could be exploited in endometrial cancer cells, we screened a panel of compounds known to target diverse metabolic pathways. Selectivity and toxicity of these compounds were compared with chemotherapeutics used to treat advanced-stage endometrial cancer. Endometrial cancer and noncancer cells displayed varying sensitivity to the DNA-damaging agent carboplatin (Fig. 4A). Notably, the noncancerous hUE cells were one of the most sensitive to carboplatin, compared with endometrial cancer and noncancerous MAD cells (Fig. 4A). In contrast, paclitaxel (a microtubule-stabilizing agent) showed greater selective toxicity toward endometrial cancer cells compared with noncancer cells (Fig. 4B). This sensitivity correlated with cell population doubling times, thereby validating the role microtubules play in cell division (Supplementary Fig. S5A and S5B; \( r = 0.796, P = 0.01 \)).

Endometrial cells were then exposed to the glycolytic inhibitors 2-DG and BrPA (Fig. 4C and D), the lipogenesis inhibitor 5-(tetradecyloxy)-2-furoic acid (TOFA; Fig. 4E), the glucose oxidation agonist dichloroacetate (DCA; Fig. 4F), the fatty acid oxidation inhibitor etomoxir (Fig. 4G), the glucose and fatty acid uptake inhibitor, phloretin (Fig. 4H), the glutaminolysis inhibitor, 6-diazo-5-oxo-l-norleucine (DON; Fig. 4I), and the pleiotropic metabolic inhibitor, metformin (Fig. 4J). These compounds displayed a range of cytotoxic effects on the panel of endometrial cells including nonsel ective toxicity to all cells (e.g., 2-DG, metformin, and etomoxir), toxicity only at supraphysiologic 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 (Fig. 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<sub>50</sub> = 34 μmol/L) and the least sensitive cells (HEC, IC<sub>50</sub> = 1.267 μmol/L; Fig. 4D). This suggested that sensitivity to BrPA was determined by specific cellular targets.
Figure 2. Cancerous endometrial cells display Warburg-like metabolism and elevated lipogenesis. Protein expression and metabolism of seven human endometrial cancer (HEC, ISH, 296, 319, AN3, KLE, RL) and noncancer stromal (MAD) and epithelial cells (hUE). A and B, glucose transporters, glycolytic/DNL enzymes, and Akt signaling pathway proteins in endometrial cells. 14-3-3 served 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 three to five technical replicates (experiments were performed at least twice).
BrPA inhibits DNL by pyruvlation of CoA

Birsoy and colleagues recently used 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 pyruvlation of GAPDH and HK2 (27). In evaluating the correlation between BrPA sensitivity and known cellular targets, we found that the expression of MCT1 (Supplementary Fig. S5C), HK2, and GAPDH (Fig. 2A), individually, did not correlate with sensitivity to BrPA (Supplementary Fig. S5D).

Glycolysis and lipogenesis were the two metabolic pathways most upregulated in endometrial tumors. We therefore investigated the effects of BrPA on these pathways in endometrial cancer cells. At a toxic dose (100 μmol/L), BrPA treatment inhibited glycolysis in 296 cells by 46% (Fig. 5A, \(P < 0.0001\)) but had a greater inhibitory effect on DNL from 3 different lipogenic precursors such as glucose (79% decrease, \(P = 0.002\); Fig. 5B), glutamine (99% decrease, \(P < 0.001\); Fig. 5C), and acetate (86% decrease, \(P = 0.0002\); Fig. 5D). DNL from these precursors requires the production of 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 (Fig. 5E; \(P < 0.002\)). The formation of acetyl-CoA requires CoA. Because BrPA can pyruvylate free thiols (R-SH; ref. 28), we examined whether BrPA reacted with the thiol on CoA using a dye that fluoresces once bound to free thiol.
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 noncancer cells (MAD, hUE) following exposure to carboplatin (A), paclitaxel (B), 2-DG (C), BrPA (D), TOFA (E), DCA (F), etomoxir (G), phloretin (H), DON (I), and metformin (J). All dose-response curves represent the mean of 3 independent experiments. Error bars are ± SEM.
groups (Fig. 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 (Fig. 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 pyrurvlation occurs at the sulfhydryl group of CoA (Supplementary Fig. S6).

BrPA-mediated cytotoxicity is independent of reactive oxygen species generation

Previous studies have indicated that the antioxidant, NAC, protects against BrPA-mediated cell death by scavenging reactive oxygen species (ROS; ref. 30). Because 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 (Fig. 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-mediated cell death. Indeed, although a toxic dose of BrPA increased ROS (Supplementary Fig. S7A and S7B), pretreatment of endometrial cancer cells with apocynin (Supplementary Fig. S7C), allopurinol (Supplementary Fig. S7D), N omega-nitro-L-arginine methyl ester (L-NAME; Supplementary Fig. S7E), manganese [III] tetrakis (4-benzoic acid) porphyrin (MnTBAP; Supplementary Fig. S7F), Mito-tempo (Supplementary Fig. S7G), resveratrol (Supplementary Fig. S7H), or ascorbic acid (Supplementary Fig. S7I) could not protect against BrPA-mediated cell death.
Because other antioxidants could not protect against BrPA-induced cell death, we proposed that excess CoA and NAC could act as “sponges” to react with BrPA and prevent cytotoxicity. Indeed, pretreatment of 296 cells with CoA (Fig. 5H) and NAC (Fig. 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, \( P < 0.0001 \); Supplementary Fig. 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 Fig. 6A and UV-irradiated cells in Supplementary Fig. S8B), DNA fragmentation (Fig. 6B), an increase in apoptotic cell populations (AV/7-AAD staining), or activation of caspases (Fig. 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; Fig. 6E and Supplementary Fig. S8C). From these observations, we conclude that BrPA-mediated cell death was due to necrosis. Because BrPA kills endometrial cancer cells in vitro, we tested the efficacy of this agent against endometrial tumors in vivo. BrPA (2.5 mg/kg) or vehicle (PBS) was administered to nude mice with palpable 296 tumors (Fig. 6F). As a single agent, BrPA dramatically inhibited tumor growth compared with vehicle controls (Fig. 6G and 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% to 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 who 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 downregulated 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 upregulation 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 upregulated 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 than their nonmalignant 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 tricarboxylic acid (TCA) cycle or oxidative phosphorylation machinery. Furthermore, our data support the concept that glucose-derived carbons are preferentially used 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 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 versus adjacent nontumor tissue. Together, our data provide 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 anticancer 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 noncancer 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 earlier (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, 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 because it is a dual glycolytic–lipogenic inhibitor. However, the pleiotropic effects of this agent may limit its administration for anticancer 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.

Disclosure of Potential Conflicts of Interest

J.M. Lancaster received speakers’ bureau honoraria from Amgen. No potential conflicts of interest were disclosed by the other authors.
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29. F.L. Byrne, D.C. Marchion, K.L. Hoehn, page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 29, 2014; revised July 28, 2014; accepted August 13, 2014; published OnlineFirst September 9, 2014.
Metabolic Vulnerabilities in Endometrial Cancer
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