Buformin exhibits anti-proliferative and anti-invasive effects in endometrial cancer cells

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Abstract: Objective: Biguanides are anti-diabetic drugs that are thought to have anti-tumorigenic effects. Most pre-clinical studies have focused on metformin for cancer treatment and prevention; however, buformin may be potentially more potent than metformin. Given this, our goal was to evaluate the effects of buformin on cell growth, adhesion and invasion in endometrial cancer cell lines. Methods: The ECC-1 and Ishikawa endometrial cancer cell lines were used. Cell proliferation was assessed by MTT assay. Apoptosis and cell cycle analysis was performed by FITC Annexin V assay and propidium iodide staining, respectively. Adhesion was analyzed using the laminin adhesion assay. Invasion was assessed using the transwell invasion assay. The effects of buformin on the AMPK/mTOR pathway were determined by Western immunoblotting. Results: Buformin and metformin inhibited cell proliferation in a dose-dependent manner in both endometrial cancer cell lines. IC50s were 1.4-1.6 mM for metformin and 8-150 μM for buformin. Buformin induced cell cycle G1 phase arrest in the ECC-1 cells and G2 phase arrest in the Ishikawa cells. For both ECC-1 and Ishikawa cells, treatment with buformin resulted in induction of apoptosis, reduction in adhesion and invasion, activation of AMPK and inhibition of phosphorylated-S6. Buformin potentiated the anti-proliferative effects of paclitaxel in both cell lines. Conclusion: Buformin has significant anti-proliferative and anti-metastatic effects in endometrial cancer cells through modulation of the AMPK/mTOR pathway. IC50 values were lower for buformin than metformin, suggesting that buformin may be more potent for endometrial cancer treatment and worthy of further investigation.

Keywords: Endometrial cancer, buformin, proliferation, invasion

Introduction

Endometrial cancer is the fourth most common cancer among women with an estimated 54,870 new cases diagnosed and 10,170 deaths in the United States in 2015 [1]. The incidence of this disease has increased over the past few decades, largely due to the growing obesity epidemic, with women now having a 1 in 38 lifetime risk of developing endometrial cancer. Obesity, diabetes and insulin resistance are well-known factors associated with both increased risk of developing endometrial cancer [2, 3] and increased risk of death [4-6]. Despite available chemotherapy, overall 5-year survival for advanced endometrial cancer remains poor at 21-56%. Thus, there is a great need for novel agents to improve endometrial cancer outcomes.

A growing body of evidence suggests that the class of drugs known as biguanides may be effective as anti-cancer agents [7]. Three biguanides, metformin, phenformin and buformin, have been used in the treatment of non-insulin-dependent diabetes mellitus. Metformin has been marketed in the United States since 1995 [8]. Buformin and phenformin are not approved for use in the United States due to increased risk of lactic acidosis. In recent years, epidemiologic evidence has shown that diabetic patients treated with biguanide drugs have a reduced risk of developing cancer compared with patients receiving sulfonylureas [7, 9]. In vitro
studies of metformin and phenformin in a variety of cancers have demonstrated that these drugs cause disruption of mitochondrial respiration leading to activation of AMP-activated protein kinase (AMPK) and inhibition of the mammalian target of rapamycin (mTOR) pathway, ultimately resulting in the inhibition of cellular proliferation, induction of apoptosis, cell cycle arrest and a reduction in protein and lipid synthesis [10-13]. In vivo studies have indicated that metformin and phenformin have promising anti-tumorigenic activity in breast cancer, colon cancer and ovarian cancer mouse models, among others [11-14]. Currently, metformin is being investigated in greater than 50 phase I, II and III clinical trials in multiple types of cancer, including endometrial cancer [15].

Looking beyond metformin at other biguanide drugs, the role for phenformin and buformin as potential anti-cancer agents has recently been investigated. Phenformin and buformin are appealing drugs compared to metformin, as they are more lipophilic and more potent inhibitors of mitochondrial complex I and cellular ATP production [16-18]. The major limitation of buformin and phenformin is their increased risk of lactic acidosis. Phenformin is associated with a 10- to 20-fold increased risk of lactic acidosis compared to metformin, and there is limited data about the incidence of buformin-associated lactic acidosis [19]. Renal secretion is required for clearance of biguanides, and nearly all episodes of lactic acidosis associated with biguanides have occurred in patients with renal dysfunction [20]. Careful patient selection and observation may allow this side effect to be minimized. Moreover, treating cells with a combination of phenformin and 2-deoxyglucose or a lactate dehydrogenase (LDH) inhibitor, can avoid development of lactic acidosis [13].

