**In vitro** metformin anti-neoplastic activity in epithelial ovarian cancer

Walter H. Gotlieb a,b,*, Julio Saumet a,b, Marie-Claude Beauchamp a,b, Jing Gu a,b, Susie Lau a,b, Michael N. Pollak b,c, Ilan Bruchim a,b

a Division of Gynecologic Oncology, Jewish General Hospital, McGill University, Montreal, Quebec, Canada
b Lady Davis Institute of Medical Research, McGill University, Montreal, Quebec, Canada
c Department of Oncology, McGill University, Montreal, Quebec, Canada

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**Abstract**

**Objective.** Metformin, a commonly used drug in the treatment of type II diabetes, may reduce cancer risk and improve cancer prognosis. We evaluated its effect on epithelial ovarian cancer cell lines.

**Methods.** The OVCAR-3 and OVCAR-4 cell lines were exposed to metformin with and without cisplatin. Cytotoxicity assays were performed in triplicates using the Alamar colorimetric assay. Levels of total and phosphorylated AMPK, p70S6K and S6K were evaluated by Western blotting following exposure to metformin.

**Results.** Metformin induces dose- and time-dependent growth inhibition of OVCAR-3 and OVCAR-4 cell lines. Metformin potentiated the effect of cisplatin in vitro. Metformin growth inhibition was partly abolished by the AMPK inhibitor, compound C. Western blotting demonstrated that metformin at cytotoxic concentrations, induced AMPK phosphorylation and decreased p70S6K and S6K phosphorylation, suggesting the mechanism for its anti-proliferative action.

**Conclusion.** Metformin significantly inhibits the growth of ovarian cancer cell lines and potentiates cisplatin. Further pre-clinical studies are being conducted to determine the applicability of metformin in the treatment of ovarian cancer.

**Keywords:** Metformin; Ovarian cancer; AMPK; p70S6K; S6K; OVCAR; Cisplatin; Translational research

**Introduction**

Ovarian cancer is the fifth most common cause of cancer related mortality in western countries [1]. The most common type is of epithelial origin. Although the majority of patients have a satisfactory initial clinical response, more than 70% will experience recurrences and ultimately die of the disease [2]. New therapeutic modalities are therefore necessary to overcome the high recurrence rate and to change response to treatment into cure.

Increasing basic science evidence and dietary findings suggests that insulin and insulin-like growth factors (IGFs) play a role in carcinogenesis and disease progression in epithelial ovarian cancer [reviewed in 3–6]. Therefore downregulation of the IGF signaling pathway attained by “caloric restriction” has been postulated as a therapeutic approach to prevent cancer development [7,8]. Among other effects, “caloric restriction” leads to the activation of the AMP-activated protein kinase (AMPK) which is the primary activator of cellular response to lowered ATP levels [9,10]. Activated AMPK has been associated with growth inhibition of human cancer cell lines [11,12].

Upon activation, the phosphorylated AMPK suppresses the mammalian target of Rapamycin (mTOR) signaling pathway, which, via its downstream molecular effectors 4E-BP1 and p70S6, ultimately affects cellular transcription and translation [13,14]. Several experimental approaches have demonstrated the therapeutic potential of mTOR inhibition, inducing drastic anti-proliferative and anti-angiogenic effects in pre-clinical models [14–17].
Metformin, a commonly used oral anti-hyperglycemic agent of the biguanide family, also activates AMPK [18]. Taken together with recent population-based studies that suggested that metformin may reduce cancer risk and improve prognosis [19,20], we evaluated the in vitro anti-neoplastic activity of metformin in epithelial ovarian cancer, and its relationship to AMPK activation and downstream pathway.

![Graph of OVCAR-3](image1)

**Fig. 1.** Metformin inhibits the proliferation of cells in a dose-dependent manner. Monolayers of cells, exposed to increasing doses of metformin for 72 h, were assessed for viability using the AlamarBlue colorimetric assay. Results indicate a dose-dependent toxicity.

