Abstract

Recent studies show that the anti-diabetic drug metformin exhibits anticancer effects against a broad spectrum of cancers including endometrial cancer. Due to its hydrophilic nature and net positive charge at physiological pH, metformin requires cation-selective transporters to enter cells. This study investigated the expression of metformin transporters in human endometrial cancer cell lines, and the role of these transporters in metformin cellular uptake and accumulation in two human endometrial cancer cell lines, Ishikawa and ECC-1. Total RNA isolated from these two cell lines was subjected to real time polymerase chain reaction (RT-PCR) to determine the expression levels of common cation-selective transporters that are known to transport metformin. MATE 1 and 2 were the predominant transporters in Ishikawa and ECC-1 cells while OCT 1-3 expression in both cell lines was relatively poor. Time-dependent [$^{14}$C] metformin uptake into Ishikawa and ECC-1 cell lines was measured by quantifying intracellular radioactivity. To demonstrate the role of transporters in the uptake of metformin in these two cell lines, metformin uptake was measured in the presence and absence of cation-selective transporter inhibitors. Metformin uptake was saturated in both cell lines, and transporter inhibitors decreased metformin uptake in Ishikawa cells. RT-PCR results demonstrate that Ishikawa and ECC-1 cells are relevant in vitro models for investigating metformin treatment in endometrial cancer because expression of metformin transporters in human endometrial cancer tissues is comparable to that observed in endometrial cancer cell lines. The finding that metformin uptake is decreased by inhibitors only in Ishikawa cells warrants further study.

Introduction

Metformin is one of the most widely prescribed anti-diabetic drugs in the United States. Recent studies suggest that it has significant anticancer effects in a wide spectrum of cancers,
including endometrial, breast, prostate, colon, ovarian, gastric, and lung cancers\textsuperscript{1-5}. Endometrial cancer, a cancerous growth in the lining of the uterus, is the most common cancer of the female reproductive organs. In 2013, the American Cancer Society estimated that over 48,500 women would be diagnosed with endometrial cancer, and over 8,000 women would die from the disease. The 1-year relative survival rate for uterine cancers is 92\% while the 5-year survival rate is 95\%, 67\%, or 16\%, depending if the cancer is diagnosed at a local, regional, or distant stage respectively\textsuperscript{6}.

Metformin is a hydrophilic drug that is positively charged at physiological pH (Figure 1).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{metformin.png}
\caption{Metformin}
\end{figure}

As a result, metformin requires transporters to pass through lipophilic cell membranes. Cation-selective transporters known to transport metformin include organic cation transporter (OCT) 1-3, plasma monoamine transporter (PMAT), and multidrug and toxin extrusion transporter (MATE) 1-2. To elicit its anti-diabetic effects, metformin is transported into hepatic cells via OCT 1 where it inhibits hepatic gluconeogenesis by first activating adenosine monophosphate-activated protein kinase (AMPK), which suppresses regulatory gluconeogenic genes\textsuperscript{7} (Figure 2).
The anti-cancer effects of metformin are proposed to occur, in part, through activation of AMPK, which inhibits the downstream mammalian target of rapamycin (mTOR) pathway that regulates cell proliferation. Previous studies have demonstrated that metformin arrests cell cycle progression and inhibits proliferation in Ishikawa and ECC-1 endometrial cancer cell lines. Therefore, to induce its anti-cancer effects in endometrial cancer, metformin uptake in endometrial cancer cells and tissues must be mediated by specific cation-selective transporters. Elucidating metformin transport mechanisms in endometrial cancer will be central to understanding the role of transporters and transporter variability in metformin anticancer effects and clinical outcomes in cancer patients. This study aims to investigate the expression of metformin transporters in human endometrial cancer cell lines and tissues, and the role of these transporters in the uptake of metformin into endometrial cancer cells.

