The role of biguanides in ovarian cancer

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ABSTRACT

Ovarian cancer is the most lethal gynecological malignancy in North America. The purpose of this study was to evaluate the role of biguanides in the treatment of epithelial ovarian cancer by testing these drugs on cell lines and to identify pathways involved in their effect. The organic cation transporter (OCT1) is known to play an important role in cellular uptake of the biguanide metformin in the liver. We aimed to determine the modulating effect of OCT1 on metformin in cancer tissue. Metformin is a widely used biguanide, commonly used for the treatment of type 2 diabetic patients. Epidemiologic and laboratory data suggest that metformin has antineoplastic activity, and this has led to ongoing clinical trials. Our laboratory recently published a study showing that metformin induced apoptosis in an AMPK-independent manner and provoked cell cycle arrest. Moreover, a synergistic effect between metformin and chemotherapy was observed on apoptosis. We observed that there is heterogeneity between primary human tumours with respect to OCT1 expression, and that knocking down OCT1expression using siRNA reduces the sensitivity of cancer cells to metformin. Interestingly, the actions of the related biguanide phenformin are less influenced by OCT1 inhibition. 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 optimum biguanide for clinical investigation.

ABRÉGÉ

Le cancer de l'ovaire est le cancer gynécologique le plus mortel en Amérique du Nord. Cette thèse évalue le rôle des biguanides dans le traitement du cancer épithélial de l'ovaire et d'identifier les voies moléculaires impliquées. Le transporteur de cations organiques (OCT1) joue un rôle important dans l'absorption cellulaire hépatique du metformin, un biguanide en grande utilisation en clinique. Nous avons cherché à déterminer l'effet de modulation de l'OCT1 sur le metformin dans les tissus cancéreux. Le metformin, fréquemment utilisé pour le traitement du diabète de type 2 a démontré une activité antinéoplasique qui a conduit à desessais cliniques présentement en cours. Notre laboratoire a récemment démontré que le metformin induit l'apoptose d'une manière indépendante de l'AMPK et provoque l'arrêt du cycle cellulaire. De plus, un effet synergique entre le metformin et la chimiothérapie a été observé sur l'induction de l'apoptose. Nous avons observé une hétérogénéité dans l'expression d'OCT1 dans les cancers ovariens primaires. L'inhibition d'OCT1 par siARN réduit la sensibilité des cellules cancéreuses au metformin. A l'inverse l'action d'un biguanide différent, le phenformin n'est pas influencé par l'inhibition d'OCT1. Ces résultats suggèrent qu'il peut y avoir des contextes où l'absorption de drogues comme les biguanides, par les cellules cancéreuses limite l'action sur les cellules

néoplasiques, ce qui permet l'hypothèse que le metformin puisse ne pas être le biguanide idéal pour des études cliniques en oncologie.

CHAPTER 1: INTRODUCTION

1. Ovarian Cancer

Ovarian cancer is the leading cause of death among all gynaecological cancers in western countries. When compared to other gynaecological cancers, the fatality rate of ovarian cancer surpasses those of cervical and endometrial cancers put together [1]. This high death rate is due to the diagnosis at an advanced stage in most patients caused by the relative lack of specific signs and symptoms of the disease and the lack of reliable tests for early detection. It is estimated that this year in North America, 24 150 women will be newly diagnosed with ovarian cancer and that 17 220 women will die of the disease [2]. Epithelial ovarian cancer (EOC) constitutes 90% of ovarian malignancies and is classified into distinct histological categories including serous, mucinous, endometrioid, clear cell, transitional, mixed, and undifferentiated subtypes [3]. Nowadays, data suggest that the cell of origin for an important proportion of high-grade pelvic serous carcinomas, including the ovary, is derived from the distal fallopian tube [2].

Although most patients with EOC experience a reasonable initial clinical response to debulking surgery and chemotherapy, the majority of these patients will not be cured. Approximately 70% will experience a recurrence and this

chemoresistance is responsible for the majority of ovarian cancer-related deaths with an overall 5-year survival rate of less than 30% [4, 5]. Presently, there are no available treatments capable of curing recurrent ovarian carcinomas due to their rapid evolution into a chemoresistant disease. It has therefore become essential to introduce new therapeutic modalities that will change response to treatment into cure and salvage these patients. Over the last decade, accumulating data suggest that the insulin/IGF pathway might be one such good therapeutic target in cancers, including for ovarian cancer.

2. Insulin and the Insulin Growth Factor Pathway

2.1 Physiological roles of insulin and insulin-like growth factor

Insulin and Insulin-like growth factor signalling (IGF) regulates cellular growth, proliferation, metabolism and survival. Insulin is a crucial regulator and is under the tight control of blood glucose levels [6]. When released by the beta-cells of the pancreas, insulin binds to receptors on the surface of most cells.

Hepatocytes, adipocytes and muscle cells are classic insulin responsive cells and express high levels of insulin receptors. On the other hand, IGF signalling plays a fundamental role in regulating embryonic growth and regulates specific differentiation in most adult tissues [7]. IGF is a major downstream target of

growth hormone (GH) and is essential for regulating growth and body size both in the prenatal and postnatal stage [8].

In addition to their role in regulating metabolism, insulin and IGF were shown to have a mitogenic effect [9]. Interestingly, the expression, signalling mechanisms, and roles of members of the insulin/IGF family such as ligands, receptors, binding proteins, and binding protein proteases and their inhibitors have been elucidated in ovarian follicle function in humans and other species. In vitro studies and genetic approaches using mouse knockout models for IGF family members have revealed that IGFs are key intraovarian regulators of follicular growth, selection, atresia, cellular differentiation, steroidogenesis, oocyte maturation, and cumulus expansion [10]. Interaction of the IGF system with other growth factor systems and ovarian peptides during follicular development is still in early investigative stages.

2.2 Insulin/IGFs in human cancers

IGF ligands, receptors and IGF binding proteins (IGFBP) have been shown to play a critical role in the development and progression of human cancers. Insulin has also been associated with increased DNA synthesis in breast cancer and aggressive cancer proliferation *in vivo* [7]. Elevated plasma concentrations of

IGF-I or IGFBP-3 have been linked to a high risk for several types of cancers including breast, prostate and lung cancer [11-13]. Some evidence has demonstrated that insulin and IGF receptor levels can influence cancer prognosis [7]. IGF/IGF-IR have been studied extensively in metastatic colon, pancreatic, prostate and breast cancer [14, 15]. In these cancers, there is a strong association with dysregulated insulin/IGF signalling pathway that has been extensively reviewed [7]. Several studies have also reported that inhibition of IGF-IR reduces metastasis of various cancer cells emphasizing the importance of IGF signalling in cancer progression. In addition, the expression levels of the IGF-IR and IR are predictive of breast cancer outcome [7]. IGF-I expression was found to be a favourable prognostic factor used in planning adjuvant treatment for breast cancer patients [16]. It was also found that IGF-IR is a good prognosticator for early breast cancer and varied among breast cancer subtypes [17]. In colon cancer, it was found that IGFBP-2 has potential prognostic implications, and loss of imprinting of IGF-II is associated with poor prognosis of colon cancer patients. However, the role of insulin/IGF in ovarian cancer warrants further description.

2.3 Insulin/IGFs in ovarian cancer

The first study showing the expression of IGF-I mRNA in ovarian cancer cells and tissues, was published back in 1991 by Yee, D et al .[18]. They also reported several IGFBPs and the IGF-IR expression by ovarian cancer cells. This study suggested that all necessary components for an IGF-I-mediated autocrine loop are present in ovarian cancer cells, an observation that was also confirmed in one of our early studies using the OVCAR-3 cell line [19]. Two other groups described the expression of the IGF and insulin receptors in ovarian tumours [20, 21]. During the same period, it was reported that IGF-I levels were higher in cyst fluid from invasive malignant neoplasms compared to benign tumours [22]. Later, another group confirmed the presence of the IGF-IR expression by immunohistochemistry (IHC) in 100% of the ovarian carcinomas samples tested [23]. These initial studies opened the door to a widespread area of research in ovarian cancer, indicating an involvement of the insulin/IGF system in ovarian tumorigenesis.

Strong support for a role of IGF-I in ovarian cancer progression also came from a recent study by Brokaw J. et al, which showed that high free IGF-I protein expression in ovarian tumour tissue was independently associated with the progression of ovarian cancer [24]. Moreover, IGF-I mRNA expression was also

associated with disease progression, implying that both endocrine and paracrine/autocrine regulations of IGF-I activity are involved in ovarian cancer [24]. Similarly, microarray expression profiles from 64 EOC patients demonstrated that individual genes including IGF-I, IGF-IR and several genes downstream of the receptor, were overexpressed in tumours associated with an unfavourable prognosis [25].

2.3.1 Targeting of the insulin/IGF pathway in ovarian cancer

Targeting of the IGF axis has emerged as a promising therapeutic approach in ovarian cancer [26]. The IGF-1 receptor (IGF-IR) pathway plays a major role in cancer growth, tumour cell survival and resistance to therapy. Inhibition of this pathway can be achieved in ovarian cancer cell lines using IGF-IR kinase inhibitors. These small molecules powerfully suppress activity of IGF-IR, IGF-IIR, Akt and ERK1/2, leading to increased apoptosis of the ovarian cancer cells [26]. The strategies used up to now to target IGF in cancer consist of blocking receptor function using receptor-specific antibodies or small-molecule tyrosine kinase inhibitors and activating AMP-activated protein kinase (AMPK).

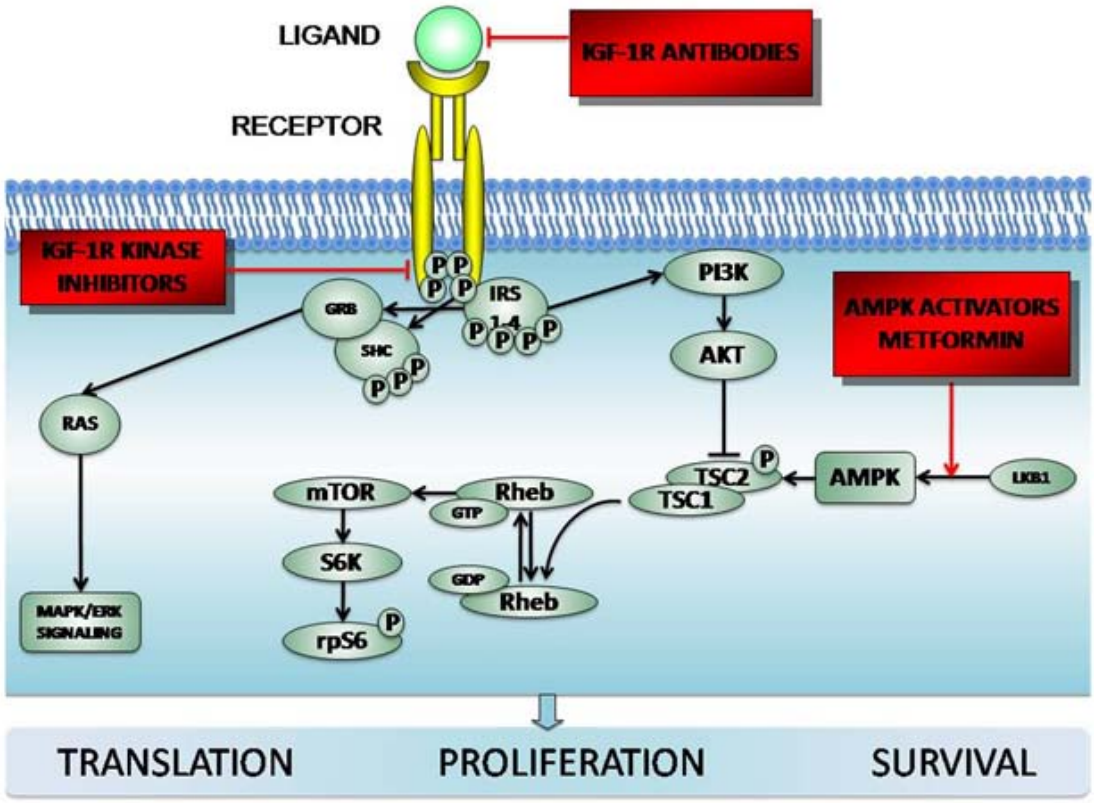


Figure 1: Insulin Receptor and Insulin Growth Factor Family signalling pathway.