Given that (1) biguanides have demonstrated beneficial chemopreventive and chemotherapeutic effects in a number of cancers and (2) buformin may be more potent than metformin in inhibition of energy metabolism in cancer cells [10, 15, 21-23], buformin warrants further evaluation as a potential drug for cancer therapy. Thus, the aim of this study was to investigate the anti-tumorigenic and anti-metastatic effects of buformin in endometrial cancer cell lines.

Materials and methods

Cell culture and reagents

Two endometrial cancer cell lines, ECC-1 and Ishikawa, were used for all experiments. The ECC-1 cells were grown in RPMI 1640 medium supplemented with 5% bovine, 100 units/ml penicillin and 100 μg/ml streptomycin under 5% CO₂. The Ishikawa cells were grown in MEM supplemented with 5% fetal bovine serum, 300 mM l-glutamine, 10,000 U/ml penicillin and 10,000 μg/ml streptomycin under 5% CO₂. Metformin, paclitaxel, RNase, and RIPA buffer was purchased from Sigma (St. Louis, MO). Buformin was purchased from Santa Cruz (Dallas, Texas). Metformin and Buformin were re-suspended in PBS. Paclitaxel was soluble in DMSO. Antibodies to phosphorylated-AMPK (Thr172), phosphorylated-S6 (Ser235/236), β-actin, pan-AMPK and pan-S6 were obtained from Cell Signaling Technology (Beverly, MA). The Annexin V FITC kit was purchased from BioVision (Mountain View, CA). Enhanced chemiluminescence western immunoblotting detection reagents were purchased from Amersham (Arlington Heights, IL). All other chemicals were purchased from Sigma.

Cell proliferation assay

The ECC-1 and Ishikawa cells were plated and grown in 96-well plates at a concentration of 5000 cells/well for 24 hours. These cells were then treated with various concentrations of buformin and metformin for a period of 72 hours. After the addition of MTT dye (5 mg/mL), the 96-well plates were incubated for 1-2 hours at 37°C. 100 μL of DMSO was added to the plates in order to terminate the MTT reaction, and the plates were read by measuring absorption at 595 nm. The effect of buformin and metformin was calculated as a percentage of control cell growth obtained from PBS (1%) treated cells grown in the same 96-well plates. Each experiment was repeated three times to assess for consistency of results.

Cell cycle analysis

The effect of buformin on cell cycle progression was assessed using Cellometer (Nexcelom, Lawrence, MA). In short, cells were plated at a density of 2 × 10⁵ cells/well in 6-well plates
overnight and then treated with varying concentrations of buformin for 48 hours. Cells were collected by 0.05% trypsin (Gibco Grand Island, NY), washed with phosphate-buffered saline (PBS) solution, fixed in a 90% methanol solution and then stored at -20°C until cell cycle analysis was performed. On the day of analysis, the cells were washed with PBS and centrifuged, resuspended in 50 ul RNase A solution (250 ug/ml) with 10 mM EDTA, followed by incubation for 30 min at 37°C. After incubation, 50 µl of propidium iodide (PI) staining solution (2 mg/ml PI, 0.1 mg/ml Azide and 0.05% Triton X-100) was added to each tube and incubated for 10 min in the dark. The cells were assessed by Cellometer. The results were analyzed using FCS4 express software (Molecular Devices, Sunnyvale, CA). Each experiment was repeated at least twice for consistency of response.

**Annexin V assay**

The effect of buformin on cell apoptosis was detected using the Annexin-V FITC kit. Briefly, 2 × 10^5 cells/well were seeded into 6-well plates overnight and then the cells were cultured in media with varying concentrations of buformin for 24 hours. The cells were collected by 0.25% trypsin without EDTA. After PBS washing, cells were resuspended in 100 ul of Annexin-V and PI dual-stain solution (0.1 ug of Annexin-V FITC and 1 ug of PI) for 15 min in the dark. Apoptotic cells were detected by Cellometer. The results were analyzed by FCS4 express software. Each experiment was repeated at least twice for consistency of response.

