Cation-selective transporters are critical to the AMPK-mediated antiproliferative effects of metformin in human breast cancer cells

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The anti-diabetic drug metformin exerts antineoplastic effects against breast cancer and other cancers. One mechanism by which metformin is believed to exert its anticancer effect involves activation of its intracellular target, adenosine monophosphate-activated protein kinase (AMPK), which is also implicated in the anti-diabetic effect of metformin. It is proposed that in cancer cells, AMPK activation leads to inhibition of the mammalian target of rapamycin (mTOR) and the downstream pS6K that regulates cell proliferation. Due to its hydrophilic and cationic nature, metformin requires cation-selective transporters to enter cells and activate AMPK. This study demonstrates that expression levels of cation-selective transporters correlate with the antiproliferative and antitumor efficacy of metformin in breast cancer. Metformin uptake and antiproliferative activity were compared between a cation-selective transporter-deficient human breast cancer cell line, BT-20, and a BT-20 cell line that was engineered to overexpress organic cation transporter 3 (OCT3), a representative of cation-selective transporters and a predominant transporter in human breast tumors. Metformin uptake was minimal in BT-20 cells, but increased by >13-fold in OCT3-BT20 cells, and its antiproliferative potency was >4-fold in OCT3-BT20 versus BT-20 cells. This increase in antiproliferative activity was associated with greater AMPK phosphorylation and decreased pS6K phosphorylation in OCT3-BT20 cells. In vitro data were corroborated by in vivo observations of significantly greater antitumor efficacy of metformin in xenograft mice bearing OCT3-overexpressing tumors versus low transporter-expressing wildtype tumors. Collectively, these findings establish a clear relationship between cation-selective transporter expression, the AMPK-mTOR-pS6K signaling cascade, and the antiproliferative activity of metformin in breast cancer.

Breast cancer is the second most common cancer and cause of cancer death among women in the United States. The American Cancer Society estimates approximately 231,840 new cases of invasive breast cancer and 40,730 breast cancer deaths in 2016. Epidemiological studies suggest that women with type 2 diabetes mellitus (T2DM) are at a greater risk of developing breast cancer. A meta-analysis study showed that T2DM is associated with 23% increased risk of breast cancer, especially in postmenopausal women. Evidence indicates that the frontline anti-diabetic drug for T2DM, namely metformin, acts as an anticancer agent in several cancers, including breast cancer. Studies have also shown that diabetic women on long-term metformin treatment have a lower risk of breast cancer compared to those not on metformin therapy, and that diabetic breast cancer patients on metformin have a lower risk of distant metastases compared to those not receiving metformin. Preoperative metformin treatment of nondiabetic women with operable invasive breast cancer results in downregulation of Ki67, a biomarker of cell proliferation and a predictive marker for clinical or pathological response to neoadjuvant therapy. Retrospective analyses showed higher pathological complete response rates (24%) in diabetic patients on metformin undergoing neoadjuvant chemotherapy for breast cancer versus diabetic patients not on metformin (8%) or nondiabetic patients (16%). An association was observed between survival in diabetic cancer patients and metformin therapy, but not between survival and sulfonylurea or insulin therapy. Preclinical studies in xenograft mouse models of breast, prostate and lung cancer.
What’s new?
Metformin, a diabetes drug, is widely known for its antitumor activities, which are effected through direct and indirect pathways. The direct pathway, which involves intracellular activation of adenosine monophosphate-activated protein kinase (AMPK), may depend on transporter-mediated uptake of metformin. Here, cation-selective transporter expression was found to correlate with transporter-mediated metformin uptake, metformin-mediated modulation of the AMPK-pS6K-mTOR pathway, and metformin antiproliferative activity in human breast cancer cell lines. Transporter expression profiles were found to vary widely among breast cancer cells and tumors, providing a rationale for transporter-expression screening in breast tumors to identify metformin-responsive patients.

showed that metformin and the chemotherapeutic agent doxorubicin (DOX) were more effective in blocking tumor growth and preventing relapse than DOX alone. In vitro studies also showed that metformin inhibited growth and colony formation of breast cancer cells, and induced cell cycle arrest and apoptosis.