Upon the binding of the ligand, the activated receptor will undergo autophosphorylation and in turn will phosphorylate IRS and SHC. Activated IRS will recruit GRB to the phosphorylated form of SHC adaptor protein. The SHC-GRB complex will induce RAS and turn on the MAPK/ERK pathway, inducing cell proliferation and survival. Phospho-IRS will also stimulate the PI3 kinase to phosphorylate AKT thus initiating its downstream effectors such as mTOR, promoting translation, proliferation and cell survival. Generally, activated AKT will have an inhibitory effect on TSC2, allowing Rheb-GDP to be converted to its GTP-bound state, thereby activating mTOR and its downstream signalling molecules to promote cellular translation. Three different potential targeted therapies are underway of investigation in ovarian cancer, including IGF-IR antibodies, IGF-IR kinase inhibitors and AMPK activators such as metformin.

Our studies and others demonstrated decreased cell survival, increased apoptosis and increased sensitivity to cisplatin in ovarian cancer cell lines exposed to various small molecule IGF-IR kinase inhibitors [19, 27, 28]. More recently, Glypican-3 (GPC-3) has been demonstrated to bind with IGF-II and activate the AKT signalling pathway to suppress cell growth, indicating its potential as a therapeutic target in ovarian clear cell carcinomas [29]. IGF-II inhibition was also suggested to reduce taxol resistance in A2780 ovarian carcinoma cell lines and may thus prove to be a useful prognosticator to predict overall disease outcome [30].

2.3.2 Receptor-specific antibodies

These agents have been designed to be highly specific for the IGF-IR i.e. they don't bind to the insulin receptor. The first study targeting IGF-IR in ovarian cancer was published in 2003 by Hongo A. et al., in which they used a soluble form dominant negative of the type I IGF-IR designated 486/STOP in CaOV-3 cells [31]. This soluble IGF-IR is a truncated receptor at the 486th amino acid, located within the extramembranous α-subunit. They showed that the 486/STOP expression could reverse transformed phenotype of the CaOV-3 in vitro and inhibit tumorigenicity *in vivo*. Likewise, the administration of the 486/STOP recombinant protein retarded the tumour growth of CaOV-3 cells *in vivo*. Simultaneously, another group tested an antagonistic monoclonal antibody

designated EM164, specific to the IGF-IR, in various cancer cell lines, including ovarian cancer [32]. They demonstrated a reduction of IGF-I-stimulated proliferation and survival of the human ovarian cancer OVCAR-5 cells.

2.3.3 Receptor kinase inhibitors

Small molecule inhibitors block IGF-IR activation by binding to the ATP-binding pocket of the receptor [27]. Most of the developed tyrosine kinase inhibitors have the side effect of attenuating insulin receptor signalling as well. However, despite this lack of specificity, they were found to be active in preclinical models and some are being evaluated in clinical trials [33-35]. There is a possibility that these agents might be more potent anti-cancer drugs since insulin receptor present on malignant cells may have an important role as well in carcinogenesis [7].

In the last couple of years, studies targeting IGF or insulin pathways in ovarian cancer mostly used small molecule IGF-IR kinase inhibitors. A recent study done showed that PTK6, a member of the Src family of tyrosine kinases often overexpressed in several tumour types, regulates IGF-IR in order to modulate anchorage-independent survival in cancer epithelial cells, suggesting its usefulness as a potential therapeutic target in both breast and ovarian cancer [36].

Moreover, our group reported an inhibition of cell survival in response to NVP-AEW541 in two human epithelial ovarian cancer cell lines, namely OVCAR-3 and OVCAR-4 [19]. Interestingly, this effect was not reversible by the addition of recombinant IGF-I. The laboratory of Dr. Gotlieb further demonstrated that this inhibitor sensitized cells to the effect of cisplatin, an effect described in other types of cancer cells as well [27]. Finally, NVP-AEW541 induced apoptosis and decreased AKT activation. Dr. Gotlieb's laboratory also performed a preliminary *in vivo* study using this small-molecule inhibitor in a human ovarian cancer xenograft model that gave promising results [79]. Indeed, the survival of these mice carrying human ovarian cancers was improved by 60% when treated with an IGF-1R kinase inhibitor (NVP-AEW541) and up to 90% when combined with cisplatin [37]. Concurrently, the Gotlieb Laboratory showed a dose and time-dependent growth inhibition of human epithelial ovarian cancer cell lines, the OVCAR-3 and OVCAR-4 in response to BMS-536924 [28], a more recently developed IGF-1R kinase inhibitor. This effect was partly mediated by AKT and the ribosomal protein S6. BMS-536924 provoked cell apoptosis as shown by the activation of PARP cleavage. Dr. Gotlieb's laboratory finally showed that this IGF-IR kinase inhibitor could sensitize cells to PARP inhibitors, possibly via the induction of DNA damage as indicated by the increased phosphorylation of histone H2AX. This study reinforced the concept that IGF-IR is a good

therapeutic target in ovarian cancer. In addition, it proposes that combination therapy using BMS-536924 with a PARP inhibitor might be an effective strategy to circumvent resistance to treatment in clinical settings.

Using OV202 cells, Haluska et al. showed an antiproliferative effect of BMS-554417 at an IC50 of 7.5μM [38]. BMS-554417 is a derivative of BMS-536924 and shares the same properties. Moreover, the drug inhibited the phosphorylation of the IGF-IR, insulin receptor, AKT and ERK1/2 and also induced apoptosis. In addition, treatment of OV202 with BMS-554417 stimulated the phosphorylation of HER-2. Inversely, treatment with the pan-HER inhibitor increased the phosphorylation of IGF-IR, suggesting a reciprocal cross-talk mechanism [39]. Therefore, the combination of BMS-536924 and a pan-HER inhibitor resulted in a synergistic antiproliferative effect in various ovarian cancer cell lines, associated with a reduction of AKT and ERK phosphorylation and apoptosis. Thus, combining targeted therapies to the HER and IGF-I family of receptors might be an effective strategy to overcome potential clinical resistance to IGF-IR inhibitors.

2.4 Metformin

Another potential drug targeting agent related to the insulin and/or IGF pathway is metformin. Metformin is an oral biguanide widely used since the 1950s for the

treatment of type 2 diabetes to lower both circulating glucose and insulin levels. The drug generally exists as a largely hydrophilic and cationic species due to acid dissociation constants (pKa) of 2.8 and 11.5 at physiological pH [40]. The latter pKa value makes metformin a stronger base than most other drugs [40]. Passive diffusion of metformin is low due to its hydrophilic nature [40], giving rise to the concept that metformin's hydrophilic property requires transporters for it to cross cell membranes in the liver [40, 41]. Interestingly, the idea that this biguanide may be a promising anti-cancer drug was first developed in the early 1970s [42]. Since then, population and cohort studies provided evidence that metformin reduces cancer risk and improves cancer prognosis in type 2 diabetic patients [43, 44]. This protective effect of metformin on cancer risk was newly confirmed in an observational cohort study comprising 4,000 patients with type II diabetes [45]. Moreover, it was recently reported that diabetic patients with breast cancer treated with metformin along with neoadjuvant chemotherapy have a better pathologic complete response (pCR) rate in comparison to patients not receiving metformin [46]. Similarly, a case-control study including 973 patients with pancreatic adenocarcinoma and 863 controls showed that metformin-treated diabetic patients had a significantly lower risk of pancreatic cancer compared to metformin-naïve patients [47]. Additionally, a prospective study comprising 1353

patients just showed that the use of metformin was associated with a lower cancer-related mortality [48].

2.4.1 Mechanism of metformin action

The mechanisms underlying this cancer protective effect are not yet completely understood but various effects of metformin may complement each other. First, metformin reduces hepatic gluconeogenesis and improves insulin sensitivity, thereby reducing endogenous insulin levels, a growth factor promoting neoplasia [49]. Second, it has been reported that the key mechanism of action of metformin is through the activation of the fuel gauge adenosine monophosphate-activated protein kinase (AMPK) [50]. This activation leads to the suppression of the mammalian Target of Rapamycin (mTOR) and the mTOR effector S6 Kinase (S6K), thus inhibiting cellular protein synthesis and growth (appendix 1) [51-54]. Furthermore, the AMPK activation suppresses gluconeogenesis, cholesterol and fatty acid synthesis, and fatty acid β-oxidation, thereby depriving the tumour from various energy sources necessary for its growth [50]. These actions of metformin, i.e. lowering blood glucose and insulin and a direct action on AMPK leading to mTOR inhibition [55-61] makes it an appealing molecule for treating human malignancies.

Other reported effects of metformin include the inhibition of neoplastic angiogenesis, halting tumour growth [49, 62]. Indeed, inhibition of mTOR impairs angiogenesis via down-regulation of expression of angiogenic factors such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) [62]. However, the effect of metformin on angiogenesis is a matter of debate since Phoenix et al. reported an increase of angiogenesis through an upregulation of VEGF in a xenograft model of ER-negative breast cancer (MDA-MB-435) [63]. Interestingly, Pearce et al. suggested that metformin could also improve the efficacy of an experimental anticancer vaccine in mice via an augmentation of CD8+ T memory cells [64]. Lastly, metformin was suggested to have anti-inflammatory properties and improve endothelial dysfunction [65]. These effects enhance the value of metformin as an adjunct to conventional chemotherapy in order to reduce the inflammatory component present in the microenvironment of most neoplastic tissues.

2.4.2 Metformin in Ovarian Cancer

Dr. Gotlieb's laboratory published the original study evaluating the anti-neoplastic effect of metformin in human epithelial ovarian cancer cell lines [54]. Dr. Gotlieb's laboratory previously demonstrated that metformin decreases ovarian cancer cell survival in a dose- and time-dependent manner, partly through AMPK activation

[54]. Moreover, Dr. Gotlieb's laboratory observed that the effect of metformin is potentiated by the addition of cisplatin. The activation of AMPK by metformin was associated with an inhibition of downstream targets of AKT, such as phospho-p70S6 and phospho-S6. The Gotlieb laboratory next evaluated whether, in addition to its anti-proliferative effect, metformin could stimulate apoptosis in human ovarian cancer cells. Metformin induced apoptosis and exhibited a synergistic effect when added to cisplatin. Moreover, Bcl-2 family proteins were involved in this effect.

These findings led to the investigation of metformin as a potentially applicable drug in the treatment of ovarian cancer, alone or in combination with cisplatin by testing it in pre-clinical animal models [66]. To the best of our knowledge, only one study so far tested metformin in an *in vivo* model of ovarian cancer [66] . In this study, A2780 ovarian cancer cells were injected intraperitoneally into nude mice, treated with metformin, alone or in combination with cisplatin for weeks after which ovarian tumour size was measured. It was found that metformin alone decreased ovarian tumour proliferation. The study also confirmed that metformin combined with cisplatin inhibited tumour growth. Metformin was found to also inhibit metastasis and angiogenesis in ovarian tumours, as well as activate AMPK and its downstream effectors [66]. During the same time period, we also

performed a preliminary *in vivo* experiment using metformin in a xenograft model where OVCAR-3 cells were injected intraperitoneally. Nude mice were treated for 4 weeks with 250mg/kg metformin, injected IP twice a week. Our results showed a significant reduced tumour weight despite the small sample size, and a lower volume of ascites (see figure 2). Taken together, these results provide a proof-of-concept that metformin could be a good alternative or additional ovarian cancer treatment.

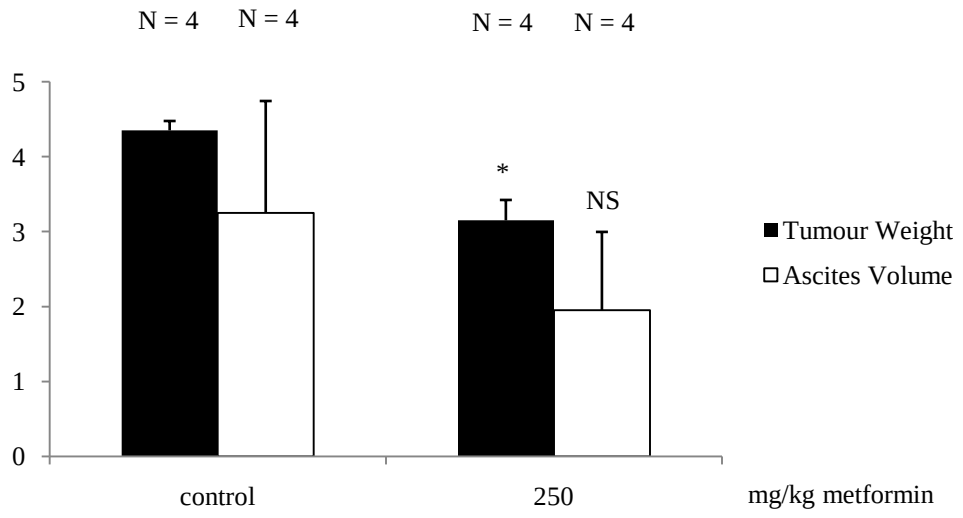


Figure 2: Tumour weight and ascites volume measured in metformin treated xenograft mice.