**Adhesion assay**

Each well in a 96-well plate was coated with 100 ul laminin-1 (10 ug/ml) and incubated at 37°C for 1 hour. This fluid was then aspirated, and 200ul blocking buffer was added to each well for 45-60 min at 37°C. The wells were then washed with PBS, and the plate was allowed to chill on ice. To each well, 2.5 × 10^5 cells were added with PBS and varying concentrations of buformin directly. The plate was then allowed to incubate at 37°C for 2 hours. After this period, the medium was aspirated, and cells were fixed by directly adding 100 ul of 5% glutaraldehyde and incubating for 30 min at room temperature. Adhered cells were then washed with PBS and stained with 100 ul of 0.1% crystal violet for 30 minutes. The cells were then washed repeatedly with water, and 100 ul of 10% acetic acid was added to each well to solubilize the dye. After 5 minutes of shaking, the absorbance was measured at 570 nm using a microplate reader from Tecan (Morrisville, NC). Each experiment was repeated at least twice for consistency of response.

**Invasion assay**

Cell invasion assays were performed using 96-well HTS transwells (Corning Life Sciences, Durham, NC) coated with 0.5-1X BME (Trevigen, Gaithersburg, MD). The ECC-1 and Ishikawa cells (50,000/well) were starved for 12 hours and then seeded in the upper chambers of the wells in 50 µl FBS-free medium. The lower chambers were filled with 150 µl regular medium with buformin. The plate was incubated for 24 hours at 37°C to allow invasion into the lower chamber. After washing the upper and lower chambers with PBS, 100 ul Calcein AM solution was added into the lower chamber and incubated at 37°C for 30-60 min. The lower chamber plate was measured by the plate reader for reading fluorescence at EX/EM 485/520 nM. Each experiment was repeated at least twice for consistency of response.

**Western immunoblotting**

The ECC-1 and Ishikawa cells were plated at 2 × 10^5 cells/well in 6 well plates in their appropriate medium and were treated for 24 hours with buformin. Cell lysates were prepared in RIPA buffer (1% NP, 0.5 sodium deoxycholate and 0.1% SDS) plus PhosStop. Equal amounts of protein were separated by gel electrophoresis and transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat dry milk and then incubated with a 1:1000 dilution of primary antibody overnight at 4°C. The membrane was then washed and incubated with a secondary peroxidase conjugated antibody for 1 hour after washing. Antibody binding was detected using an enhanced chemiluminescence detection buffer by Alpha Innotech imaging system (San Leandro, CA). Each experiment was repeated three times to assess for consistency of results.

**Statistical analysis**

Data were presented as mean ± standard error of the mean. Comparisons between groups...
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Figure 1. Effect of buformin and metformin on cell proliferation in endometrial cancer cells. The ECC-1 (A) and Ishikawa (B) cell lines were cultured in the presence of varying concentrations of buformin and metformin for 72 h. Cell proliferation was determined by MTT assay. The results are shown as the mean ± SE of triplicate samples and are representative of three independent experiments.

Figure 2. Effect of buformin on cell cycle and apoptosis in the Ishikawa and ECC-1 cell lines. The cells were treated with buformin at different doses for 48 h and cell cycle was analyzed by Cellometer. Buformin induced cell cycle arrest in G1 phase in the ECC-1 (A) cells and in G2 phase in the Ishikawa (B) cells. The ECC-1 and Ishikawa cells were grown for 24 h and then treated with buformin at the indicated concentrations for an additional 24 h. Annexin V expression was detected by Cellometer. Buformin induced apoptosis in the Ishikawa cells (D), but not in the ECC-1 cells (C). The results are shown as the mean ± SD and are representative of three independent experiments. **p<0.01.

were determined with the two-sided unpaired student’s t-test using GraphPad software (La Jolla, CA USA). A value of p<0.05 was considered as significant.
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Results

Comparison of buformin with metformin in the inhibition of cell proliferation in endometrial cancer cells

The effects of buformin and metformin on cell proliferation were examined in the endometrial cancer cell lines, ECC-1 and Ishikawa. Both cell lines were exposed to varying doses of buformin and metformin for 72 hours. Both buformin and metformin inhibited cell growth in a dose-dependent manner in both endometrial cancer cell lines. The mean IC50 value for metformin was 1.6 mM in the ECC-1 cells and 1.4 mM in the Ishikawa cells, after 72 hours of treatment. For the cells treated with buformin, the mean IC50 value was approximately 150 μM and 8 μM for the ECC-1 and Ishikawa cells at 72 hours, respectively (Figure 1A and 1B). These results suggest that both metformin and buformin effectively inhibit cell proliferation in endometrial cancer cells; however, buformin appeared more potent than metformin, given the lower IC50 values for buformin over metformin.