As an anticancer agent, metformin is thought to exert its antiproliferative effects via an extracellular indirect pathway (insulin-dependent) and an intracellular direct pathway (insulin-independent). Insulin can bind to the insulin receptor that is highly expressed in cancer cells, and induce cell proliferation. It is suggested that metformin, by lowering circulating insulin levels, can induce anticancer effects by intercepting insulin-dependent tumor growth. The direct antiproliferative effects of metformin in cancer cells are thought to be mediated via activation of intracellular adenosine monophosphate-activated protein kinase (AMPK), which leads to down regulation of the mammalian target of rapamycin (mTOR) and its downstream target, p70S6K (pS6K). In hepatocytes, AMPK and its upstream regulator liver kinase B1 (LKB1) are key mediators in the glucose-lowering effect of metformin. Metformin activates AMPK via LKB1, leading to inhibition of liver gluconeogenesis and lowering of circulating glucose and insulin levels. Hence, AMPK appears to be a common intracellular target both for the antidiabetic and anticancer effects of metformin.

Metformin is hydrophilic (logD of −6.13 at pH 6.0) and charged at all physiological pH values (pKa 12.4). Therefore, it cannot enter cells via passive diffusion across the cell membrane and relies on cation-selective transporters to enter the cell where it can activate its intracellular target, AMPK. Transport proteins such as organic cation transporters (OCT 1–3) (SLC22A1-3), plasma monoamine transporter (PMAT) (SLC29A4) and multidrug and toxin extrusion proteins (MATE1 and 2) (SLC47A1 and SLC47A2) facilitate metformin trafficking in different organs and tissues. Such as the intestine, liver and kidney, and thus drive the disposition of metformin. In the liver, metformin is taken up into hepatocytes predominantly via OCT1, and thus this transporter plays a critical role in the antidiabetic effect of metformin. We have recently reported that as many as four cation-selective transporters, namely OCT1, PMAT, serotonin reuptake transporter (SERT) and a high affinity choline transporter (CHT) contribute to the intestinal uptake and absorption of metformin. These four transporters define the systemic exposure to orally administered metformin, and consequently, its pharmacologic behavior.

Emerging literature on the antiproliferative and anticancer efficacy of metformin in cancer cell lines and preclinical models of the disease either ignores the role of transporters or often suggests that a single transporter is responsible for metformin trafficking through tumor cells/tissues. Based on our studies on metformin transport in the intestinal tissue, we anticipate that multiple transporters, and interplay among them, may affect the uptake of metformin into tumor cells and tissues, and therefore influence its antiproliferative and antitumor efficacy. Hence, in any preclinical or clinical study in which the anticancer efficacy of metformin is evaluated, one must consider the expression of one or more metformin transporters in tumor cells for appropriate interpretation of the mechanisms underlying the antitumor effect of metformin.

In the present study, we have characterized the expression of cation-selective transporters in human breast cancer tissues and in nine commonly studied human breast cancer cell lines. The nine human breast cancer cell lines analyzed in this study were selected based on the two main subtypes of breast cancer, namely luminal and basal. Luminal breast cancer accounts for >70% of tumors that express estrogen receptors (ERs) and/or progesterone receptors (PRs), and low or no human epidermal growth factor receptors (EGFRs) 1 and 2, the latter also known as HER2. Basal-like breast cancers are generally triple negative as they lack ER, PR and HER2, but express EGFR1 and exhibit enhanced hypoxia and high tumor grade. Further, we have compared transporter expression profiles between breast tissues and breast cancer cell lines and evaluated an association between transporter expression levels and the antiproliferative efficacy of metformin in human breast cancer cells. The most definitive role of cation-selective transporters in the uptake and antiproliferative efficacy of metformin was obtained in this study by engineering an OCT3-overexpressing cell line (i.e., OCT3-BT20) from the BT-20 cell line that does not express detectable levels of cation-selective transporter genes. Gene and protein expression of OCT3 in BT-20 and OCT3-BT20 cells are related to metformin uptake, its antiproliferative efficacy, and its modulation of the AMPK-mTOR signaling pathway. It is
important to emphasize that OCT3 was chosen for overexpression simply as a representative of cation-selective transporters. The role of transporters in the antitumor efficacy of metformin was also assessed in xenograft mouse models of breast cancer. Thus, this is the first comprehensive study in which the expression of cation-selective transporter genes and proteins has been characterized in several commonly used human breast cancer cell lines, and an unequivocal relationship has been established between cation transporter expression in human breast cancer cells and the antiproliferative efficacy of metformin.

Material and Methods

Materials

The human breast cancer cell lines analyzed in this study were obtained from the Tissue Culture Facility (TCF) at the University of North Carolina at Chapel Hill, and were authenticated by TCF through forensic Short Tandem Repeat Analysis techniques. Snap-frozen breast tissues were purchased from the UNC Tissue Procurement Facility with IRB exemption.