**Methods**

*Cell Culture*

ECC-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium and
Ishikawa cells were cultured in minimum essential medium (MEM) with 5% fetal bovine serum (FBS), 5% L-Glutamine 200mM, and 1% antibiotic-antimycotic. Both cell lines were cultured at 37°C in 5% CO2 and 90% relative humidity.

**RT-PCR**

Total RNA was isolated from endometrial cancer cell lines and tissues using the Qiagen RNeasy Plus kit, and cDNA was synthesized using a FirstStrand Synthesis kit (Invitrogen). The mRNA levels for each transporter were determined by Taqman assay (Applied Biosystems), and normalized to the 18s rRNA eukaryotic housekeeping gene.

**Uptake Studies**

Cells were seeded at 250,000 cells/cm² and cultured for 4-7 days. Growth medium was changed every other day and the day before experimentation. Cells were incubated with transport buffer (HBSS with 10mM HEPES and 25mM D-glucose) for 30 minutes prior to each study. For time-dependent studies, cells were exposed to 50µM [¹⁴C] metformin for 1-30 minutes. For concentration-dependent studies, cells were exposed to [¹⁴C] metformin concentrations ranging from 100µM to 20mM for 5 minutes. Chemical inhibition studies utilized 50µM [¹⁴C] metformin alone and in combination with the transporter inhibitors quinidine (Quin, 500µM), pyrimethamine (Pyr, 0.4µM), mitoxantrone (Mito, 25µM), corticosterone (Cort, 1µM), desipramine (Des, 200µM), and MPP⁺ (MPP⁺, 5mM). Transporters inhibited by these chemical inhibitors are shown in Figure 3. In each study, cells were washed 3 times with ice cold buffer after the desired time point, lysed (0.1M NaOH with 1% SDS), and analyzed by liquid scintillation spectrometry. Metformin uptake was normalized to protein content that was determined by the BCA protein assay.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibited Transporters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinidine 500µM</td>
<td>All transporters</td>
</tr>
<tr>
<td>Pyrimethamine 0.4µM</td>
<td>MATE 1 &amp; 2</td>
</tr>
<tr>
<td>Mitoxantrone 25µM</td>
<td>OCT 1 &amp; MATE 2</td>
</tr>
<tr>
<td>Corticosterone 1µM</td>
<td>OCT 3</td>
</tr>
<tr>
<td>Desipramine 200µM</td>
<td>OCT 1-3 &amp; PMAT</td>
</tr>
<tr>
<td>MPP+ 5mM</td>
<td>All transporters</td>
</tr>
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**Figure 3:** Transporters inhibited by each inhibitor.

*Statistical Analysis:* Data was analyzed by one-way ANOVA and Bonferroni post-hoc test. Data represents mean ± SD; n=3, *p<0.05 compared to control.

**Results**

**RT-PCR**

All six transporters of interest were present in both Ishikawa and ECC-1 cell lines (Figure 4). MATE 1 and 2 showed the highest expression in both cell lines, with MATE 2 expression being 6-fold higher than MATE 1 expression in Ishikawa cells and 5-fold higher in ECC-1 cells. Expression of OCT 1-3 was relatively poor in both cell lines.

**Figure 4:** Relative transporter expression in ECC-1 and Ishikawa cell lines
Uptake Studies

Uptake of [14C]metformin was linear in both Ishikawa and ECC-1 cell lines, and reached saturation at 10 minutes (Figure 5).

![Figure 5: Time-dependent uptake of metformin in (A) Ishikawa cells and (B) ECC-1 cells.](image)

Concentration-dependent uptake of metformin at 5 minutes in Ishikawa cells was linear up to 1mM and saturated at 2mM (Figure 6A), while in ECC-1 cells uptake of metformin at 5 minutes was linear up to 5mM and saturated at 7.5mM (Figure 6B).

