Relevance of the OCT1 transporter to the antineoplastic effect of biguanides

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A B S T R A C T

Epidemiologic and laboratory data suggesting that metformin has antineoplastic activity have led to ongoing clinical trials. However, pharmacokinetic issues that may influence metformin activity have not been studied in detail. The organic cation transporter 1 (OCT1) is known to play an important role in cellular uptake of metformin in the liver. We show that siRNA knockdown of OCT1 reduced sensitivity of epithelial ovarian cancer cells to metformin, but interestingly not to another biguanide, phenformin, with respect to both activation of AMP kinase and inhibition of proliferation. We observed that there is heterogeneity between primary human tumors with respect to OCT1 expression. These results suggest that there may be settings where drug uptake limits direct action of metformin on neoplastic cells, raising the possibility that metformin may not be the optimal biguanide for clinical investigation.

1. Introduction

Metformin is an orally active biguanide that lowers systemic glucose and insulin and is commonly used for the treatment of type II diabetes. Retrospective studies suggest that metformin may have a protective role against cancer, possibly by reducing elevated systemic insulin levels and/or by directly inhibiting cellular proliferation via AMPK pathway activation within neoplastic cells [1]. Studies have demonstrated induction of apoptosis by metformin in pancreatic [2], prostate, and colon cancer [3]. Several publications reported a potential therapeutic effect of metformin in epithelial ovarian cancer (EOC) [4–6]. We previously demonstrated that metformin enhances ovarian cancer cell cytotoxicity in a dose- and time-dependent manner, an effect potentiated by cisplatin [5]. Metformin induces apoptosis through the modulation of the Bcl-2 family of proteins in some experimental systems [7], but other mechanisms including AMPK-dependent inhibition of mTOR may also play a role [8,9].

Phenformin is another biguanide with anti-diabetic activity [10]. It was withdrawn from the market in the late 1970s due to a small risk of lactic acidosis in patients treated for diabetes [11]. Phenformin has been shown to have anti-neoplastic activity, including p21 cell-cycle inhibition leading to apoptosis. Moreover, phenformin was shown to reduce tumor growth in several animal models [12–16]. While risk/benefit considerations clearly favor use of metformin over phenformin for treatment of diabetes, the risk of phenformin-associated lactic acidosis is low enough that this agent certainly would not be contraindicated for cancer treatment if it had demonstrated superior antineoplastic activity.

The organic cation transporter 1 (OCT1) is responsible for organic cation uptake into hepatic cells via facilitated diffusion as well as active transport [17]. It is well known that OCT1 is highly expressed particularly in hepatic cells, where many drugs such as metformin and phenformin act [17,18]. OCT1 has previously been reported to have several polymorphisms that can influence the cellular uptake of metformin [19]. The most common mutation, OCT1-420del, occurs with an allelic frequency of 16% [20] and was found to be present in 20% of Caucasian Americans displaying a reduced response to metformin [19]. While germ-line OCT1 polymorphisms may have modest but detectable effects on metformin efficacy in diabetes, we conducted *in vitro* studies to investigate whether differences in OCT1 expression amongst neoplastic tissues may be a more important consideration for potential applications in oncology.

2. Materials and methods

2.1. Cell culture

The ovarian cancer cell lines OVCAR-3 and SKOV-3 (American Tissue Culture Collection, Manassas, VA, USA) were grown in RPMI-1640 medium (Wisent Bioproducts, Saint-Bruno, QC, Canada) supplemented with 10% fetal bovine serum (FBS), 2 mM

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glutamine, and 10 μg/ml gentamicin. Each cell line was passaged every 5–7 days and maintained at 37 °C in a 5% CO_2, 95% air atmosphere incubator.

2.2. Chemicals and antibodies

Metformin (catalog# D150959), Phenformin (catalog# P7045) and Anti-OCT1 (catalog# AV41516) were purchased from Sigma–Aldrich (Oakville, ON, Canada) OCT1 siRNA (sc-42552) was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Anti-phospho-AMPK (Thr^{172}), and anti-β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.3. Cytotoxicity assays

Cells were treated separately with increasing doses of metformin and phenformin in the presence or absence of OCT1 siRNA, and then incubated for 72 h. AlamarBlue colorimetric assay was performed in medium containing 1% FBS in triplicates as described earlier [21].