Cell culture

Cells were cultured at 37°C, passaged at 90% confluency and plated in 75-cm² T-flasks. For uptake studies, MDA-MB-231 and BT-20 cells were seeded on 24-well plates at a density of 37,500 cells/cm², and MCF-7 cells at 75,000 cells/cm².

Generation of OCT3-BT20 cells

OCT3 from the pSPORT1 vector was cloned into a pcDNA3.1 vector. BT-20 cells (1 × 10⁶) were transfected with 2 g of vector, and cultured in 6-well plates. Single OCT3-BT20 colonies were isolated in selection medium containing 200 μg/ml Geneticin® and [¹⁴C]metformin (50 μM) uptake (5 min) was evaluated in the presence/absence of 50 μM famotidine (OCT3 and MATE1 inhibitor) or 500 μM quinidine (pan transporter inhibitor) to confirm functional activity of OCT3 in OCT3-BT20 cells. OCT3-expressing MCF-7 (OCT3-MCF7) cells were generated from MCF-7 cells by the same method utilized to generate OCT3-BT20 cells.

Determination of transporter gene expression

Total RNA from cells/tissues was isolated and synthesized into cDNA. Transporter gene expression was determined by real-time polymerase chain reaction (RT-PCR) using Taqman® assays, and normalized to endogenous 18s rRNA.

Determination of transporter protein expression

Cells were lysed and protein content was measured with a bicinchoninic acid (BCA) protein assay kit. Proteins (20 μg) were subjected to gel electrophoresis, transferred to a nitrocellulose membrane and probed with a primary OCT1, OCT2, OCT3, PMAT or MATE1 antibody and a secondary goat anti-rabbit IgG-horseradish peroxidase antibody. Protein bands were detected with SuperSignal® West Dura Extended Duration Substrate Kit and imaged. Membranes were stripped and analyzed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

To determine metformin-mediated AMPK activation and pS6K inhibition (assessed by their phosphorylation status), cells were incubated with culture medium in the presence/absence of 5 mM metformin for 2 days. Protein was extracted and subjected to Western blotting as described above using primary antibodies against p-AMPKα (Thr172) and p-pS6K. Densitometry of protein bands from three Western blots was performed, and the percent change in p-AMPK and p-pS6K between metformin-treated and control cells was calculated using the formula:

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\text{Percent change in phosphorylated protein} = \frac{- \text{Intensity of phosphorylated protein (control)} / \text{Intensity of GAPDH (control)}}{- \text{Intensity of phosphorylated protein (treatment)} / \text{Intensity of GAPDH (treatment)}}
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Cellular uptake of metformin

Uptake studies were conducted using methods previously reported with minor deviations.³⁶ Cells were preincubated for 30 min in transport buffer (0.5 ml), which was replaced with transport buffer containing varying concentrations of [¹⁴C]metformin or [¹⁴C]metformin plus transporter inhibitors (500 μM quinidine, 200 μM MPP⁺ or 50 μM famotidine). Metformin uptake was determined over 5 min (within linear uptake range). Cells were lysed with 500 μl of 1 M NaOH–0.1% SDS (3 h with shaking). [¹⁴C]Metformin in lysates was measured using liquid scintillation spectrometry. Protein content was determined by the BCA protein assay.

Cell proliferation assay

MDA-MB-231, BT-20, BT-549 and OCT3-BT20 cells were seeded in 96-well plates. After 24 hrs, cells were incubated in medium containing metformin (1 μM to 100 mM) for 5 days and cell viability was assessed by the Alamar Blue® assay. To demonstrate that AMPK activation is required for the antiproliferative activity of metformin, cells were pretreated with 2 μM of the selective AMPK inhibitor, dorsomorphin (Compound C), followed by incubation with 10 mM metformin in the presence/absence of 2 μM Compound C for 48 hrs. Cell viability was determined by the Alamar Blue® assay.
Tumor studies

Xenograft mice bearing OCT3-MCF7 tumors and MCF-7 tumors were generated by subcutaneously injecting 2 × 10^6 OCT3-MCF7 cells or MCF-7 cells into the right flank of athymic nude mice. Metformin (50 mg/kg) or saline was administered daily via intraperitoneal injections for 20 days after which mice were euthanized and tumor tissues were isolated and weighed.