![Graph of OVCAR-4](image2)

**Fig. 2.** Metformin inhibits the proliferation of cells in a time-dependent manner. Monolayers of cells, exposed to 50 mM of metformin for 24 h, 48 h, 72 h and 96 h, were assessed for viability using the AlamarBlue colorimetric assay. Results indicate a time-dependent toxicity.
Materials and methods

Cell lines and treatments

The human ovarian cancer cell lines OVCAR-3 (American tissue culture collection, Manassas, VA) and OVCAR-4 were grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 10 μg/ml of garamycin at 37 °C in 5% CO2. The cell cultures were routinely passaged every 3–5 days. Assays were conducted under serum-free conditions as previously described [3]. Metformin was obtained from Sigma Laboratories (Oakville, Ontario). Cisplatin was obtained from Mayne Pharmaceuticals (Montreal, Quebec) as a stock solution of 1 mg/ml.

Western blotting

Cells were lysed in a radioimmunoprecipitation assay buffer (RIPA) (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and aprotinin at 0.2 U/ml). Clarified protein lysates (50 g) were resolved electrophoretically on denaturing SDS-polyacrylamide gels (8–10%), and transferred to nitrocellulose membranes. Membranes were probed with the following primary antibodies specific for: phospho-AMPK (Thr 172), total AMPK (AMPKα), phospho-p70S6K (Thr389), total p70S6K, phospho-S6K (Ser240/244) (Cell Signaling Technology, Beverly MA). Proteins were visualized with Horseradish peroxidase (HRP)-conjugated secondary antibodies (Amershan Biosciences, Baie-d’Urfe, QC). To corroborate equal loading, membranes were stripped and reprobed using an antibody specific for actin (Millipore, Billerica, MA). Antigen–antibody complexes were detected using the ELC system (Amershan Biosciences, Baie-d’Urfe, QC).

Cytotoxicity assays

Monolayers of 2000 cells were plated into 96-well flat-bottom cell culture plates (Corning Incorporated, NY, USA) in medium containing 10% FBS. 24 h after plating, when cells had already attached and reached a ~40% confluency, the cells were washed and the medium replaced with serum-free RPMI-1640 supplemented with 2 mM L-glutamine, and 10 μg/ml of garamycin. All experiments were performed for 72 h in serum-free conditions. Controls included 0.2% DMSO. Metformin was used at concentrations ranging from 1 mM to 100 mM. For time course, cells were incubated under similar conditions for variable times ranging from 24 h to 96 h. For the cisplatin experiments, cells were incubated for 4 h at 37 °C with cisplatin ranging from 5 to 10 μg/ml, then the medium was changed where indicated and the cells were further exposed to DMSO as control or to metformin at concentrations ranging from 1–100 mM. In order to assess the role of AMPK, cells were pretreated with compound C or vehicle for 30 min followed by metformin for 24 h. All experiments were performed in triplicates and were reproduced and confirmed. Cell viability was assessed by visual inspection of the plates and the use of the AlamarBlue colorimetric assay. AlamarBlue (Biosource, Camarillo, CA) assay allows quantitative analysis of cell viability via the innate metabolic activity that results in a chemical reduction of AlamarBlue that changes from the oxidized (blue) form to the reduced pink form. After cells were treated, AlamarBlue was added into the plates. When the color of the dye changed from blue to pink (around 6–18 h), plates were read in an ELISA plate reader at 2 different wavelengths, 562 nm and 620 nm to plot the graph. Percentage of reduced AlamarBlue was calculated as described previously [3]. We compared our results using the AlamarBlue technique with the MTT assay and confirmed the validity of our findings (data not shown).

Results

Metformin inhibits the proliferation of OVCAR-3 and OVCAR-4 cells in a dose- and time-dependent manner

Metformin induced significant proliferation inhibition on both the OVCAR-3 and OVCAR-4 cells lines in a dose- (Fig. 1) and time-dependent manner (Fig. 2).

Metformin potentiates the effect of cisplatin

Cisplatin remains the most active treatment for epithelial ovarian carcinoma, however sensitivity of the tumor cells to cisplatin varies. Here we demonstrate that metformin could increase the cytotoxicity of cisplatin on OVCAR-3 cells (Fig. 3).