8 mice were randomized into two experimental groups: one metformin treated group and control. At 6 weeks old, 4 mice were injected intraperitoneally with $1x10^7$ OVCAR-3 cells. Mice were treated for 4 weeks with 250mg/kg of metformin delivered by intraperitoneal injection two times per week. At the end of the treatment period, mice were sacrificed at which point blood, ascites and tumour tissues were collected and measured. * $P < 0.05$ vs. control.

3. Organic Cation Transporter 1

The physiological role of OCT1 involves the hepatic excretion of organic cations [67]. Other functions of OCT1 include the uptake of xenobiotics and endogenous compounds in systemic circulation, maintaining body fluid homeostasis, and acting as a defense system against toxic agents [41]. Human organic cation transporter 1 (OCT1) was identified by homology screening of human cDNA against rat OCT1 and was found to be mainly expressed in the liver, and to a lower degree in the proximal distal tubules of the kidney [67]. OCT1 was also found to be expressed on the luminal membrane of ciliated epithelial cells in bronchial tissue [68]. The hOCT1 gene is localized on chromosome 6q26 [69] and is activated by HNF-4alpha, a homodimeric nuclear receptor found in the OCT1 promoter region [70]. OCT1 is also responsible for the hepatic uptake of metformin, an oral biguanide, utilized for treating type II diabetes mellitus [71].

3.1 OCT1 polymorphisms

OCT1 mediates the first step in the response to metformin, thus the genetic variability in SLC22A1, the gene encoding for OCT1 protein, may modulate human response to metformin [72]. OCT1 has been reported to have several polymorphisms that can limit the cellular uptake of metformin [71, 73, 74]. Known human OCT1 polymorphisms are summarized in Table 1. The variability of OCT1

can modulate drug response by modifying hepatic cell drug clearance [75]. A study screening mutations in 57 Caucasians found 25 single nucleotide polymorphisms (SNPs) within OCT1 [76]. The most common mutation, OCT1-420del, occurs with an allelic frequency of 16% and [76] was found to be present in 20% of white Americans and showed reduced affinity for metformin [71]. Two mutations, F160L and M420del, were found to show similar transport activity to the OCT1 wild-type using a neurotoxin, 1-methyl-4-phenylpyridinium (MPP+). The less frequent mutations R61C, C88R and G401S displayed reduced MPP+ activity, altering the duration and intensity of drugs and neurotransmitter substrates of OCT1 [76]. Two anti-emetic agents, tropisetron and ondansetron, are used as serotonin receptor type antagonists in cancer treatment and have increased efficacy in patients with certain OCT1 polymorphisms limiting drug uptake in hepatic cells [77]. Studies investigating the effects of SLC22A1 gene polymorphisms on A1C measurements in type 2 diabetes mellitus demonstrated reduced metformin uptake in SNPs similar to the abovementioned data [78, 79].

Table 1: Locations of commonly studied polymorphisms influencing OCT1 transport ability.

Decreased activity	Increased activity	Organism	Citation
MPP+: R61C, (9.1%), C88R (0.6%), G401S (3.2%)	F160L (22%) and M420del (16%) normal MPP+, C88R, G401S mediate TEA+ and serotonin uptake	Xenopus oocytes	[76]
R61C, G220V, P341L, G401S, G465R	M408V, M420del are most common, exhibit normal function, S14F exhibits increased function	Xenopus laevis oocytes	[80]
M420del (19% frequency in Caucasians, 5% African Americans), S189L Reduced metformin: R61C, S189L, G220V, G401S, M420del, G465R	Normal metformin uptake: F160L, P341L, R342H, V408M, R488M	Xenopus oocytes	[71]
R61C, C88R, G410S, M420del, G465R		HEK293 cells	[81]

3.2 OCT1 and biguanides

The genetic variability of OCT1 is a determinant of the rate and uptake ability of metformin [80], as well as the therapeutic action of the drug [71]. Organic cation transporters are present in the liver where biguanides such as metformin inhibit complex 1 of mitochondrial respiration in hepatocytes [41]. OCT1 specific for the liver is necessary for metformin to exert its antidiabetic effect [72]. When Oct1 deficient mouse hepatocytes were treated with metformin, AMPK

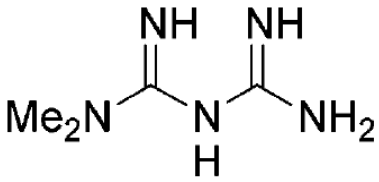
phosphorylation and gluconeogenesis were reduced, while metformin's glucose-lowering effect was eliminated [71]. The OCT1 genotype in polycystic ovarian syndrome (PCOS) was also determined to be a lipid and insulin modulator in response to metformin, but metformin's response during menses, hyperandrogenemia, or glucose tolerance was unaffected [82]. In renal cells, metformin clearance requires the presence of at least one functional OCT1 allele [81]. Pharmacokinetic studies showed that liver concentration of metformin in the presence of OCT1 is 30 fold higher than OCT1 deficient hepatocytes [41]. The plasma lactate level in OCT1 deficient cells was also found to be lower due to lower metformin uptake and the liver being the target organ for lactic acidosis [83]. As some patients may be taking several drugs for different illnesses, drug-drug interactions also merit investigation. It was found that co-administration of the cardiovascular drug verapamil significantly inhibited the effect of metformin uptake by OCT1 in M420del variants, whereas V408M was much less affected when compared to OCT1-reference [74].

Phenformin is a biguanide analog to metformin with anti-diabetic activity. It is structurally analogous to metformin with the addition of a phenyl group [84]. Phenformin was removed from the American and European markets in 1977 because of some toxicity concerns [85]. Phenformin exhibits a higher affinity and

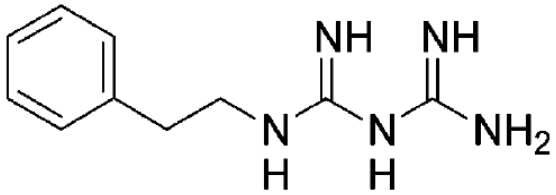
transport activity than metformin for human OCT1 [86]. It also exhibits a higher affinity and transport activity than metformin in rat OCT1 and is capable of reducing oxygen consumption in rat hepatocytes [83]. The biguanide structures of metformin and phenformin are composed of amine groups that make them highly basic (Figure 3) [84]. Biguanides generally exist as positively charged species at physiological pH [41, 84]. They can form dibasic salts, a property that is essential in the production of oral metformin [84]. Biguanides can also be found as acidic species and create complexes with transition metals when they are in their anionic form [84]. Metformin is less lipophilic than its predecessor phenformin due to phenformin's phenylethyl side group, prompting ongoing investigations to construct a prodrug that is more lipophilic and demonstrates better oral absorption than metformin [40].

Studies have shown anti-hyperglycemic behaviour, including increased insulin activity and decreased lipid formation, as well as anti-tumour activity in several animal models of brain, breast and lung cancers, nerve tissue and prostate cancer cell lines [87]. AMPK phosphorylation is reduced when metformin is administered in hepatic mouse cells expressing reduced or non-functional variants of OCT1 as compared to wild-type OCT1 [71]. Because of the higher affinity of Phenformin for OCT1, it is hypothesized to be an appropriate substitute

to treat patients carrying OCT1 polymorphisms that are minimally or completely unresponsive to metformin.



Metformin



Phenformin

Figure 3: Chemical structure of analogous biguanides metformin and phenformin.

4. Hypothesis and Objectives

We hypothesized that metformin is acting on cancer cells through OCT1 whereas phenformin's anti-cancer activity would be less dependent on OCT1 polymorphisms, and could represent a suitable alternative in patients carrying OCT polymorphisms.

Our general objective is to evaluate the role OCT1 in the anticancer effect of biguanides.

Our specific objectives are:

- To evaluate the expression of OCT1 in EOC
- To evaluate the effect of OCT1 on the reduction of cell viability by metformin and phenformin
- To evaluate the effect of OCT1 on the modulation of downstream regulators of the IGF pathway in response to biguanides

CHAPTER 2: Relevance of the OCT1 transporter to the antineoplastic effect of biguanides.

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RUNNING TITLE: Anti-cancer effect of biguanides: Involvement of OCT1

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Abstract

BACKGROUND: Epidemiologic and laboratory data suggesting that metformin has antineoplastic activity have led to ongoing clinical trials. However, pharmacokinetic issues that may influence metformin activity in cancer have not been studied in detail. The organic cation transporter (OCT1) is known to play an important role in cellular uptake of metformin in the liver. We sought to evaluate the involvement of OCT1 in the anti-cancer effects of biguanides.

METHODS: siRNA transfection targeting OCT1 expression in OVCAR-3 and SKOV-3 cell lines followed by cell survival assays and western blot analysis were performed. 105 tumours in TMA were analysed for OCT1 expression.

RESULTS: 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 tumours with respect to OCT1 expression.

CONCLUSION: 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.

KEYWORDS: cancer, biguanides, metformin, phenformin, organic cation transporter 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 [7]. Studies have demonstrated induction of apoptosis by metformin in pancreatic cancer [88], prostate and colon cancer [89], and endometrial cancer [90]. Several publications reported a potential therapeutic effect of metformin in epithelial ovarian cancer (EOC) [54, 66, 91]. We previously demonstrated that metformin enhances ovarian cancer cell cytotoxicity in a dose- and time-dependent manner, an effect potentiated by cisplatin [54]. Metformin induces apoptosis through the modulation of the Bcl-2 family of proteins in some experimental systems [92], but

other mechanisms including AMPK-dependent inhibition of mTOR may also play a role [51, 93].

Phenformin is another biguanide with anti-diabetic activity [94]. It was withdrawn from the market in the late 1970s due to a small risk of lactic acidosis in patients treated for diabetes [85]. Phenformin has been shown to have anti-neoplastic activity, including p21 cell-cycle inhibition leading to apoptosis. Moreover, phenformin was shown to reduce tumour growth in several animal models [87, 95-98]. While risk/benefit considerations clearly favour 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 [99]. It is well known that OCT1 is highly expressed particularly in hepatic cells, where many drugs such as metformin and phenformin act [86, 99]. OCT1 has previously been reported to have several polymorphisms that can influence the cellular uptake of metformin [71]. The most common mutation, OCT1-420del, occurs with an allelic frequency of 16% [76] and was found to be present in 20% of Caucasian

Americans displaying a reduced response to metformin [71]. 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.

Materials and Methods

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), 2mM glutamine, and 10ug/ml gentamicin. Each cell line was passaged every 5 to 7 days and maintained at 37˚C in a 5% CO_2, 95% air atmosphere incubator.

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).

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 hours. AlamarBlue colorimetric assay was performed in medium containing 1% FBS in triplicates as described earlier [28].

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 minutes 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 hours and medium was changed containing increasing doses of phenformin and metformin in 1% FBS, respectively.

Protein assay: Total protein content was determined by the Lowry method [100] using a colorimetric assay (Bio-Rad, Mississauga, ON, Canada).

Protein extraction and Western blot analysis: Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (25mM Tris-HCl pH7.6, 150mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS) supplemented with

protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany). Protein lysates (110µg) 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. $P < 0.05$.

Tissue Microarray: TMA was kindly provided by the Terry Fox Research Institute and contains 105 patients with serous papillary epithelial ovarian 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 32min, 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.

Statistical Analysis: All values are expressed as means ± SEM. Data were analyzed by one-way ANOVA followed by the Newman-Keuls test for multiple comparisons. $P < 0.05$ was considered significant. Data was analyzed using Prism (GraphPad Software, La Jolla, USA).

Results

OCT1 is expressed in epithelial ovarian cancer cell lines.