Statistical analyses

All data are expressed as mean ± SD. Statistical differences in transporter expression between human breast tumor tissues (n = 15) and the corresponding adjacent nonmalignant tissues (n = 15) from the same subject were determined by Wilcoxon signed-rank test. Statistical significance for difference in mean transporter expression in normal human breast tissues from mammoplasty surgeries (n = 5) and in human breast tumor tissues (n = 15) or nonmalignant breast tissues adjacent to tumors (n = 15) was determined by Mann–Whitney U test. Tukey’s test was used to analyze data from chemical inhibition studies in OCT3-BT20 cells and BT-20 cells, and for percent change in the phosphorylation of AMPK and pS6K. For chemical inhibition studies in MCF-7, MDA-MB-231, BT-20, OCT3-BT20 and BT-549 cells, an independent t test was used to compare control and treated groups. Analysis of variance (ANOVA) followed by Tukey’s test was performed to determine statistical significance in tumor weights between OCT3-MCF7 tumors and MCF-7 tumors.

Results

Gene and protein expression of cation-selective transporters in human breast cancer cell lines

The four luminal human breast cancer cell lines (MCF-7, SK-BR-3, ZR-75-1 and BT-474) and two basal cell lines, BT-20 and MDA-MB-435S, expressed negligible levels of OCT1, OCT2, OCT3, PMAT, MATE1 and MATE2 transporter genes, whereas three basal cell lines, MDA-MB-231, MDA-MB-468 and BT-549 had relatively higher levels of transporter gene expression, with MATE1 being the predominant transporter (Fig. 1a). MATE1 was also the predominant transporter in MCF-7 and MDA-MB-435S cell lines, although its expression was relatively low compared to that in MDA-MB-231, MDA-MB-468 and BT-549 cells. OCT3, the second most highly expressed transporter gene in MDA-MB-231 cells, showed negligible expression in the other breast cancer cell lines. Expression of OCT1, OCT3, PMAT and MATE1 transporter proteins in BT-20, MCF-7, MDA-MB-231, BT-549, MDA-MB-468 and MDA-MB-435S cell lines was assessed by Western blot analyses. Corresponding to gene expression, OCT1, OCT3, PMAT and MATE1 proteins were detected in MDA-MB-468, MDA-MB-435S and MDA-MB-231 cells (Fig. 1b). No transporter proteins were detected in the BT-20 cell line which had negligible transporter gene expression (Fig. 1b). Thus, transporter protein expression reflected transporter gene expression in the human breast cancer cell lines analyzed. The variability in metformin transporter expression profiles among these cell lines suggests that cells within breast cancer tissues are also likely to show heterogeneity in metformin transporter expression.

Gene expression of cation-selective transporters in human breast tissues

The expression of OCT1, OCT2, OCT3, PMAT, MATE1 and MATE2 genes was assessed by RT-PCR in human breast tumor tissues, their corresponding adjacent nonmalignant tissues, and in normal breast tissues obtained from mammoplasty surgeries. OCT3 and PMAT were the predominant transporter genes expressed in all three tissue types (Fig. 2), with lower expression of OCT1 and MATE1 genes, and negligible expression of the OCT2 gene. MATE2 gene expression was negligible in breast tumor tissues, and was low in normal breast tissues and in tissues adjacent to breast tumors. The expression of OCT1, OCT3, PMAT and MATE1 genes was downregulated in all 15 pairs of breast tumor tissues analyzed compared to the corresponding adjacent nonmalignant tissues, although this decrease was not statistically significant. No comparison was made between OCT2 and MATE2 gene expression in breast tumor tissues and their corresponding adjacent nonmalignant tissues as these transporters were below detectable levels in several tissues examined.