Buformin induced cell cycle arrest

To evaluate the underlying mechanism of growth inhibition by buformin, the cell cycle profile was analyzed after treating the ECC-1 and Ishikawa cells with varying doses (1-1000 μM) of buformin for 48 hours. As illustrated in Figure 2A and 2B, buformin treatment resulted in G1 cell cycle arrest in the ECC-1 cells and increased G2 phase in the Ishikawa cells in a dose-dependent manner, suggesting that

Figure 3. Effect of buformin on AMPK/mTOR/S6 pathway in ECC-1 and Ishikawa cells. The ECC-1 and Ishikawa (A) cell lines were treated with buformin (10 μM) in a time course fashion as indicated. Phosphorylated-AMPK, pan-AMPK, phosphorylated-S6 and pan S-6 were detected by Western immunoblotting. Buformin increased phosphorylation of AMPK and decreased phosphorylation of S6 expression as determined in time course study. The ECC-1 and Ishikawa (B) cells were treated with buformin at different concentrations for 24 h. Western blot results showed that buformin reduced phosphorylation of S6 expression in a dose dependent manner. The results are shown one of three independent experiments.
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Buformin induced cell cycle arrest through different checkpoints among these two cell lines.

**Buformin induced apoptosis**

We assessed the effects of buformin on cell apoptosis using the Annexin V assay. This assay detects apoptotic cells by monitoring fluorescently labeled Annexin V, which binds to the phosphatidylserine (PS) externalized on the surface of cell membrane that is a distinct phenomenon of early apoptosis [24]. The ECC-1 and Ishikawa cells were treated with buformin at varying concentrations (1-1000 μM) overnight. The percentage of apoptotic cells increased in a dose-dependent manner in the Ishikawa cells (Figure 2D). Buformin had no effect on the expression of Annexin V in the ECC-1 cells (Figure 2C). When combined with the cell cycle results, these data indicate that inhibition of cell proliferation by buformin may involve divergent pathways in different endometrial cancer cell lines.

**Effect of buformin on the AMPK/mTOR pathway**

It is well known that activation of AMPK and inhibition of the mTOR pathway play a crucial role in control of cell growth survival in endometrial cancer, and targeting of these pathways leads to the inhibition of endometrial cancer growth [10, 15]. To investigate the mechanisms underlying the inhibition of cell growth by buformin, we characterized the effect of buformin on these signaling pathways in a time course fashion. Buformin increased phosphorylation of AMPK and decreased phosphorylation of S6 expression in both endometrial cancer cell lines (Figure 3A). The greatest effects were seen in the ECC-1 cell line after 48 hours of exposure to buformin for both increased activation of AMPK and decreased phosphorylation of S6. For the Ishikawa cell line, maximal effects were seen after 12 hours of exposure to buformin. Following this, we treated cells with buformin at varying concentrations for 24 hours and evaluated the effect of different concentrations of buformin on the mTOR pathway. Buformin decreased phosphorylation of ribosomal protein S6 in a dose-dependent manner in both cancer cell lines (Figure 3B). These data suggest that buformin exerts its anti-tumorigenic activity via activation of AMPK and inhibition of the mTOR signaling pathways in endometrial cancer cells.