OCT1 has been reported to be mainly expressed in liver, kidney and small intestine [81]. 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.

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

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 anti-proliferative 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.

AMPK activates the anti-proliferative pathway in response to metformin in the presence of OCT1.

The activation of AMPK by metformin led to decreased phosphorylation of downstream proteins phospho-p70S6 Kinase and phospho-S6 in the presence of OCT1 (Fig. 4a). A recovery of both downstream protein levels was noted in the presence of OCT1 siRNA. Phenformin-activated AMPK exhibited a decrease in phosphorylation of downstream effectors in the anti-proliferative cascade with or without OCT1 siRNA (Fig. 4b).

OCT1 expression is variable in EOC tumours. To evaluate the potential clinical relevance of our findings, we immunostained a tissue microarray of primary human EOC for OCT1. We observed variable expression of OCT1 amongst the 105 tumours (Fig. 5).

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 [101]. The resulting AMPK activation with secondary suppression of gluconeogenesis [102] 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 tumours are insulin sensitive [103], there is also current interest in the hypothesis that metformin can act directly on tumour cells [103]. 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 findings suggest that direct actions of metformin may be limited by low OCT1 expression by some tumours, 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 [104]. The results reported here predict that apart from these considerations, expression levels of the OCT1 transporter in neoplastic tissue may influence efficacy of metformin. If further *in vivo* and clinical studies confirm our *in vitro* observations and demonstrate that poor tumour uptake of metformin commonly limits efficacy, other biguanides such as phenformin may become preferred agents for clinical trials.

Legends and Figures

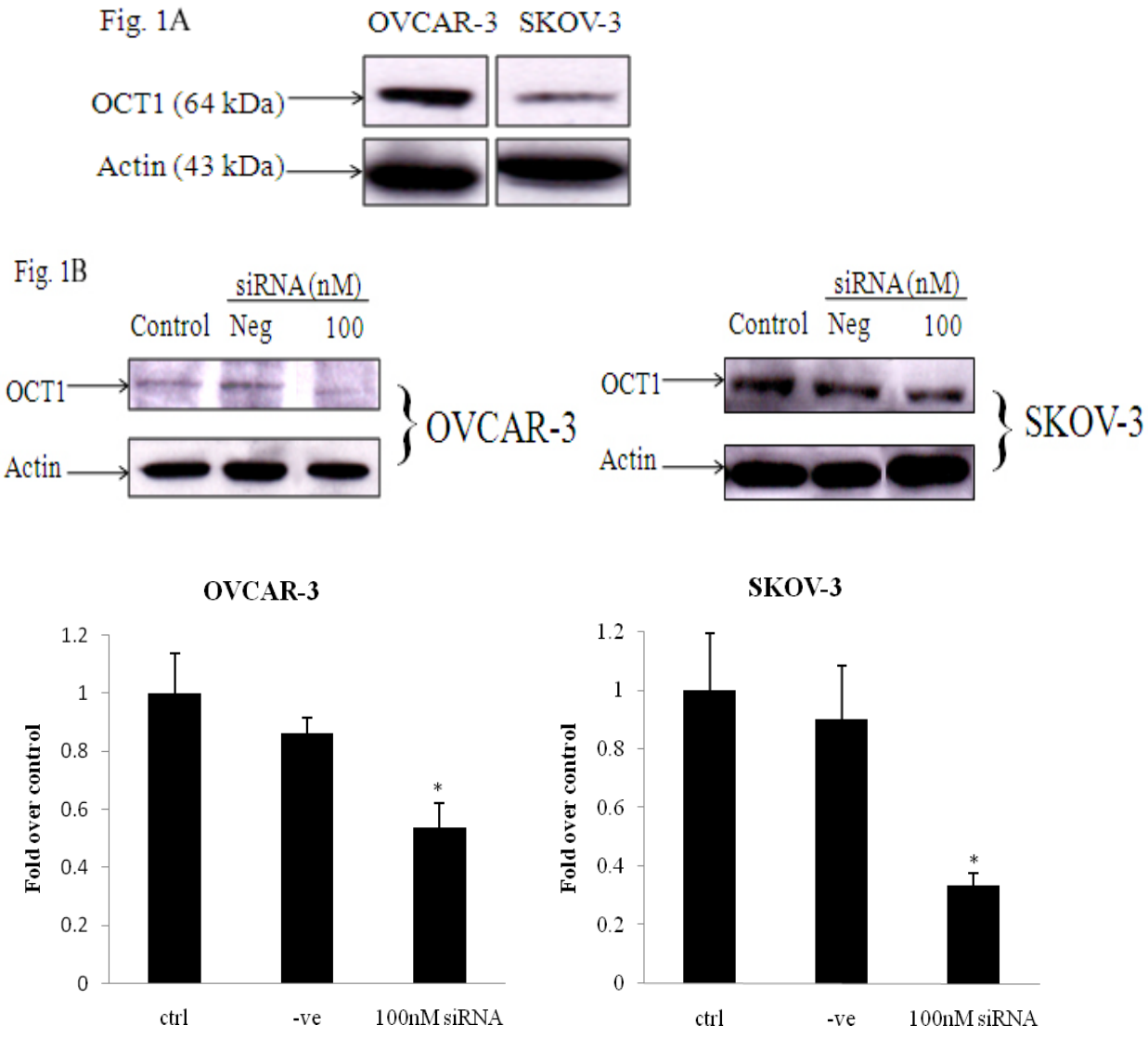

Fig. 1: OCT1 expression in human EOC cell lines.

(A) OCT1 protein levels. (B) Inhibition of OCT1 protein expression with increasing doses of siRNA in OVCAR-3 and SKOV-3 cell lines. * $P < 0.05$ vs. positive and negative controls.

Fig. 2A

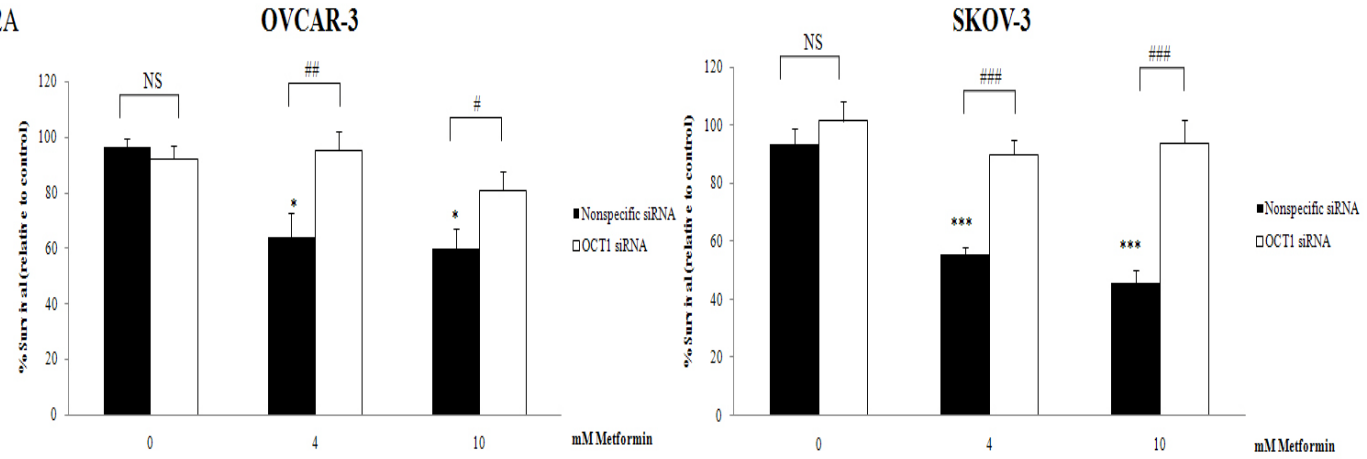

Fig. 2B

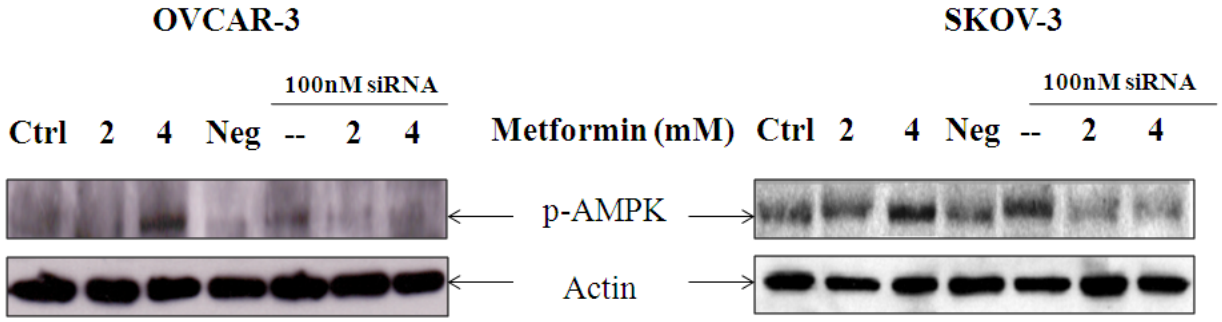

Fig. 2: Metformin requires OCT1 to reduce EOC cell survival and initiate the first step in the AMPK signalling cascade.

(A) Cells were incubated with unspecific and OCT1 siRNA as described in materials and methods for 72h in RPMI without FBS, in the presence or absence of metformin 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. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. nonspecific siRNA.

Fig. 3A

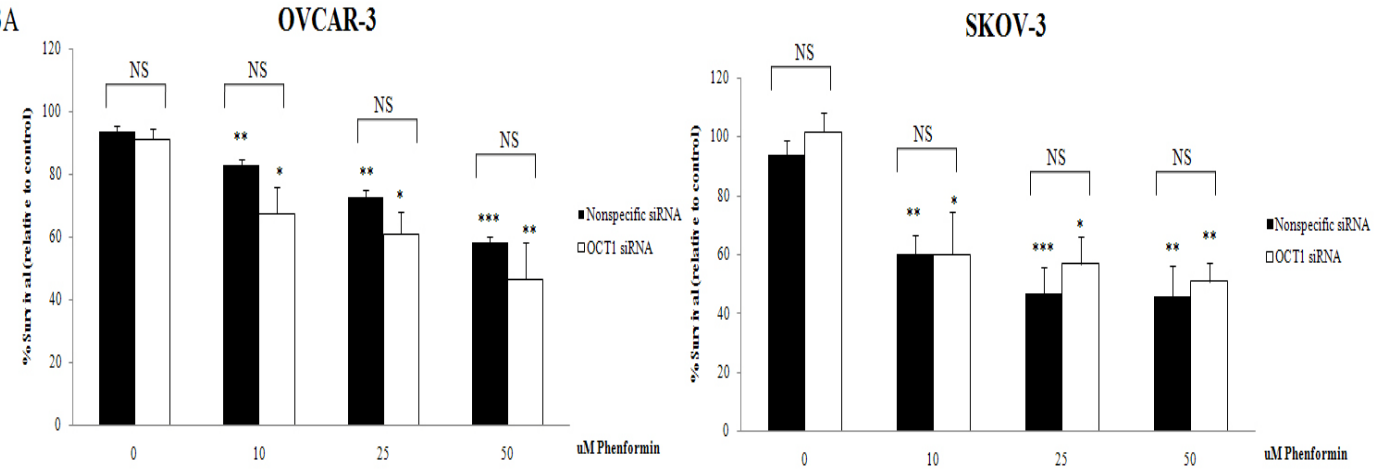

Fig. 3B

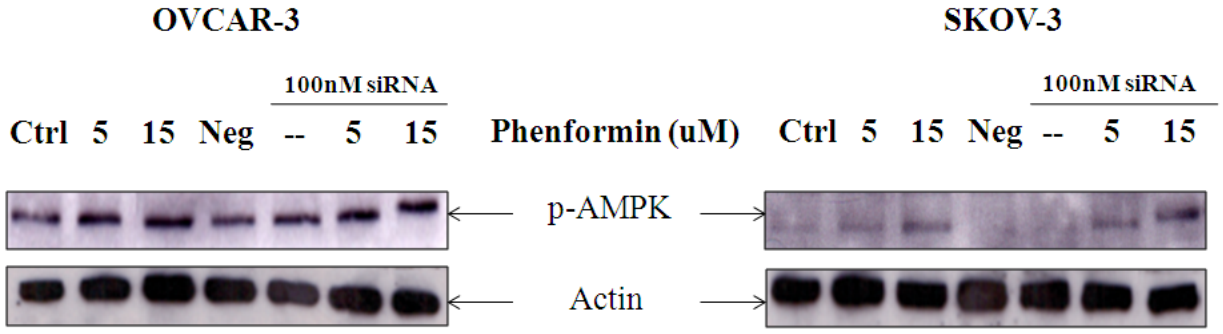

Fig. 3: Phenformin does not require OCT1 to reduce EOC cell survival and initiate the first step in the AMPK signalling cascade.