Compound C inhibits the anti-proliferative effect induced by metformin

Compound C is known to function as an ATP-competitive inhibitor of AMPK [21]. Here we demonstrate that OVCAR-3 cells pretreated with compound C were partly rescued from proliferation inhibition induced by metformin (Fig. 4).
Increased phosphorylation of AMPK and decreased phosphorylation of p70S6K and S6K following metformin treatment

Western blotting was conducted to assess the effect of metformin on AMPK, p70S6K, and S6K phosphorylation. OVCAR-3 cell lines were treated with 20 mM of metformin for 1, 2, 4, or 8 h. Increased phosphorylation of AMPK together with decreased phosphorylation of p70S6K and its downstream target S6K, was observed over time (Fig. 5a). Moreover, a dose-dependent increase of phospho-AMPK together with a decrease of phospho-p70S6K and phospho-S6K was revealed when cells were treated over 8 h with different doses of metformin (1–32 mM) (Fig. 5b).

Discussion

The potential link between Insulin/IGF-I signaling pathways and cancer has attracted substantial attention during the last years [22]. As a result there is a growing interest in targeting this pathway for cancer treatment. AMPK is a conserved serine/threonine protein kinase regulator of cellular metabolism that is activated in response to nutrient deprivation and pathological stresses [23]. Interestingly, several in vitro and in vivo studies show cancer growth inhibition following AMPK activation [11,12].

Normal cell growth depends on the balance between various intracellular pathways influencing mTOR phosphorylation. Their deregulation leads to metabolic growth related diseases, resistance to apoptosis, and increased proliferation [13–16,24].

Metformin is an oral biguanide introduced into the clinical practice in the 1950s for the treatment of type II diabetes mellitus [25]. Metformin has been increasingly used for the treatment of other conditions associated with insulin resistance, especially polycystic ovarian syndrome [26]. Metformin activates AMPK by mimicking the intracellular metabolic changes of nutritional deprivation [10]. Despite earlier reports that metformin induces changes in the tyrosine kinase activity of the insulin receptor [27,28], recent work demonstrates that a major mechanism of metformin as an agent that reduces insulin and glucose levels in type II diabetes, involves the reduction of hepatic glucose output as a consequence of AMPK pathway activation in hepatocytes [10]. Other groups have reported that metformin targets respiratory chain complex I of the mitochondria, or activates AMPK via changes in mitochondrial reactive nitrogen species [29]. Rodent studies have suggested that biguanides could protect against malignant tumor development in rats exposed to carcinogens [30], and also against breast cancer in HER-2/neu transgenic mice [31].

In the present manuscript we present evidence that treatment with metformin induces significant growth inhibition of the OVCAR-3 and OVCAR-4 cell lines in a time- and dose-dependent manner. Moreover, besides its anti-neoplastic activity as a single agent, metformin co-incubation with cisplatin demonstrated an increased cytotoxicity as compared to each agent alone.

We investigated which downstream pathways could be related to the effect of metformin, and observed that metformin induces an increase of AMPK activity in a time- and dose-dependent manner, consistent with the hypothesis that metformin acts through the stimulation of AMPK in peripheral tissues [10]. Moreover this upregulation of P-AMPK was associated with a reduced phosphorylation of p70S6K and S6 kinase. These downregulations in turn are known to decrease mRNA translation and protein synthesis, that would explain the anti-neoplastic effect observed. In addition metformin anti-proliferative effect was partly reversed in cells pretreated with the known AMPK inhibitor compound C.

In summary, in addition to the well known glucose and insulin lowering effects of metformin, useful for type II diabetes, it also acts as a growth inhibitor of the epithelial ovarian cancer cell lines OVCAR-3 and OVCAR-4 in vitro. Growth inhibition was associated in the ovarian cancer model with upregulation of AMPK activity resulting in downstream signaling inhibition. These findings support the development of pre-clinical experiments in animal models to further evaluate the potential role of metformin in the treatment of epithelial ovarian carcinoma.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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