2.4. siRNA transfection

Cells were seeded in 6- and 96-well flat-bottom cell culture plates (Corning Incorporated, NY, USA). Lipofectamine for siRNA treatment was obtained from Invitrogen (Burlington, Ontario, Canada). Lipofectamine (1:1) was mixed with negative siRNA and OCT1 siRNA separately in RPMI-1640 with no FBS. Following 30 min of incubation at room temperature, both negative and OCT1 siRNA were added to their respective wells. The cells were incubated at 37 °C for 5 h and medium was changed containing increasing doses of phenformin and metformin in 1% FBS, respectively.

2.5. Protein assay

Total protein content was determined by the Lowry method [22] using a colorimetric assay (Bio-Rad, Mississauga, ON, Canada).

2.6. Protein extraction and Western blot analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris·HCl pH 7.6, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany). Protein lysates (110 μg) were resolved electrophoretically on 10% denaturing SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in 5% milk and probed with antibodies specific for anti-OCT1 anti-phospho-AMPK (Thr^{172}) and anti-β-actin. Following incubation with horseradish peroxidise (HRP)-conjugated secondary antibodies, immunoblotted proteins were visualized by enhanced chemoluminescence (ECL). Relative levels of negative and positive siRNA for OCT1 were quantified by scanning densitometry and normalized by β-actin. The level of positive siRNA for OCT1 was significantly different from the level under control conditions $P < 0.05$.

2.7. Tissue microarray

TMA was kindly provided by the Terry Fox Research Institute and contains 105 patients with serous papillary epithelial ovarian