(A) Cells were incubated with or without siRNA for 72h 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. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. 0mM metformin.

Fig. 4A

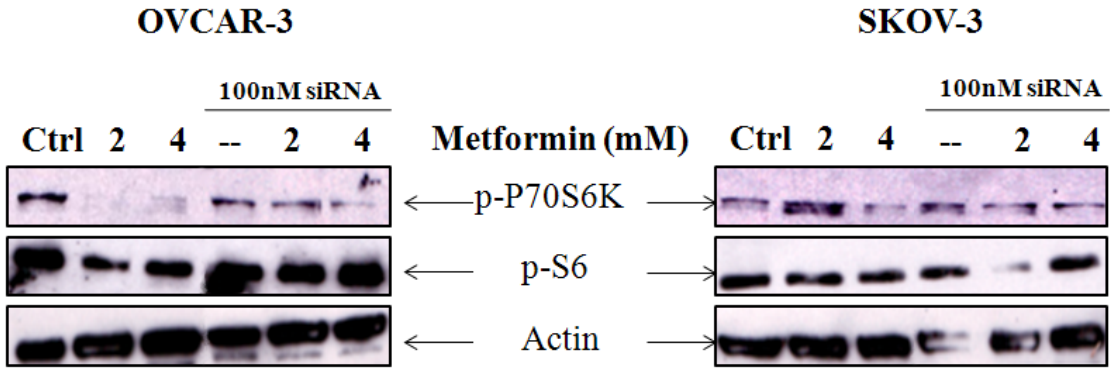

Fig. 4B

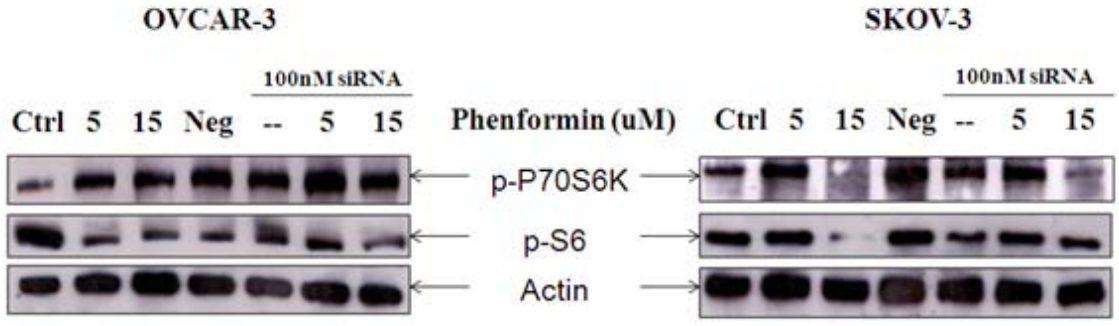

Fig. 4: AMPK activation in the presence of metformin leads to activation of the anti-proliferative downstream cascade of events when OCT1 is functional.

Cell lysates were subjected to western blot for phospho-p70S6K, phospho-S6 and actin. (A) In cells treated with metformin, AMPK activates the downstream cascade of proteins leading to tumour anti-proliferation in both cell lines when OCT1 is present only. (B) Phenformin decreased phosphorylation of downstream effectors regardless of OCT1 expression.

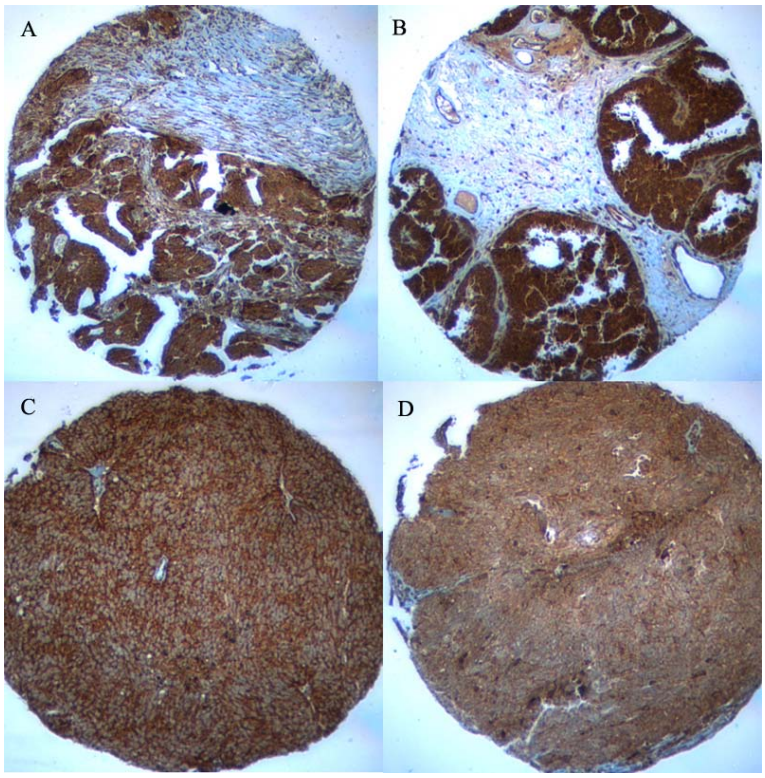

Fig. 5: Tissue microarray exhibiting variation of expression in patient derived samples from human EOC tumour tissues.

All samples are high grade serous ovarian cancer. 4 representative sections out of 105 are shown.

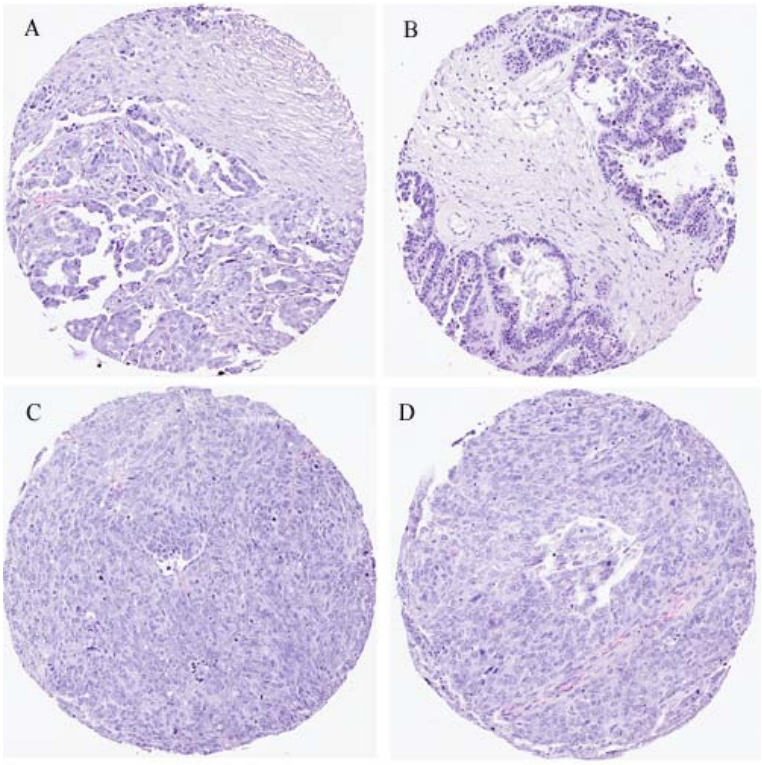

Fig. 6: Hematoxylin and eosin stain of patient derived EOC tumour samples.

4 representative sections out of 105 are shown.

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CHAPTER 3: DISCUSSION AND CONCLUSION

Discussion

Ovarian cancer is the fifth leading cause of cancer death among females in North America [105]. Although improvements in chemotherapy regimens over the last 30 years [106] have enabled to prolong survival of patients with ovarian cancer, the overall cure rate has remained unchanged around 30% [107]. Thus, alternative treatments in combination with conventional chemotherapy are currently being investigated to treat cancer. Epidemiologic and laboratory data have provided evidence that metformin, a commonly used biguanide to treat diabetes, has anti-cancer effects. We recently published a study demonstrating one of metformin's anti-cancer ability was to induce apoptosis in ovarian cancer. We next provided evidence for the involvement of OCT1 in metformin's effect on tumour cells.

In our first study, we determined that metformin modulated the Bcl-2 protein family to downregulate anti-apoptotic protein expression and increased caspase activity associated with cell cycle arrest. Additionally, we found that metformin combined with cisplatin induced a synergistic effect on apoptosis (see Appendix).

In the same study, we observed that metformin increased the ratio of pro-apoptotic Bcl-2 family members, such as Bax and Bad, to the anti-apoptotic Bcl-2 family members Bcl-2, Bcl-xL, and Mcl-1. In accordance with our observations, it was reported that metformin stimulated mitochondrial proteins leading to elevated caspase activity resulting in apoptosis [92]. Similarly, other investigators found that pro-survival members of the Bcl-2 family are involved in the pathogenesis of ovarian cancer [108].

Despite a previous report that AMPK was partially involved in metformin's anti-proliferative effect in ovarian cell lines [54], we found that inhibition of AMPK did not modulate the induction of apoptosis by metformin. Metformin however affected cell cycle proteins in two ovarian cancer cell lines, OVCAR-3 and OVCAR-4, with increased levels of cyclin A and B in response to increasing doses of metformin. We also observed an increase in the accumulation of cells in the S and G2/M phases. This was consistent with findings in endometrial cancer cells that demonstrated G0/G1 phase cell cycle arrest and in pancreatic cancer cells in which arrest in the S phase [88] was described following metformin. Rattan et al. also reported the effect of metformin on various ovarian cancer cell lines [109], showing G0/G1 cell cycle arrest, decrease in cyclin D1 and the proportion of cells in S phase.

Current clinical practice involves treatment of ovarian cancer patients with conventional chemotherapy ultimately resulting in chemoresistance. To evaluate whether metformin could decrease chemoresistance, we performed experiments evaluating the combined effects of metformin and cisplatin, a conventionally used chemotherapeutic drug. We observed a synergistic effect between metformin and cisplatin by isobolometric analysis in two ovarian cancer cell lines, OVCAR-3 and OVCAR-4 [92], associated with an increase in apoptosis. We identified a difference between the two cell lines with OVCAR-3 exhibiting a much weaker inhibition of anti-apoptotic Bcl-2 proteins and a lower induction of the pro-apoptotic Bcl-2 protein than in OVCAR-4. In order to evaluate the difference in response to metformin between the two cell lines, we investigated the OCT1 transporter in cancer cells. OCT1 is a biguanide transporter and is polymorphic in nature. We hypothesized that tumours expressing less OCT1 or polymorphic variations of the OCT1 transporter might yield lower levels of metformin uptake, with subsequently lower activation of apoptotic pathway proteins.

Our in vivo results, in combination with the results of the prior in vivo study performed by Rattan et al. suggest that metformin deserves significant consideration to treat ovarian cancer either in addition, or as a substitute to current chemotherapy regimens. In the Rattan et al. study, metformin was fed to

mice by dissolving in water, thus maintaining a constant effect and prolonged activation of AMPK [66]. On the other hand, our study was based on intraperitoneal injections of metformin yielding a shorter antitumor effect due to systemic clearance. Despite this disparity in administration of metformin, the ovarian cell line treated in our study yielded a significant decrease in tumour weight. However, whether or not metformin response can be modulated by OCT1 expression *in vivo* warrants further investigation. What also remains to be evaluated is the response to treatment of mice with a metformin analog such as phenformin, whether modulation of OCT1 can affect phenformin's anti-cancer properties, and whether phenformin can potentiate the effect of cisplatin in a similar manner to metformin. Although many studies had examined the effects of OCT1 expression and pharmacokinetics in the context of renal and hepatic physiology, as well as the impacts of OCT1 in chronic illness [82], limited attention had been given to its role in oncologic treatment. In the second part of our study, we evaluated, for the first time, the role of OCT1 on metformin's action in cancer.