![Figure 6: Concentration dependent uptake of metformin in (A) Ishikawa cells and (B) ECC-1 cells.](image)

Metformin uptake was significantly decreased in the presence of the pan transporter inhibitor quinidine (66% decrease) and the OCT 1 and MATE 1 inhibitor, mitoxantrone (54% decrease)
(Figure 7A). There was a trend toward a decrease in metformin uptake with the use of the OCT 3 inhibitor, corticosterone (36% decrease) and another pan transporter inhibitor, MPP+ (38% decrease). Treatment with the MATE 1 and 2 inhibitor pyrimethamine and the OCT 1-3 and PMAT inhibitor desipramine did not inhibit metformin uptake. Metformin uptake was not significantly decreased in ECC-1 cells with any inhibitor used (Figure 7B).

![Graph showing metformin uptake in the presence of inhibitors in Ishikawa and ECC-1 cells.](image)

**Figure 7:** Metformin uptake in the presence of inhibitors in (A) Ishikawa cells and (B) ECC-1 cells.

**Discussion**

RT-PCR results suggest that cation-selective transporters known to transport metformin are present in both the Ishikawa and ECC-1 cell lines. Studies by others in the Thakker laboratory showed that MATE 1 was the primary transporter in RT-PCR analysis of 15 human endometrial tumor tissues and 5 adjacent non-malignant tissues, with PMAT and OCT 3 also expressed in significant amounts. The predominant expression of MATE 1 in both human endometrial tissues types, and its high expression in the two endometrial cancer cell lines analyzed suggest that the Ishikawa and ECC-1 cell lines are relevant *in vitro* models to evaluate the role of transporters in the anticancer efficacy of metformin in endometrial cancer.
The saturation of metformin uptake in the Ishikawa cell line with respect to time and concentration suggests that uptake was transporter mediated. The significant decrease in metformin uptake in the presence of transporter inhibitors in Ishikawa cells further establishes the role of transporters in metformin uptake in this cell line. The significant decrease observed with quinidine (500µM), a pan inhibitor at the concentration used, and with mitoxantrone (25µM), which inhibits MATE 1 and OCT 3 suggests that these two transporters play an important role in the uptake of metformin into Ishikawa cells. Metformin uptake was also decreased in the presence of corticosterone (1µM) which also inhibits OCT 3, although this decrease was not statistically significant. Interestingly, pyrimethamine (0.4µM), which inhibits MATE 1 and MATE 2 did not significantly decrease metformin uptake. The wide variability observed in metformin uptake in the presence of pyrimethamine has made it challenging to accurately interpret this result.

The uptake of metformin in the ECC-1 cell line was also saturated with respect to time and concentration, which suggests that uptake was mediated by transporters. However, transporter inhibitors did not decrease metformin uptake, which was unexpected. The chemical inhibition studies in both cell lines were performed the same day, using the same dosing solutions, suggesting that this finding is not due to procedural error. Furthermore, the compact standard deviations observed in this experiment, minimize the possibility that a significant decrease in metformin uptake in the presence of chemical inhibitors was obscured by an outlying result. Therefore, these chemical inhibition studies in ECC-1 cells require further investigation.

The current cornerstone of endometrial cancer treatment is hysterectomy, although patients may also receive radiation, chemotherapy, and/or hormonal therapy based on the cancer stage and other patient specific factors\textsuperscript{6,9}. Metformin is a safe and tolerable drug with primary
side effects that include diarrhea and nausea, which can be minimized with dose titrations. The safety profile of metformin, compared to chemotherapy and radiation, makes it an attractive agent for the treatment of endometrial cancer. Understanding the relative contributions of cation-selective transporters to the uptake of metformin in endometrial cancer cells is important for conducting future in vivo studies which will be crucial to optimizing metformin therapy in endometrial cancer, and understanding potential variability in response to metformin treatment due to genetic and physiologic variability of transporters.

In conclusion, the Ishikawa and ECC-1 cell lines are reasonable endometrial cancer models in which to study the critical role of transporters in the anticancer efficacy of metformin in endometrial cancer.

References