**Buformin inhibits cell adhesion and invasion**

Adhesion and invasion are important steps leading to metastasis in endometrial cancer. In order to determine the effect of buformin on adhesion and invasion of endometrial cancer cells, an *in vitro* laminin adhesion assay and transwell invasion system were employed, respectively. Incubation of the ECC-1 and Ishikawa cell lines with buformin (10 and 100 μM) for 2 hours showed significant inhibition of cell adhesion (22-31% in ECC-1 cells and 4-21% in Ishikawa cells, p<0.05) (Figure 4A). Buformin (10 and 100 μM) significantly blocked cell invasion after 24 hours of treatment in both cell lines (15-28% in ECC-1 cells and 17-25% in Ishikawa cells, p<0.05) (Figure 4B). Inhibition
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Synergistic anti-proliferative effects of buformin and paclitaxel

We have previously confirmed that metformin increased the sensitivity of endometrial cancer cells to paclitaxel [25]. We sought to determine if buformin had similar effects when combined with paclitaxel. We evaluated the effects of buformin and paclitaxel on cell proliferation in the ECC-1 and Ishikawa cells. Both cell lines were treated with serial dilutions of paclitaxel in combination with 0.1 or 1 μM of buformin for 48 hours. Their individual and combined effects on growth inhibition were evaluated using Chou-Talalay method [25]. The addition of buformin at 0.1 or 1 μM to paclitaxel led to a greater inhibition of cell proliferation than that of paclitaxel alone (Figure 5A-D). The analysis of synergy quantification showed that the combination buformin with paclitaxel resulted in significant synergistic anti-proliferative effects (95% CI of 0.104-0.561 for both cell lines).

Discussion

Based on pre-clinical and epidemiological data, biguanides are thought to have potentially beneficial chemo-preventative and chemotherapeutic effects. Buformin has broader tissue availability and greater potency compared to metformin but few studies have explored its anti-tumorigenic activity [7]. Early animal studies showed that long term use of buformin significantly decreased the total spontaneous tumor incidence in rats by 49.5% [16]. Postnatal
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treatment with buformin beginning at 2 months of age significantly reduced the incidence of malignant neurogenic tumors in rats exposed to N-nitrosomethylurea (NMU) transplacentally [18]. A recent study showed that buformin had greater inhibition of colony formation in human colon cancer HT-29 cells than metformin in both normal and glucose-free medium [26]. Additionally, buformin significantly decreased the incidence of adenocarcinoma of the mammary gland in female rats induced by 7,12-dimethylbenz(a)anthracene (DMBA) and increased the mean latent period of detection of all neoplasms [27], indicating the efficacy of buformin in vivo. Lastly, buformin, but not phenformin or metformin, decreased cancer incidence, multiplicity and burden in the 1-methyl-1-nitrosourea-induced mammary carcinogenesis rat model, suggesting that inherent differences in biological action may exist among biguanides [28].

Our results find that buformin strongly inhibited the proliferation of both ECC-1 and Ishikawa cells in a dose-dependent manner. Buformin also showed greater potential in the inhibition of ECC-1 and Ishikawa cell growth than metformin, as evidenced by lower IC50 values for buformin over metformin. Growth inhibition was accompanied by decreased cell adhesion and invasion, induction of cell cycle arrest, activation of AMPK and inhibition of the mTOR pathway. Moreover, buformin significantly increased the sensitivity of paclitaxel in the inhibition of cell proliferation in both cell lines. The results are in agreement with our previous studies examining the anti-tumorigenic activity of metformin in endometrial cancer cells [10, 25], suggesting that buformin may be an effective agent in the treatment of endometrial cancer.

Several possible mechanisms related to the anti-tumorigenic activity of biguanides could be explained by the functional activation of AMPK [10, 11]. AMPK has pleiotropic effects on cellular metabolism and growth in most cancer cells. The mTOR signaling pathway is a key regulatory step in cellular growth in response to nutrients and growth factor changes. Through AMPK-dependent or -independent mechanisms, metformin and phenformin have been shown to inhibit mTOR signaling and induce anti-tumorigenic effects [9, 15]. Buformin and metformin exhibit similar effects in the inhibition of phosphorylation of 4E-BP1 (a downstream target of mTOR) in fibrosarcoma HT1080 cells, and immunohistochemical analysis showed a reduction in the expression of phosphorylated-4E-BP1 in xenografts of gastric cancer after intra-tumoral injection of buformin [29]. In this study, AMPK activation and inhibition of phosphorylated S6 expression were observed by Western immunoblotting in a dose-dependent and time-dependent manner in endometrial cancer cell lines after treatment with buformin. This is consistent with the proposed mechanism of action of metformin and phenformin involving AMPK activation leading to mTOR inhibition. To our knowledge, this is the first study to evaluate the role of buformin on AMPK/mTOR signaling in endometrial cancer cells.