By using OCT1 siRNA, we demonstrated that metformin reduced cell survival exclusively in the presence of OCT1, while phenformin reduced cell survival in an OCT1-independent manner. Similar results were observed in both EOC cell lines.

These findings differ from observations by Sogame et al. that both metformin and phenformin are transported into human hepatic cells through OCT1 [86]. This discrepancy in tissue sensitivity to biguanides is relevant to cancer treatment, where the primary goal is to have an impact directly on the tumour itself, rather than via a secondary effect of reducing blood insulin levels. Next, we evaluated the pathway activated by both biguanides, and the implication of OCT1. It was previously showed that metformin activates the AMPK pathway via OCT1 in mouse hepatocytes [71]. Similarly to our cell survival results, we showed that phenformin activated the AMPK pathway independently of OCT1, as opposed to metformin, which required OCT1 presence to activate AMPK. Activation of the AMPK pathway has an antagonistic effect on mTOR, leading to decreased phospho-S6K and subsequent cell proliferation [7].

Our observation that phenformin has the ability to exert its anti-proliferative effect independently of OCT1 in ovarian cancer cell lines suggests that this differential uptake between normal OCT1 and polymorphisms of the transporter might provide therapeutic ratio advantages in the treatment of cancer. In accordance with this concept, our tissue microarray analysis suggests that OCT1 expression is patient-specific (Fig. 4). These data also put forth the idea of OCT1's utility as

a predictive factor for biguanide responsiveness in the clinical treatment of cancer, notably epithelial ovarian cancer.

In the current study, we showed that both biguanides dose-dependently decreased epithelial ovarian cancer cell survival. The required dose of phenformin was three orders of magnitude less than that of metformin. Doses in our previous study and in most literature are found to range between 1 and 100 mM metformin [92], as opposed to typical type 2 diabetic patients whose blood concentrations approach 50μM when treated with metformin [110]. However, studies have shown the accumulation of metformin in tissues where it can reach concentrations used in our studies. In mouse hepatocytes, it was found that metformin levels reached 8mM after treatment [111].

Interestingly, OCT1 may play a role in chemoresistance to conventional chemotherapy drugs. It was shown that the presence of OCT1 determines picoplatin transport ability into HEK293 cells transfected with OCT1 [112]. Structural differences in platinum compounds such as oxaliplatin are major determinants of interaction with OCT1 in colon cancer cell lines [113]. These findings not only suggest that variation of OCT1 can alter the uptake and resistance of platinum chemotherapy drugs, in addition to metformin, but also

substantiate the need for additional drug treatment to cancer chemotherapy in general. If biguanides find their way in cancer treatment, as suggested by some [87, 109, 114], evaluation of OCT1 polymorphisms in the individual patient might direct us to the most appropriate drug, e.g. metformin or phenformin, avoiding unnecessary treatment and allowing targeted personalized therapeutic interventions.

Conclusion

The first part of our study demonstrating that metformin induced apoptosis through the involvement of Bcl-2 family proteins strengthens the concept that metformin is worth evaluating in a clinical setting for ovarian cancer treatment. The variable expression pattern of OCT1 in patients from different high grade serous EOC tumours suggests a concomitant variation in response to treatment with metformin. The substitution of metformin for phenformin constitutes an example of potential personalized treatment regimens for patients suffering from cancer, specifically ovarian cancer.

FUTURE STUDIES

Our work will lay the basis for future studies using patient-derived EOC cell lines obtained from surgical specimens to describe their OCT1 polymorphism status, if any. These studies will also include combinatorial effects with conventional chemotherapeutic drugs such as cisplatin. This is a unique opportunity to use clinical samples and data for our future studies. Future experiments will also involve testing of metformin and phenformin *in vivo* by using selected OCT1 knockout mice [115] and injecting them with human EOC cells knocked down for OCT1 expression and wild-type OCT1. Injecting these mice with human EOC cell lines will allow us to evaluate the efficacy of each drug in slowing cancer progression and correlate with OCT1 status. The administration method of metformin and phenformin should also be compared in order to find the optimal route for delivery to maximize the antitumor effect of these drugs. In the case of phenformin, it would be interesting to know if prolonged administration of the drug through drinking water would achieve the desired oncologic effect despite its known toxic side effects. Finally, it was recently reported that OCT1 might play a role in platinum compounds uptake, widely used chemotherapeutic agents for EOC. We can therefore envision combinatorial studies involving metformin, phenformin and platinum agent, such as cisplatin, carboplatin and picoplatin in our patient's cell lines in relation to their OCT1 status. This pilot project

evaluating the role of OCT1 in biguanides uptake could offer innovative therapeutic approaches in the future for EOC patients, based on their unique phenotype.

APPENDIX

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Induction of apoptosis by metformin in epithelial ovarian cancer: Involvement of the Bcl-2 family proteins

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ABSTRACT

Objective. The aims of the study were to evaluate the ability of metformin to induce apoptosis in epithelial ovarian cancer cell lines and to identify the pathways involved in this effect.

Methods. After treatment with metformin and/or cisplatin, OVCAR-3 and OVCAR-4 cellular apoptosis was assessed by flow cytometry and caspase 3/7 activity. Cell cycle analysis was also performed by flow cytometry as well. Modulation of protein expression of the Bcl-2 family after treatment with metformin and/or cisplatin was determined by Western blotting.

Results. Metformin induced apoptosis in OVCAR-3 and OVCAR-4 cell lines in an AMPK-independent manner and provoked a cell cycle arrest in the S and G2/M phase. Moreover, we established that metformin can induce apoptosis in OVCAR-3 and OVCAR-4 cells by activating caspases 3/7, down-regulating Bcl-2 and Bcl-xL expression, and up-regulating Bax and Bad expression. The induction of apoptosis by metformin was also enhanced by cisplatin and combination of these drugs did not modulate the expression of Bcl-2 family proteins in OVCAR-3 cell line, whereas the effect was enhanced in OVCAR-4 cell line.

Conclusion. Bcl-xL and Bcl-2 targeted strategies were suggested to constitute an effective therapeutic tool for the treatment of chemoresistant ovarian carcinoma, in conjunction with conventional chemotherapy. These data are relevant to ongoing translational research efforts and clinical trials exploring a possible protective effect of metformin against ovarian cancer, including Bcl-2 inhibition.

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Introduction

Ovarian cancer is the leading cause of death among all gynecological cancers and the fifth most common cause of cancer-related death in western countries [1]. The lack of symptoms of this disease in its early stages makes early diagnosis extremely difficult. Patients with advanced ovarian cancer are initially treated by a combination of debulking surgery and standard chemotherapy [2]. Despite an initial 70–80% response rate, most patients will relapse within 1–2 years and develop resistance to chemotherapy. In fact, the overall 5-year survival rate is less than 30% [3]. The identification of new drugs or novel therapeutic strategies with the ability to re-sensitize ovarian carcinoma cells to existing chemotherapy has become a major challenge.

Metformin is an oral biguanide which lowers circulating levels of glucose and insulin and is commonly used for the treatment of type II diabetes. Two population studies provided preliminary evidence that metformin may reduce cancer risk and improve prognosis in patients with type II diabetes [4,5]. This protective effect of metformin on cancer risk was recently confirmed in a cohort study comprising 4000 patients with type II diabetes [6]. Moreover, it was recently reported that diabetic patients with breast cancer treated with metformin along with neoadjuvant chemotherapy have a better pathologic complete response (pCR) rate in comparison to patients not receiving metformin [7]. Recent data further demonstrated that the key mechanism of action of metformin is by activating the AMPK–LKB1 pathway [8,9]. Other AMPK activators have displayed growth inhibitory effects in various cancer cell types [10–12]. Therefore, metformin might exhibit two potential anti-neoplastic effects: reducing circulating insulin levels and directly inhibiting growth through the AMPK–LKB1 pathway.

We have previously demonstrated that metformin decreases ovarian cancer cell survival in a dose- and time-dependent manner, partly through AMPK activation [13]. Moreover, we observed that the effect of metformin is potentiated by the addition of cisplatin. We therefore decided to evaluate whether, in addition to its antiproliferative effect, metformin could stimulate apoptosis in human ovarian

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cancer cells. We also sought out to identify the pathways involved in this effect.

Bcl-2 family proteins are crucial for apoptosis commitment, mainly via the control of the mitochondrial pathway which is frequently triggered in response to chemotherapeutic agents. Elevated levels of Bcl-2 in tumor cells may contribute to chemoresistance by stabilizing the mitochondrial membrane against apoptotic insult. Thus, Bcl-2 or Bcl-xl may be good therapeutic targets [14,15]. Although controversial, the majority of studies exhibit evidence of an increased expression of Bcl-2 and Bcl-xL in ovarian cancer [16–18]. Moreover, Anderson et al. recently demonstrated that Bcl-2 levels are elevated in the urine of patients with epithelial ovarian cancer in two different cohorts [19]. We next examined whether metformin, alone or in combination with cisplatin, modulates the pro- and anti-apoptotic protein members of the Bcl-2 family.

Materials and methods

Cells lines and treatment

The 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 glutamine, and 10 μg/ml gentamicin. The cells were routinely passaged every 5–7 days. All cells were maintained at 37 °C in a 5% CO_2, 95% air atmosphere incubator. Assays were performed in medium containing 1% FBS. Metformin was obtained from Sigma-Aldrich (cat#D150959) and kept as a stock solution of 1 M in RPMI without serum. Cisplatin was obtained from the hospital pharmacy.

Chemicals and antibodies

Cell culture materials were obtained from Invitrogen (Burlington, Ontario, Canada). Anti-phospho-AMPK (Thr^{172}), anti-AMPKα1, anti-phospho-P70S6K (Thr^{389}), anti-phospho-Bcl2 (Ser^{70}), anti-Bcl2, anti-Bcl-xL, anti-Mcl-1, anti-phospho-Bad (Ser^{112}), anti-Bax, caspase-3, cleaved caspase-3, and anti-β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG, and enhanced chemiluminescence (ECL) reagents were obtained from Pharmacia-Amersham (Baie-d'Urfe, Quebec,Canada).

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). Briefly, clarified protein lysates (50 μg) were resolved electrophoretically on 10% denaturing SDS–polyacrylamide gels and transferred to nitrocellulose membranes. After blocking in 5% milk, membranes were probed with the following primary antibodies specific for phospho-$AMPK^{Thr172}$, AMPKα1, anti-phospho-$Bcl2^{Ser70}$, Bcl-2, Bcl-xL, Bax, phospho-Bad^{Ser112}, caspase-3, cleaved caspase-3, and β-actin. Immunobloted proteins were visualized using horseradish peroxidise (HRP)-conjugated secondary antibodies and antigen-antibody complexes were detected using the ECL system.

Determination of protein concentrations

Total protein content was measured according to the Lowry method [20] using a colorimetric assay (Bio-Rad, Mississauga, Ontario).

Caspase-3/7 activity

Caspase-3/7 activity was assayed in cell culture. We used the Caspase-Glo 3/7 assay (Promega, Madison, WI). The assay provides a proluminescent caspase-3/7 substrate that is cleaved to aminoluciferin. The released aminoluciferin is a substrate that is consumed by the luciferase, generating a luminescent signal. The signal is proportional to caspase-3/7 activity. The protocol provided by the manufacturer was adapted for use in 96-well tissue culture plates. Briefly, the cells were seeded in 96-well plates under the indicated treatment conditions, after which reagents from the assay kit were added to the culture medium for 1 h. At the end of the incubation period, luciferase activity was measured with a luminometer, giving the relative caspase-3/7 activity.

Flow cytometry

After treatment, adherent cells were collected using trypsin-EDTA while floating cells were collected by centrifugation. The cells were combined and washed twice with ice-cold phosphate-buffered saline (PBS). To determine the percentage of apoptotic cells, collected cells were resuspended in propidium iodide and annexin V along with annexin V binding buffer. After 15 minutes at room temperature in the dark, the proportion of apoptotic cells was measured by flow cytometry with a FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ).

For cell cycle analysis, after collection and washing, cells were fixed in 70% ethanol. The cells were then washed twice with ice-cold PBS and resuspended in propidium iodide buffer (PBS, 0.1% Triton X-100, 0.1 mM EDTA, 0.05 mg/ml ribonuclease A, and 50 mM propidium iodide). After 30 minutes at room temperature, the cell cycle distribution was determined by flow cytometry with a FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ).