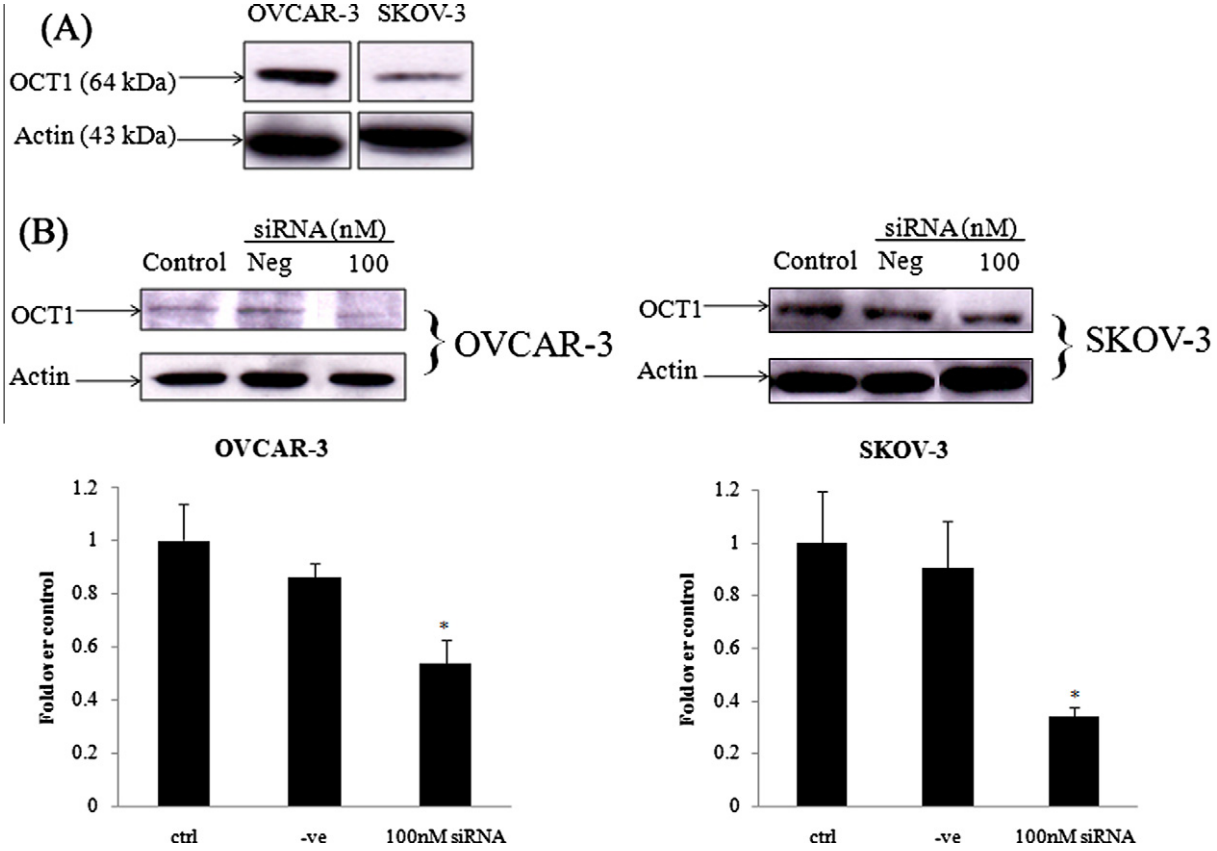

Fig. 1. OCT1 expression in human EOC cell lines. (A) Cell lysates from OVCAR-3 and SKOV-3 cell lines were subjected to western blot for OCT1 and actin. One representative experiment out of three is shown. (B) Inhibition of OCT1 protein expression with increasing doses of siRNA in OVCAR-3 and SKOV-3 cell lines. Cells were incubated with nonspecific and OCT1 siRNA for 72 h in RPMI without FBS. Densitometric analysis is shown. *$P < 0.05$ when compared to positive and negative controls.

carcinoma. Immunohistochemistry was performed at the Segal Cancer Centre Research Pathology Facility (Jewish General Hospital). Tissue samples were cut at 4-μm, placed on SuperFrost/Plus slides (Fisher), and dried overnight at 37 °C. The slides were then loaded onto the Discovery XT Autostainer (Ventana Medical System). All solutions used for automated immunohistochemistry were from Ventana Medical System unless otherwise specified. Briefly, rabbit polyclonal anti-OCT1(AB1) (Sigma–Aldrich) diluted 1:100 in Antibody diluent solution, was manually applied for 32 min, then followed by the appropriate detection kit (Omnimap anti-Rabbit HRP). A negative control was performed by the omission of the primary antibody. Sections were analyzed by conventional light microscopy.

2.8. Statistical analysis

All values are expressed as means ± SEM. Data were analyzed by one-way ANOVA followed by the Newman–Keuls test for multiple

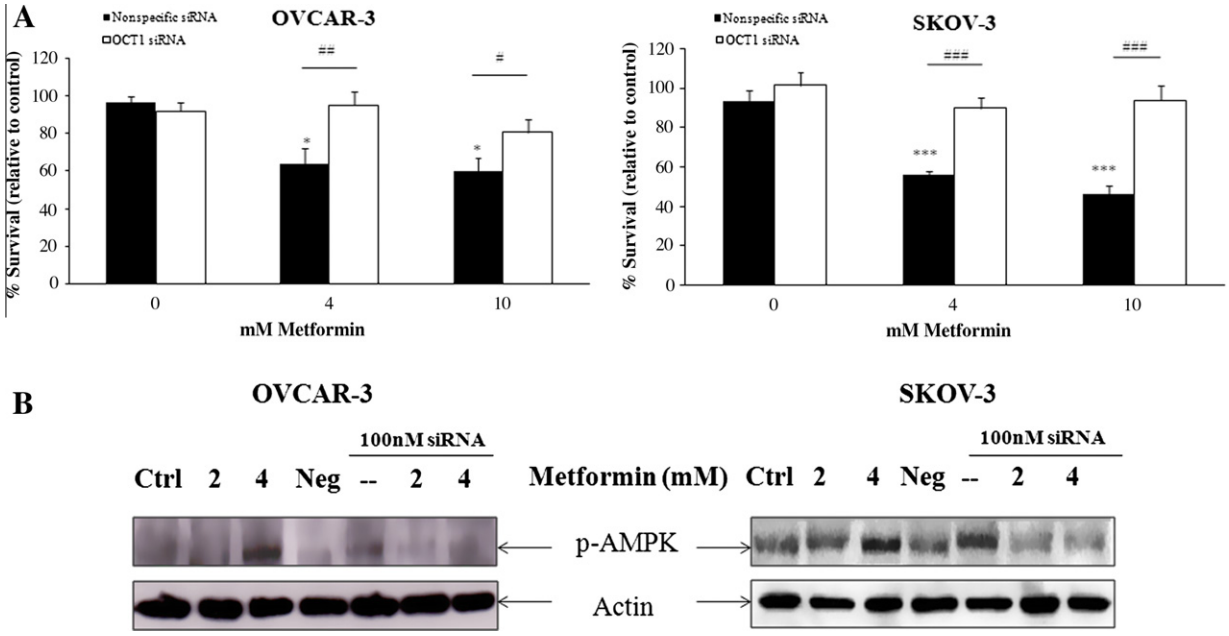

Fig. 2. Metformin requires OCT1 to initiate the first step in the AMPK signaling cascade and decrease cell survival. (A) Cells were incubated with nonspecific and OCT1 siRNA for 72 h in RPMI without FBS, in the presence or absence of metformin. Cell viability was determined using Alamar Blue reagent. (B) Cell lysates were subjected to western blot for phospho-AMPK (Thr^{389}) and actin. One representative experiment out of three is shown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared to experiments with 0 mM metformin.$^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ when compared to nonspecific siRNA.