The most common route of metastasis in endometrial cancer is the direct extension of a tumor to the myometrium [30]. Cell adhesion and invasion are early steps in metastasis that involve tumor cell interaction, extracellular matrix (ECM) degradation and cell migration [31]. Clinically it is necessary to identify specific agents that may target the process of cell adhesion and invasion, in addition to proliferation. Only a few studies have focused on the role of biguanides in the process of adhesion and invasion in cancer. It has been shown that metformin inhibits melanoma invasion by regulating the EMT-like regulatory factors through AMPK/p53 pathway [32]. Additionally, Tan et al found that in vitro invasion of endometrial cancer cells was significantly attenuated by sera from women with polycystic ovary syndrome (PCOS) after 6 months of metformin treatment (850 mg twice daily) compared to matched controls. These effects appeared to be mediated through important regulators of inflammation including NF-kB, MMP-2 and MMP-9, as well as activation of Akt and Erk1/2 signaling pathways [33]. Our study is the first to demonstrate that buformin inhibits cell adhesion and invasion in endometrial cancer.

A concern surrounding the clinical use of biguanides is the risk of lactic acidosis as a potential side effect. Biguanides impair mitochondrial respiration via inhibition of complex I, which can result in a compensatory acceleration of glycolysis to counteract the reduced ATP pro-
duction via oxidative phosphorylation and a resultant increase in the production of lactic acid. Each biguanide is associated with varying risk of lactic acidosis with buformin and phenformin having an increased risk compared to metformin. In vivo data shows the IC50 values for development of lactic acidosis in rats to be 5 μM for phenformin, 199 μM for buformin and 735 μM for metformin [19]. Biguanides reduce oxygen consumption in isolated rat hepatocytes in a concentration-dependent manner, and the EC50 (the concentration of a drug that gives half-maximal response) values of biguanides determined in vivo correlate with the increase of blood lactate, suggesting that oxygen consumption may be used as an index for the incidence of lactic acidosis [21]. While the liver is largely responsible for lactic acid production, the kidneys are critical to the clearance of lactic acid. Patients with diabetes may be susceptible to developing lactic acidosis due to reduced renal dysfunction resulting in impaired clearance of biguanides [20, 34]. The incidence of lactic acidosis due to buformin is not known. There are case reports of buformin-induced lactic acidosis, as buformin is approved for use in many European and Asian countries [35]. However, despite this potential side effect, we believe that buformin could be safely applied for use in cancer patients with appropriate patient selection (i.e normal renal function).

We hypothesize that the efficacy of biguanides will be best appreciated when used in combination with traditional cytotoxic therapies. The administration of metformin together with paclitaxel, carboplatin or doxorubicin has been shown to block tumor growth and prolong relapse rate in a variety of mouse xenografts [36]. The combination of phenformin and PLX4720 (a BRAF inhibitor) synergistically induced tumor regression in nude mice bearing melanoma xenografts and in a genetically engineered BRAFV600E/PTEN null-driven mouse model of melanoma [37]. Our previous study found that metformin potentiated the effects of paclitaxel in endometrial cancer cells through modulation of the mTOR pathway [25]. In this study, we treated both ECC-1 and Ishikawa cells with varying doses of paclitaxel alone, in combination with 0.1 or 1 μM buformin. The addition of buformin at 0.1 and 1 μM to paclitaxel led to a greater inhibition of cell proliferation than that of paclitaxel alone in both cell lines (CI<1). It is not surprising that low doses of buformin sensitize the cytotoxicity of paclitaxel in endometrial cancer cell lines given that it shares the same targeting pathways as metformin. Clinically, the combination of buformin and paclitaxel would allow for the use of lower doses of paclitaxel and a resultant decrease in toxicity associated with paclitaxel, without impacting the efficacy of treatment.

In summary, we find that buformin has anti-proliferative and anti-metastatic effects in endometrial cancer cells. Although the risk/benefit ratio clearly favors metformin over buformin for the treatment of diabetes, this may not hold true for the treatment of cancer, especially if buformin is found to have greater anti-tumorigenic activity than metformin. Given our promising results, future studies conducted in our laboratory will focus on evaluating the effects of buformin versus metformin on tumor growth in genetically engineered models of endometrial cancer.

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Disclosure of conflict of interest

None.

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