Statistical analysis

All values are expressed as means ± SEM. For multiple comparisons, data were analyzed by one-way ANOVA followed by the Student–Newman–Keuls test. $P<0.05$ was considered significant.

Results

Metformin induces apoptosis of epithelial ovarian cancer cells in an AMPK-independent manner

Flow cytometry analysis using annexin V labeling was carried out to measure apoptosis in our cell lines in the presence of metformin. As shown in Fig. 1A, metformin induces apoptosis dose-dependently in both cell lines with a more pronounced effect observed in OVCAR-3 cells. As an additional indication of apoptosis occurring in those cells, caspases-3/7 activity, which play key effector roles in apoptosis, were measured. As shown in Fig. 1B, caspases-3/7 activity was also increased in a dose-dependent manner and to a maximum of 9 fold in response to metformin compared to control. Moreover, these results were confirmed by western blots showing an increase of its activated form, the cleaved caspase-3, in both cell lines (data not shown). We next evaluated the implication of AMPK, a well-known signaling molecule induced by metformin, in the induction of apoptosis by metformin using compound C (Fig. 2). Our results demonstrated an AMPK-independent activation of apoptosis in human epithelial ovarian cancer (EOC) cells.

Effect of metformin on cell cycle

Next, we tested the effect of metformin on cell cycle in each cell lines. When treating OVCAR-3 and OVCAR-4 cells with 10 mM metformin, a slight decrease was observed in cells arrested in the

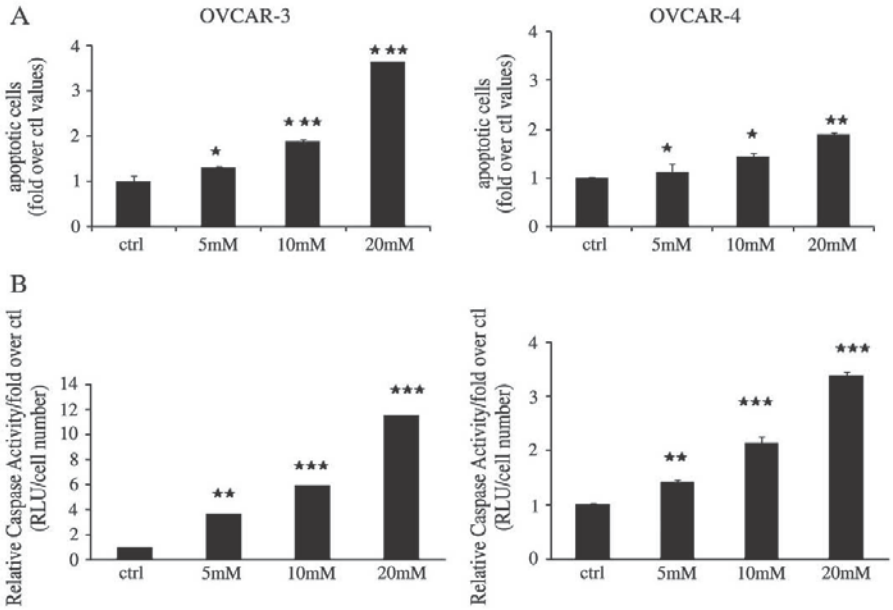

Fig. 1. Dose-dependent effect of metformin on (A) cellular apoptosis and (B) caspase-3/7 activity. OVCAR-3 and OVCAR-4 cells were incubated with 5, 10 and 20 mM metformin for 72 h in 1% FBS and (A) apoptosis levels were determined by flow cytometry. Results represent the mean of three independent experiments. (B) After cells treatment, we measured caspase-3/7 activity using the Caspase-Glo 3/7 assay. Results represent the mean of three independent experiments. *$P<0.05$ versus control, **$P<0.01$ versus control, ***$P<0.001$ versus control.

G0/G1 phase in both cell lines (Fig. 3A and C). Concurrently, there was an increase in cells arrested in the S and G2/M phases of the cell cycle. To confirm these data, we measured the levels of cyclins D1, A and B, which are associated with G0/G1, S, and G2/M phases, respectively. Levels of cyclins A and B increased in response to metformin in a dose-dependent manner, while cyclin D1 levels were not modulated (Fig. 3B and D). Taken together, these results suggest that the cells stopped proliferating and that, when they attempt to replicate, they undergo apoptosis instead of mitosis, causing their accumulation in the S phase.

Effect of metformin on pro-survival proteins of the bcl-2 family in OVCAR-3 and OVCAR-4 cell lines

Then, we attempted to elucidate a pathway involved in apoptosis induction by metformin. To do so, we measured the levels of pro-survival proteins of the Bcl-2 family in the presence of increasing doses of metformin. Levels of phospho-Bcl-2, Bcl-2, Bcl-xL, and Mcl-1 were all downregulated in both human EOC cell lines tested in a dose-dependent manner (Fig. 4A).

Effect of metformin on pro-apoptotic proteins from the Bcl-2 family in OVCAR-3 and OVCAR-4 cell lines

The ratio between the pro- and anti-apoptotic proteins may influence the susceptibility of cells to apoptosis. Western blots demonstrated a dose-dependent induction of Bax and phospho-Bad protein levels, both pro-apoptotic proteins of the Bcl-2 family, in response to treatment with metformin (Fig. 4B).

Effect of the combination of metformin and cisplatin on apoptosis

We next tested the combination effect of metformin with cisplatin, one of the most widely used chemotherapeutic agents for

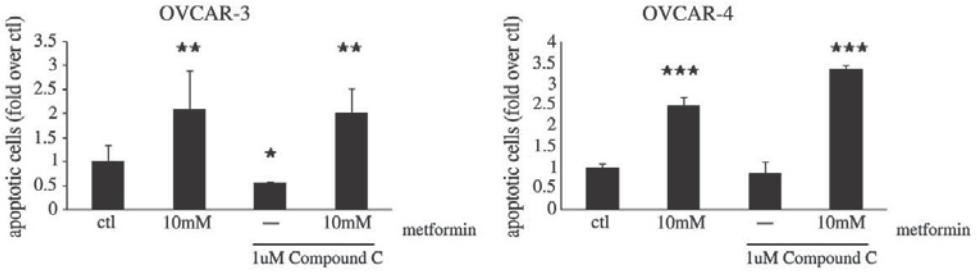

Fig. 2. Metformin induces apoptosis in an AMPK-independent manner in OVCAR-3 and OVCAR-4 cell lines. Cells were incubated for 72 h in 1% FBS in the presence of 10 mM metformin and/or 1 μM compound C and apoptosis levels assessed by flow cytometry. Results represent the mean of three independent experiments. *$P<0.05$ versus control, **$P<0.01$ versus control, ***$P<0.001$ versus control.

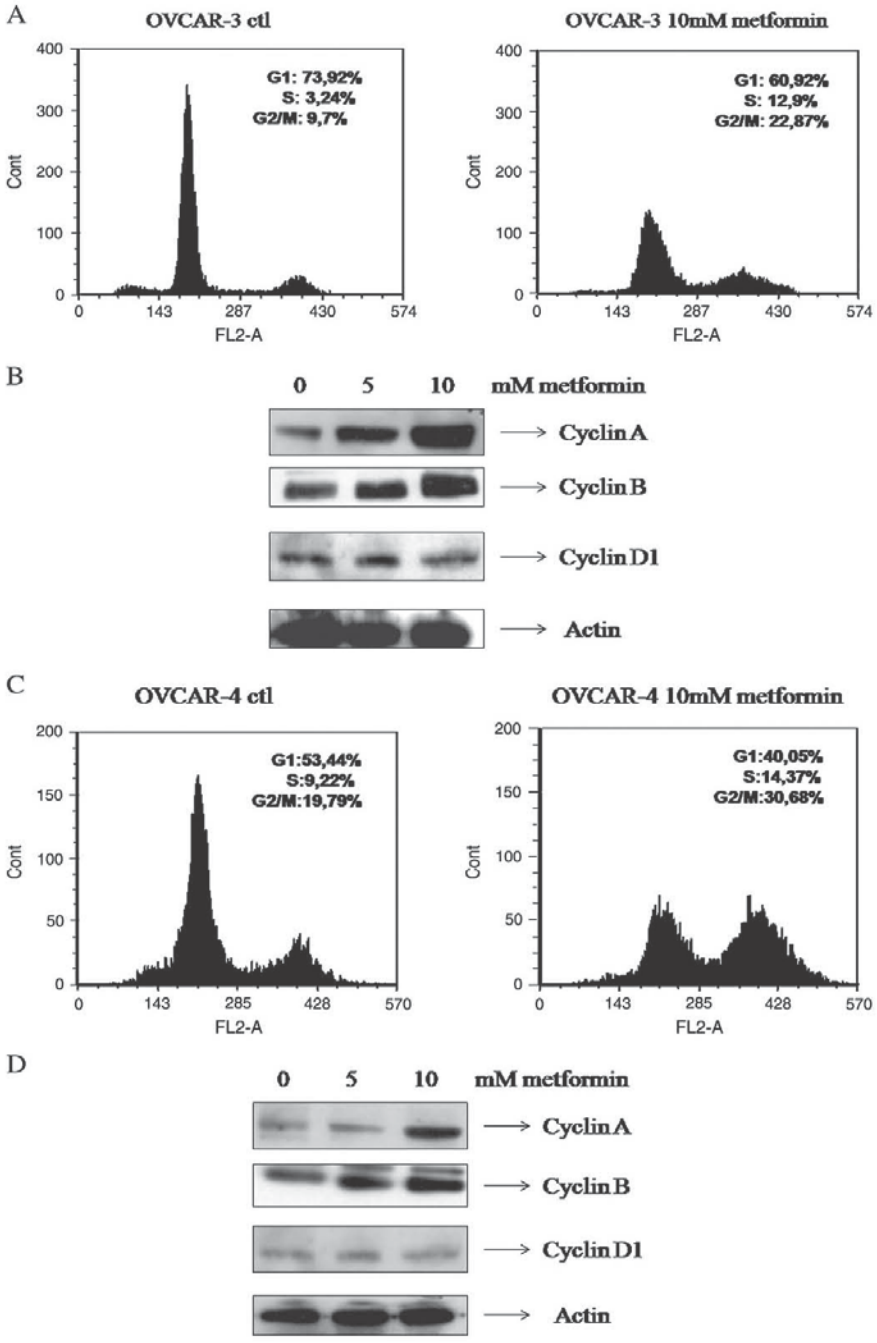

Fig. 3. Metformin blocks cell cycle progression in S phase. (A) OVCAR-3 and (C) OVCAR-4 cell lines were incubated with or without 10 mM metformin for 72 h in 1% FBS and cell cycle distributions were analyzed by flow cytometry. OVCAR-3 (B) and OVCAR-4 (D) cell lysates were subjected to Western blot for cyclins A, B, D1 and actin. One representative experiment out of three is shown.

ovarian cancer. The combination of both drugs exhibited a substantial synergistic effect on the induction of apoptosis compared to treatment of each drug alone in both cell lines (Fig. 5A). Combination index (CI) values were calculated using the classic isobologram equation and indicate synergy (CI<1) or additivity (CI approximately 1) [21]. We found a synergistic interaction between

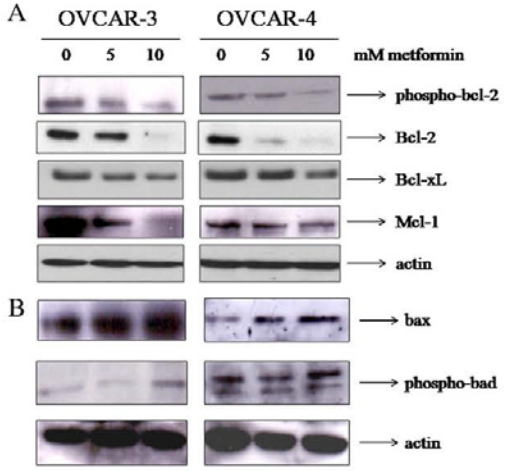

Fig. 4. Metformin inhibits anti-apoptotic (A) and induces pro-apoptotic (B) proteins from the bcl-2 family in a dose-dependent manner. OVCAR-3 and OVCAR-4 cells were treated with 10 mM metformin for 72 h in 1% FBS. Cell lysates were subjected to Western blot for phospho-AMPK, AMPK, phosphor-bcl-2, bcl-2, bcl-xL, Mcl-1, bax, phospho-bad and actin. One representative blot out of three is shown.

metformin and cisplatin on apoptosis induction with a CI of 0.81 and 0.67 for OVCAR-3 and OVCAR-4, respectively. Again, caspase-3/7 activity paralleled the effect of metformin, alone or in combination with cisplatin (Fig. 5B).