Metformin uptake in human breast cancer cell lines

[^14C]Metformin (50 μM) uptake was first assessed in the low transporter-expressing MCF-7 cell line, a widely used in vitro model for breast cancer. Uptake was inefficient and was not inhibited by the pan cation-selective transporter inhibitor MPP⁺ (200 μM). Metformin uptake in transporter-deficient BT-20 cells was also low and comparable to its uptake in MCF-7 cells, and uptake was not inhibited by MPP⁺ (Fig. 3a). This result suggests that transporter-mediated metformin uptake in MCF-7 and BT-20 cells is negligible, and corresponds with low transporter expression levels in these cell lines. However, metformin uptake in MDA-MB-231 cells, which express OCT3 and MATE1, was ~12-fold higher compared to BT-20 cells (22.78 vs. 1.97 pmol/mg protein/min) (Fig. 3a). Similarly, metformin uptake in BT-549 cells, which express high levels of MATE1, was ~14-fold higher than uptake in BT-20 cells (27.43 vs. 1.97 pmol/mg protein/min, respectively; p < 0.001) (Fig. 3a). Overexpression of OCT3 in BT-20 cells (OCT3-BT20) increased metformin uptake by >13-fold compared to wild-type BT-20 cells (108.38 vs. 8.09 pmol/mg protein/min; p < 0.001) (Fig. 3b). Treatment of OCT3-BT20 cells with the OCT3-selective inhibitor, famotidine (50 μM), and the pan cation-selective transporter inhibitor, quinidine (500 μM), decreased metformin uptake by 88% and 96%, respectively, confirming that almost all metformin uptake in these cells is mediated by OCT3. RT-PCR and Western blot analyses confirmed the overexpression of OCT3 gene and protein in OCT3-BT20 cells. There were no
Figure 1. Expression of cation-selective transporters OCT1-3, PMAT and MATE1-2 in human breast cancer cell lines. (a) Relative gene expression of known metformin transporters OCT1-3, PMAT and MATE1-2 in four luminal human breast cancer cell lines (MCF-7, SK-BR-3, ZR-75-1 and BT-474) and five basal human breast cancer cell lines (BT-20, MDA-MB-435S, MDA-MB-231, MDA-MB-468 and BT-549) was determined by RT-PCR and normalized to 18s rRNA. (b) Expression of OCT1, OCT3, PMAT and MATE1 transporter proteins in MDA-MB-468, MDA-MB-435S, BT-549, MDA-MB-231, MCF-7 and BT-20 breast cancer cell lines was assessed by Western blot analyses, using primary antibodies specific for human OCT1, OCT3, PMAT and MATE1. GAPDH was used as a loading control.
detectable levels of OCT3 mRNA in wild-type BT-20 cells (p < 0.001) (Fig. 3c).

**Role of cation-selective transporters in the antiproliferative efficacy of metformin in human breast cancer cell lines**

Cation-selective transporter-mediated increase in metformin uptake in OCT3-BT20 cells translated into greater potency of the antiproliferative effect of metformin in this cell line compared to that in BT-20 cells; the extracellular metformin concentrations required to inhibit 50% cell growth (IC$_{50}$) of OCT3-BT20 cells was 2.13 mM, and IC$_{50}$ of BT-20 cells was 9.06 mM (p < 0.001) (Fig. 4a). Further, the transporter-competent BT-549 cells, which express high levels of MATE1, were also more sensitive to the antiproliferative activity of metformin compared to transporter-deficient BT-20 cells (IC$_{50}$ 2.93 mM and IC$_{50}$ 9.06 mM, respectively; p < 0.001) (Fig. 4b). These data establish a direct relationship between cation-selective transporter expression levels and metformin-mediated inhibition of cell growth in human breast cancer cell lines. Interestingly, despite high expression of OCT3 and MATE1 in MDA-MB-231 cells and a corresponding ~12-fold higher metformin uptake compared to BT-20 cells, the IC$_{50}$ for MDA-MB-231 was comparable to the IC$_{50}$ for BT-20 cells (10.56 and 9.06 mM, respectively) (Fig. 4b). This discrepancy between high metformin uptake and its poor antiproliferative efficacy in MDA-MB-231 cells appears to be due to the lack of a functional intracellular AMPK-mTOR-pS6K signaling cascade that is required for the antiproliferative activity of metformin. It has been hypothesized that metformin suppresses mTOR/pS6K signaling by activating AMPK in cancer cells$^{19}$; since MDA-MB-231 cells do not express LKB1$^{35}$ that is required for AMPK activation, metformin does not exert antiproliferative effects in MDA-MB-231 cells despite achieving high intracellular concentrations. To further demonstrate that AMPK activation is required for the antiproliferative activity of metformin, breast cancer cell lines with varying transporter expression profiles (i.e., BT-20, OCT3-BT20, MCF-7, BT-549 and MBA-MB-231 cells) were treated with metformin in the presence or absence of the selective AMPK inhibitor, Compound C. Our results show that Compound C attenuates the antiproliferative activity of metformin in all the breast cancer cell lines tested (Supporting Information Fig. 1). As expected, Compound C did not have any effect on the antiproliferative activity of metformin in MDA-MB-231 cells, since the mechanism for AMPK activation is dysfunctional in this cell line.

**Interplay of cation-selective transporter expression and AMPK/pS6K modulation in BT-20, OCT3-BT20 and MDA-MB-231 cell lines**

To further test the hypothesis that metformin transporters, as well as AMPK activation by metformin, are essential for its antiproliferative activity in breast cancer cell lines, we utilized a transporter-deficient cell line with a functional AMPK/pS6K/mTOR signaling cascade (BT-20), an engineered BT-20 cell line that overexpresses a metformin transporter and has a functional...
AMPK/pS6K/mTOR pathway (OCT3-BT20), and a cell line that expresses metformin transporters but lacks LKB1 that is required for AMPK activation by metformin (MDA-MB-231). Cells were treated with 5 mM metformin for 48 hr and the phosphorylation status of the two intracellular signaling targets of metformin, namely AMPK and pS6K, was evaluated. Additional breast cancer cell lines with varying transporter expression profiles (i.e., low transporter-expressing MCF-7 cells and transporter-competent MDA-MB-468, MDA-MB-435S and BT-549 cells) were also assayed for comparison.