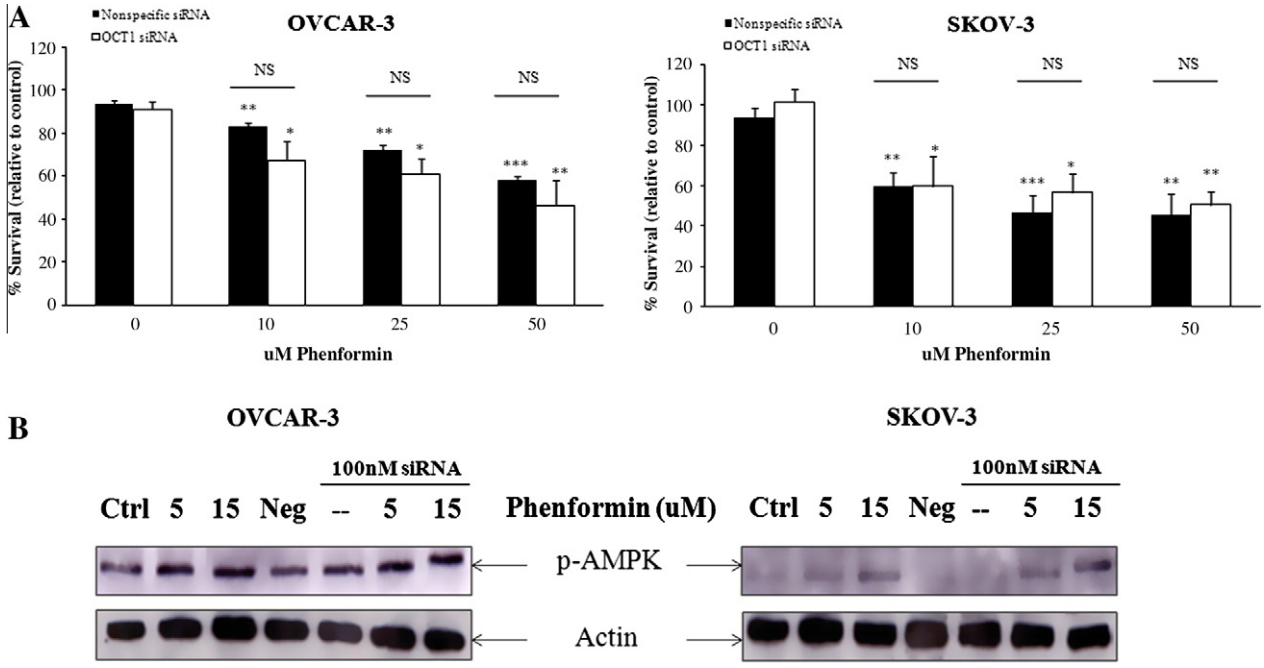

Fig. 3. Phenformin does not require OCT1 to initiate the first step in the AMPK signaling cascade or to reduce EOC cell survival. (A) Cells were incubated with or without siRNA for 72 h in RPMI without FBS, in the presence or absence of phenformin and cell viability was determined using Alamar Blue reagent. (B) Cell lysates were subjected to western blot for phospho-AMPK (Thr^{389}) and actin. One representative experiment out of three is shown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared to 0 mM metformin.

comparisons. $P < 0.05$ was considered significant. Data was analyzed using Prism (GraphPad Software, La Jolla, USA).

3. Results

3.1. OCT1 is expressed in epithelial ovarian cancer cell lines

OCT1 has been reported to be mainly expressed in liver, kidney and small intestine [23]. We investigated the expression of OCT1 in the human EOC cell lines OVCAR-3 and SKOV-3. We found the presence of detectable levels of OCT1 protein expression (Fig. 1A) in both human EOC cell lines.

3.2. OCT1 is crucial for the metformin effect in OVCAR-3 and SKOV-3 cell lines

In order to evaluate the role of OCT1 on the effect of metformin on human EOC, we first exposed the cell lines to OCT1 siRNA and demonstrated a dose-dependent inhibition of OCT1 protein expression in both cell lines (Fig. 1B). The inhibition of OCT1 by siRNA was associated with a significant reversal of the cytotoxicity induced by metformin in both cell lines in a dose-dependent manner (Fig. 2A), suggesting a crucial role for OCT1 in the antiproliferative effect of metformin. An increase in phospho-AMPK (Thr^{172}) protein expression was observed in both cell lines in

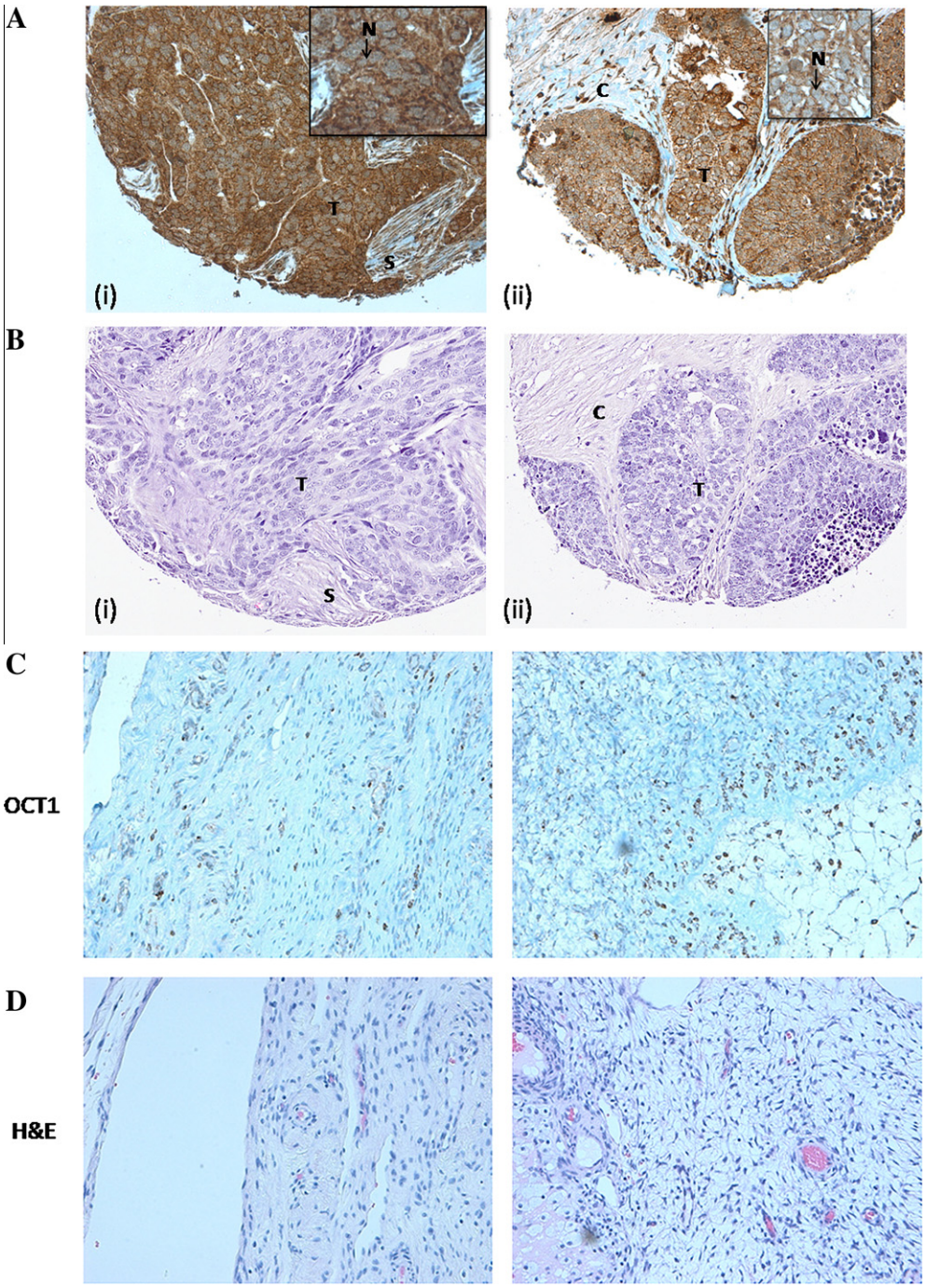

Fig. 4. Tissue microarray exhibiting high (i) and low (ii) expression of OCT1(A) in cores from human serous EOC tumour tissues. 2 representative cores out of 105 are shown. The corresponding H&E staining are shown in B(i) and (ii), respectively. S: stromal cells, C: collagen, T: tumor cells, N: nucleus. (C) OCT1 expression in normal ovaries with their corresponding H&E staining (D).