The increase in AMPK phosphorylation within transporter-deficient BT-20 cells in response to metformin treatment was low, as was the corresponding decrease in pS6K phosphorylation. In contrast, a noticeably higher AMPK phosphorylation and decreased pS6K phosphorylation was observed in OCT3-BT20 cells following metformin treatment (Fig. 5b). Metformin treatment had no effect on the phosphorylation status of AMPK and pS6K in the MDA-MB-231 cell line despite high transporter expression and high metformin cellular uptake, presumably due to a defective AMPK pathway (Fig. 5a). As expected, the transporter-competent MDA-MB-468, MDA-MB-435S and BT-549 cells showed a metformin-mediated increase in AMPK phosphorylation and decrease in pS6K phosphorylation (Fig. 5a). The MCF-7 cell line, despite relatively low expression of cation-selective transporters, also showed an increase in AMPK phosphorylation following metformin treatment (Fig. 5b), which could suggest a higher sensitivity of this cell line to metformin as reported in the literature, or the presence of an unidentified metformin transporter. Notably, the decrease in pS6K phosphorylation in MCF-7 cells was relatively small and did not correspond with the extent of AMPK activation (Fig. 5b).

Western blot data on metformin-mediated changes in AMPK and pS6K phosphorylation in MCF-7 cells (low transporter expression with a functional LKB1), MDA-MB-231 cells (transporter-competent without LKB1), BT-20 cells (transporter-deficient with a functional LKB1) and OCT3-BT20 cells (transporter-competent with a functional LKB1) were also assayed for comparison.

Figure 3. Metformin uptake in human breast cancer cell lines with high and low cation-selective transporter expression. Metformin uptake was assessed in the presence or absence of the pan cation-selective transporter inhibitors MPP⁺ (200 μM) and quinidine (500 μM), or the OCT3 and MATE1 inhibitor famotidine (50 μM) in (a) low transporter-expressing MCF-7 cells, transporter-competent MDA-MB-231 and BT-549 cells, and transporter-deficient BT-20 cells, and (b) transporter-competent OCT3-BT20 and transporter-deficient BT-20 cells. Data represent mean ± SD; n = 4. *p < 0.05 for (a) and *p < 0.001 for (b). (c) Expression of OCT2 and OCT3 genes in OCT3-BT20 and BT-20 cells was analyzed by RT-PCR and normalized to 18s rRNA. OCT3 protein expression in OCT3-BT20 and BT-20 cells, evaluated by Western blot analysis, is shown as an insert. Results are shown as the mean ± SD; n = 3. *p < 0.001.
cells resulted in ~87% increase in AMPK phosphorylation and ~17% decrease in pS6K phosphorylation. In the low-transporter expressing MCF-7 cells, metformin increased AMPK phosphorylation by ~50%, with ~9% decrease in pS6K phosphorylation (Fig. 5c). Metformin had a minimal effect on AMPK and pS6K phosphorylation (~15 and <1%, respectively) in MDA-MB-231 cells. Collectively, these data clearly establish an association between cation-selective transporter expression, modulation of AMPK/pS6K phosphorylation and the antiproliferative potency of metformin.

Correlation of cation-selective transporter expression with the antitumor efficacy of metformin in xenograft tumors

For in vivo studies, the tumorigenic, fast-growing low transporter-expressing MCF-7 cells were engineered to generate OCT3-overexpressing MCF-7 cells because the BT-20 and OCT3-BT20 cells produced measurable tumors (>50 mm³ tumors) in only two out of >60 animals being tested. However, using MCF-7 and OCT3-MCF7 cells, xenograft mouse models of breast cancer (with >50 mm³ tumors) were successfully generated by subcutaneous injections of these two cell types into athymic nude mice. Metformin treatment (50 mg/kg, intraperitoneal) significantly reduced the weights of OCT3-MCF7 tumors compared to saline treatment (62.3 mg with metformin treatment compared to 252.5 mg with saline treatment, p < 0.001) (Fig. 6). Interestingly, metformin was less efficacious in reducing the weights of MCF-7 tumors (182.5 mg with metformin treatment compared to 245.0 mg with saline treatment; p < 0.05). Expressed another way, the sensitivity of OCT3-MCF7 tumors to the antitumor effects of metformin was threefold higher compared to MCF-7 tumors (62.3 vs. 182.5 mg; p < 0.001). The similar weights of OCT3-MCF7 tumors and MCF-7 tumors in saline-treated mice (245.0 vs. 252.5 mg) suggest that the overexpression of OCT3 in OCT3-MCF7 tumors did not alter the endogenous growth pattern of these cells/tumors (Fig. 6).

Discussion

Initial observations on the unexpected anticancer efficacy of metformin, the leading antidiabetic drug, in T2DM patients with breast cancer1,2 have been subsequently supported by other clinical and preclinical studies on the antineoplastic effects of this drug.3–9,11–15 It is well established that the hepatic and renal disposition of metformin is mediated by cation-selective transporters, and our own data implicate OCT1, PMAT, SERT and CHT in its intestinal absorption.38 Our studies also demonstrated that following metformin uptake into intestinal epithelial cells across the apical membrane, the drug is unable to egress across the basolateral cell membrane as this membrane lacks metformin transporters.38 These data provide strong evidence that metformin trafficking across cell membranes is transporter-dependent. Others have shown a decrease in the glucose-lowering effect of metformin in mOct1-knockout mice, implicating an important role for cation-selective transporters in the antidiabetic efficacy of metformin.39 The interaction of metformin with cation-selective transporters has also been implicated in tumor cells. In a study by Patel et al.,40 siRNA-mediated attenuation of OCT3 expression in human head and neck squamous cell carcinoma cells decreased the effect of metformin on the phosphorylation of pS6K; however, the study did not investigate a relationship between OCT3 expression and the antiproliferative activity of metformin. In the present study, we have characterized the expression of cation-selective transporter genes and proteins in human breast cancer tissues, and in human breast cancer cell lines that are commonly used in breast cancer research. We have then correlated transporter expression profiles with the cellular uptake and antiproliferative activity of metformin. We have also provided a mechanistic basis for this correlation by demonstrating that activation of intracellular...
AMPK and down regulation of pS6K are associated with transporter-mediated uptake and antiproliferative activity of metformin.

Our results showed that OCT3 and PMAT are the predominant transporters expressed in breast tumor tissues (Fig. 2). Notably, OCT3 gene expression in breast tumor tissues was 13,000-fold higher than its expression in normal breast tissues, suggesting that OCT3 could play an important role in the antitumor efficacy of metformin in breast cancer. Other transporter genes expressed in breast tumors are MATE1, MATE2.
transporter in a transporter-deficient cell line would be 20 cells (Fig. 3).

The first evidence that MATE1 acts as an uptake transporter rather than an efflux transporter in breast cancer cells.

To characterize the expression profiles of OCT1-3, PMAT and MATE1-2 in luminal and basal human breast cancer cell lines, four luminal breast cancer cell lines (MCF-7, SK-BR-3, ZR-75–1 and BT-474) and five basal cell lines (MDA-MB-231, MDA-MB-435S, MDA-MB-468, BT-20 and BT-549) were analyzed. Transporter expression varied among the nine breast cancer cell lines, with low or undetectable cation-selective transporter expression in MCF-7, SK-BR-3, ZR-75–1, BT-474 and BT-20 cells and multiple transporters expressed in MDA-MB-231, MDA-MB-435S and MDA-MB-468 cells (Fig. 1). Generally, there was good correspondence between transporter gene and protein expression (Fig. 1).

The dependence of metformin uptake into human breast cancer cell lines on cation-selective transporters was demonstrated by a head-to-head comparison of metformin uptake in two cell lines with the same origin (i.e., the BT-20 cell line), and hence the same genetic background, but with one cell line expressing a metformin transporter (OCT3-BT20 cell line) and the other cell line practically devoid of the transporter (BT-20 cell line) (Fig. 1). Metformin uptake in OCT3-BT20 cells was >13-fold higher compared to uptake in BT-20 cells (Fig. 3b). These data from two cell lines that differ only in the presence or absence of a cation-selective transporter provide the most direct evidence that metformin uptake in breast cancer cell lines is transporter-dependent.