response to metformin (Fig. 2B). Similarly, reduction of OCT1 by siRNA reduced the AMPK activation induced by metformin.

3.3. The effect of phenformin on OVCAR-3 and SKOV-3 cell lines is independent of OCT1 expression

Phenformin was shown to exhibit dose-dependent antiproliferative effect in both cell lines at lower concentrations than metformin (Fig. 3A). Unlike metformin, phenformin maintained its antiproliferative effect in a dose-dependent manner when OCT1 was reduced by siRNA in both cell lines (Fig. 3A). Likewise, protein levels of phospho-AMPK (Thr^{172}) were unaffected by treatment with OCT1 siRNA in phenformin-treated cells (Fig. 3B). Taken together, these results suggest that OCT1 is not necessary for phenformin, in contrast to metformin, to elicit its effect in EOC cells.

3.4. OCT1 expression is variable in EOC tumors

To evaluate the potential clinical relevance of our findings, we immunostained a tissue microarray of human serous EOC for OCT1. As shown in Fig. 4, OCT1 was specific to tumor cells as stromal cells (S) or collagen (C) remained unstained. Moreover, the OCT1 staining was exclusively cytoplasmic. Most of the cores were showing low positivity for OCT1 staining (64.3 ± 2.4%) (Fig. 4Aii), or high positivity (35.7 ± 2.4%) (Fig. 4Ai) but interestingly, among the 105 EOC samples, none of them showed negativity. In addition, we evaluated the expression of OCT1 in two non-cancerous ovaries and found almost no staining, as demonstrated previously in rodent [24] (Fig. 4C and D).

4. Discussion

The relevance of germ-line polymorphisms of OCT1 to efficacy of metformin has been studied in diabetes. Much of metformin's activity in diabetes treatment takes place in the liver, where metformin is thought to initially reduce oxidative phosphorylation [25]. The resulting AMPK activation with secondary suppression of gluconeogenesis [26] leads to decreases in circulating glucose and insulin levels. Germ-line polymorphisms in the OCT1 gene have been correlated with metformin activity, although these effects are modest in magnitude. While the suppression of gluconeogenesis with secondary reductions in hyperglycemia and hyperinsulinemia may contribute to antineoplastic action of metformin in some contexts where tumors are insulin sensitive [27], there is also current interest in the hypothesis that metformin can act directly on tumor cells [27]. This latter activity will depend critically on whole organism and cellular pharmacokinetic factors, but little is known about the levels of the relevant transport molecules in neoplastic tissue. Our observation that non-cancerous tissue showed a virtual absence of OCT1 expression suggests that metformin's effect could be specific to tumor cells in ovarian tissue. Moreover, our *in vitro* findings suggest that direct actions of metformin may be limited by low OCT1 expression by some tumors, and that this could be minimized by the use of other biguanides such as phenformin.

Further *in vivo* studies using OCT1 knock-out models will be required to address this issue in more detail. Our observations suggest the relevance of measuring OCT1 expression in neoplastic tissue in current clinical trials. It is likely that if biguanides are found to be useful for indications in oncology, their optimum use will depend on patient selection by using appropriate predictive biomarkers. It has already been recognized that some of these biomarkers may be related to host characteristics such as degree of hyperinsulinemia, while others may be related to mechanism of cellular action, such as functionality of the LKB1-AMPK signaling system [28]. The results reported here predict that apart from these considerations, expression levels of the OCT1 transporter in neoplastic tissue may influence efficacy of metformin. Indeed, we demonstrated high and low expression of OCT1 in patients with serous ovarian cancer. If further *in vivo* and clinical studies confirm our *in vitro* observations and demonstrate that poor tumor uptake of metformin commonly limits efficacy, other biguanides such as phenformin may become preferred agents for clinical trials.

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