An alternative experimental approach to overexpressing a transporter in a transporter-deficient cell line would be siRNA-mediated knockdown of a transporter in a transporter-competent cell line, and comparison of metformin uptake and its antiproliferative activity between the wild-type and transporter-knockdown cells. However, because metformin uptake into cells is mediated by multiple transporters, this approach would not have been as effective as the one employed in our study. The advantage of our strategy utilizing two cell lines with the same genetic background to demonstrate the critical role of transporters in the uptake and antiproliferative efficacy of metformin is evident from our data comparing these parameters between two cell lines with different genetic backgrounds, namely BT-20 (transporter-deficient) and MDA-MB-231 (transporter-competent) cells. As expected, metformin uptake in MDA-MB-231 cells was ~12-fold higher compared to uptake in BT-20 cells (Fig. 3a), but its antiproliferative activity was similar in both these cell lines (Fig. 4b). This discrepancy between metformin uptake and its antiproliferative activity in the MDA-MB-231 cells is due to an intrinsic deficiency in LKB1,20 the kinase that is required for the phosphorylation of AMPK, resulting in a defective AMPK-pS6K-mTOR pathway and subsequent inability of metformin to exert its antiproliferative effect in this cell line. The dependence of the antiproliferative activity of metformin on AMPK activation was confirmed by our results showing attenuation of the antiproliferative effects of metformin in BT-20, OCT3-BT20, BT-549 and MCF-7 cells in the presence of the AMPK inhibitor, Compound C, but not in MDA-MB-231 cells (Supporting Information Fig. 1).

Others have reported the involvement of cation-selective transporters, such as OCT1, in the antiproliferative activity of metformin in epithelial ovarian cancer cells, and the contribution of OCT3 to metformin-mediated decrease in cell viability in head and neck squamous cell carcinoma cell lines.40,42 However, our study represents the most comprehensive approach to evaluating the role of transporters in the antiproliferative activity of metformin in cancer cell lines.

Our results underscore the importance of cation-selective transporter expression as a criterion for selecting breast cancer cell lines to investigate the antiproliferative or antitumor activity of metformin. A review of the literature suggests that little or no attention has been paid to cation-selective transporter expression in selecting relevant in vitro (cellular) and in vivo (xenograft) models of breast cancer for such studies.

To demonstrate that low/no cation-selective transporter expression in breast tumors could significantly restrict the antineoplastic effects of metformin, we conducted an in vivo proof-of-concept study. Xenograft mouse models of breast cancer were developed by subcutaneous injections of BT-20 and OCT3-BT20 cells into athymic nude mice. However, due to the limited tumorigenicity of BT-20 cells and the inherent slow growth rate of BT-20 tumors (as also reported in the literature) and of OCT3-BT20 tumors, only a small number of mice developed measurable tumors by the end of Week 10 post injection. Hence, we used the highly tumorigenic, fast-growing and low transporter-expressing MCF-7 cells to...
generate an OCT3-overexpressing MCF-7 (OCT3-MCF7) cell line, and used these two cell types to develop xenograft mice bearing MCF-7 and OCT3-MCF7 tumors. Our results show that metformin is significantly more potent against OCT3-MCF7 tumors than against MCF-7 tumors (Fig. 6). These preliminary in vivo results support our hypothesis that transporters are central to the anticancer efficacy of metformin.

Clinical studies have shown that transporter polymorphisms alter the pharmacokinetics and glucose-lowering efficacy of metformin. Healthy volunteers with reduced function OCT1 alleles had a significantly higher area under the plasma concentration–time curve of glucose.43 It has also been reported that renal clearance and net secretion of metformin were significantly altered in individuals heterozygous for an OCT2 variant allele compared to individuals homozygous for the OCT2 reference allele, and altered metformin disposition and response were observed in patients carrying MATE1 and MATE2 promoter variants.44,45 Additionally, a wide variability in hepatic expression of OCT1 (113-fold variation) and OCT3 (27-fold variability) in Caucasians has been previously reported.28 Taken together, these data suggest that genetic variants among cation-selective transporters impact the antidiabetic efficacy of metformin, and could explain the subtherapeutic efficacy of this drug in 36% of diabetic patients on metformin therapy.40,42,46,47 Therefore, it is likely that transporter variants could also affect the anticancer efficacy of metformin in breast cancer. Our in vitro data showing variability in cation-selective transporter expression profiles in human breast cancer cell lines reflect the degree of variability in transporter expression among cells comprising human breast tumors, and suggest that treatment outcomes to metformin therapy in breast cancer could be highly variable. Thus, to predict the efficacy of metformin as an anticancer agent in breast cancer or other cancers, screening patients for transporter and LKB1 expression in neoplastic tissues may be necessary. Alternatively, new drug design and formulation strategies could be developed to deliver metformin or its analogs into tumor tissue/cells without the need for cation-selective transporters, so that all cancer patients can avail of this efficacious and cost-effective drug regardless of transporter expression profiles or transporter variants.

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References

27. Han TK, Proctor WR, Costales CL, et al. Four cation-selective transporters contribute to apical...