Effect of the combination of metformin and cisplatin on pro-survival and pro-apoptotic proteins of the Bcl-2 family in OVCAR-3 and OVCAR-4 cell lines

We next evaluated the combined effect of metformin and cisplatin on the levels of pro-survival Bcl-2 family proteins. In OVCAR-3, the addition of cisplatin to metformin did not significantly modulate the levels of any pro-survival (Fig. 6A) and pro-apoptotic (Fig. 6B) proteins of the Bcl-2 family compared to metformin alone, whereas in OVCAR-4, the combination of both drugs enhanced their effect on the pro-and anti-apoptotic proteins of the Bcl-2 family.

Discussion

Epithelial ovarian cancer is the leading cause of death among gynecological cancers and close to 70% of patients with advanced-stage disease will experience recurrence [22,23]. This is caused by the development of resistance to current therapies, implying the need to develop novel therapeutic modalities with innovative mechanisms of action.

Metformin has been used for several decades for the treatment of type 2 diabetes and has a proven track record of being highly effective with minimal toxicity. It is available as a stable, oral drug and is remarkably inexpensive. Interestingly, the idea that this biguanide may be a promising anti-cancer drug was first developed in the early 1970s [24]. Later on, two population studies provided preliminary evidence that metformin may reduce cancer risk and improve prognosis in type 2 diabetic patients [4,5]. We have previously reported, for the first time, that metformin reduces epithelial ovarian cancer cell proliferation, partly through AMPK activation [13].

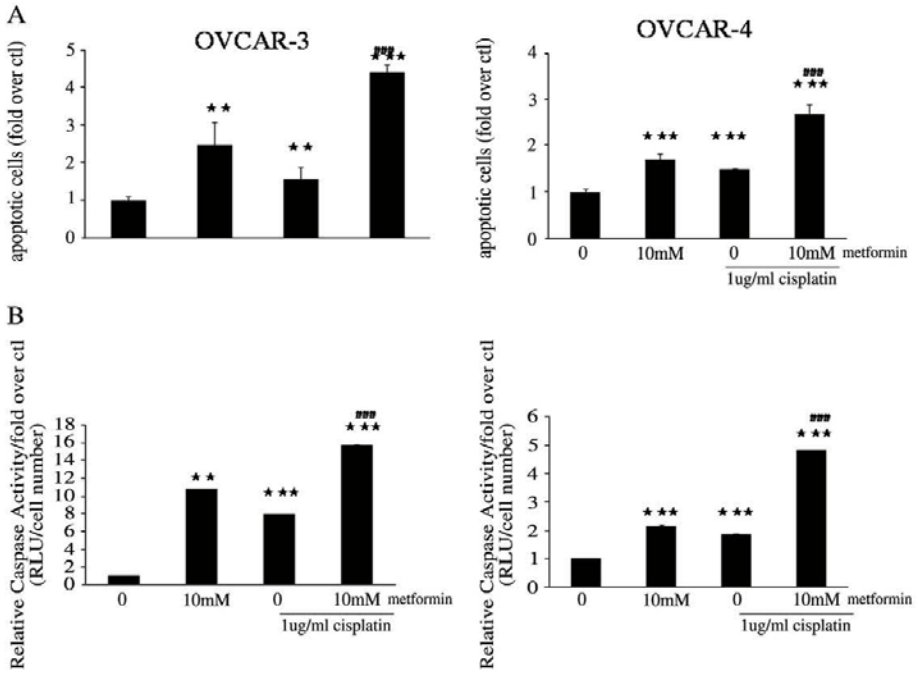

Fig. 5. Combination effect of metformin and cisplatin on (A) cellular apoptosis and (B) caspase-3 activity. OVCAR-3 and OVCAR-4 cells were incubated in the presence of 10 mM metformin and/or 1 μg/ml cisplatin for 48 h in 1% FBS and (A) apoptosis levels were determined by flow cytometry. Results represent the mean of three independent experiments. (B) After cells treatment, we measured caspase-3/7 activity using the Caspase-Glo 3/7 assay. Results represent the mean of three independent experiments. *$P<0.05$ versus control, **$P<0.01$ versus control, ***$P<0.001$ versus control; ###$P<0.001$ versus 10 mM metformin.

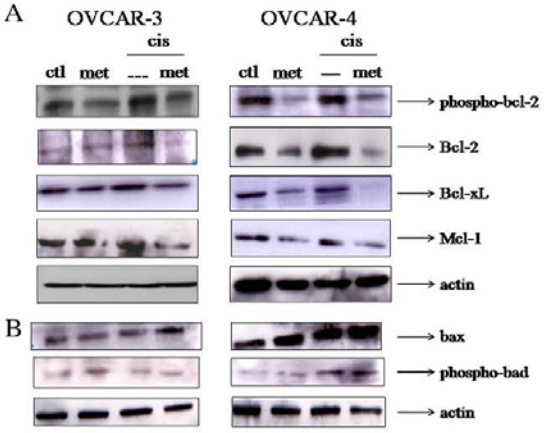

Fig. 6. Combination effect of metformin and cisplatin on the expression of anti-apoptotic (A) and pro-apoptotic (B) proteins from the bcl-2 family. OVCAR-3 and OVCAR-4 cells were treated with 10 mM metformin and/or 1 μg/ml cisplatin for 72 h in 1% FBS. Cell lysates were subjected to Western blot for phospho-AMPK, AMPK, phospho-bcl-2, bcl-2, bcl-xL, Mcl-1, bax, phospho-bad and actin. One representative blot out of three is shown.

Recently, these results were confirmed in both cisplatin-resistant and cisplatin-sensitive ovarian cancer cells [25].

In this article, we have examined whether metformin stimulates apoptosis in addition to its anti-proliferative action [13], thereby contributing to its anti-neoplastic effect. Our flow cytometry results demonstrate that metformin induces apoptosis in both cell lines in a dose-dependent manner (Fig. 1A). These findings were further confirmed by our results showing activation of caspase-3 by metformin in both cell lines (Fig. 1B). Data concerning the effect of metformin on apoptosis in cancer cells are limited and somewhat inconsistent. Ben Sahra et al. have shown that metformin blocked the cell cycle in the G0/G1 phase in prostate cancer cells and did not induce apoptosis [26]. Similarly, breast cancer cells did not undergo apoptosis in response to metformin [27]. In contrast, metformin has been shown to stimulate apoptosis in pancreatic cancer cells [28]. The discrepancy observed between studies on the effect of metformin on apoptosis may be the result of variations in experimental conditions and/or cell-specific functions [29–31] and will require further investigation.

We then investigated the implication of AMPK in the induction of apoptosis by metformin using compound C. As shown in Fig. 2, the inhibition of AMPK did not modulate the apoptosis induction by metformin although we have previously reported that AMPK was, at least partly, involved in the antiproliferative effect of metformin in ovarian cell lines [13]. Conflicting data exist in the literature showing an AMPK-dependent [30,31] or independent [26] effect of metformin on proliferation as well as on apoptosis. Interestingly, only one other study evaluated the antiproliferative effect of metformin on ovarian cancer cell lines and found that the activation of AMPK was not essential [25]. It is possible that metformin modulates other oncogenic pathways through the action of LKB1, but this warrants further examination.

Next, we evaluated the effects of metformin on cell cycle distribution and progression. As shown in Fig. 3A, metformin marginally reduced the number of cells in the G1 phase. Concurrently, ovarian cancer cells were blocked in S and G2/M phases when exposed to metformin for 72 h. Our flow cytometry results were confirmed by testing various cyclin levels. We found a striking elevation of cyclin A and B levels in both cell lines in response to increasing doses of metformin (Fig. 3B), suggesting an accumulation of cells in the S and G2/M phases. Correspondingly to our flow cytometry data, no modulation of cyclin D1 was observed. Again, differences exist between studies regarding the effect of metformin on cell cycle distribution. A cell cycle arrest was described in the G0/G1 phase in breast [27], prostate [30] and endometrial [32] cancer cells whereas others found a cell cycle arrest in the S phase of prostate cancer cells, as we did [28]. These data suggest that metformin could sensitize the response of patients to DNA damaging agents (chemotherapy or radiotherapy) due to their extended arrest in the S phase [28]. Only one publication reported the effect of metformin on diverse ovarian cancer cell lines [25], showing a cell cycle arrest in G0/G1 phase along with a reduction of cyclin D1 and a reduction of the percentages of cells in S phase. One possible explanation for the variations of the metformin effect in different ovarian cancer cells is the existing polymorphisms of the metformin transporter, OCT1 (organic cation transporter) [33]. The role of OCT1 in metformin uptake by ovarian cancer cells is unknown at the moment but is under investigation.

Many death and survival genes, such as Bcl-2 or Bax, which are regulated by extracellular factors, are involved in apoptosis [34]. When the ratio of pro-apoptotic Bcl-2 family members (bax, bad) to anti-apoptotic bcl-2 family (bcl-2, Bcl-XL and Mcl-1) members increases, pores form in the outer mitochondrial membrane, liberating apoptogenic mitochondrial proteins to activate caspases and induce apoptosis [35]. Thus, we next sought to evaluate the effect of metformin on various pro or anti-apoptotic proteins of the bcl-2 family. Our results have shown a decrease in the expression of phospho-Bcl-2, Bcl-2, Bcl-xL and Mcl-1 anti-apoptotic proteins in cells treated with metformin (Fig. 4A). Concomitantly, we have observed that the pro-apoptotic proteins, Bax and phospho-Bad, are induced in the cells exposed to metformin (Fig. 4B).

In this study, we have demonstrated not only the additional pro-apoptotic effect to the previously described anti-proliferative metformin effect but also the beneficial effect of combining metformin with the cytotoxic drug, cisplatin, commonly used in the treatment of ovarian cancer. Both metformin and cisplatin stimulated apoptosis. The increase in apoptosis was significantly greater when metformin was added to cells treated with cisplatin when compared to the action of each of the drugs alone as shown by our FACS analysis as well as caspases-3/7 activity (Fig. 5). The combination index (CI) was 0.81 and 0.67 for OVCAR-3 and OVCAR-4, respectively, suggesting a synergistic effect between the drugs. In OVCAR-3 cell line, our results demonstrated decreased expression of Bcl-xL, Bcl-2 and phospho-Bcl-2 in cells treated with metformin alone, with no amplification of this effect when cisplatin was added (Fig. 6A). Similarly, the two drugs together did not induce the pro-apoptotic proteins of the bcl-2 family, bax and bad, compared to each one alone (Fig. 6B). Taken together, these results suggest that although there is a synergistic effect of cisplatin and metformin on ovarian cancer cell apoptosis, it seems that the mechanism of action differs as compared to when the cells are exposed to metformin alone. As suggested earlier, it is possible that when cells are exposed to metformin and arrested in the S phase, they become more susceptible to chemotherapeutic agents such as cisplatin. On the other hand, in OVCAR-4, the combination of the drugs induced a stronger inhibition of the anti-apoptotic proteins and a greater induction of the pro-apoptotic proteins (Fig. 6), suggesting a cell specific effect of the combined drugs.

Although the doses used in this study are similar to most in vitro and pre-clinical studies found in the literature, ranging from 1 to 100 mM, one can argue that it is still supra-physiological levels. Indeed, the concentration of metformin in the blood of type 2 diabetic patients treated with the drug approximates 50 μM [36], meaning that we used 200-fold excess over the therapeutic levels. However, it has been reported that metformin accumulates in tissues where it can reach concentrations at which our in vitro observations might occur.

For instance, it was demonstrated that metformin levels attained 8 mM in the mice liver after treatment [37].

Therefore, targeted inhibition of Bcl-2 and Bcl-xL expression has the potential to facilitate tumor cell apoptosis. This study describes the correlation between metformin treatment, the inhibition of the anti-apoptotic Bcl-2 family proteins, and the increase in apoptotis when metformin is added to the treatment with cytotoxic drugs. This in vitro study offers a basis for further pre-clinical studies on the benefits of metformin treatment for ovarian cancer.

Conflict of interest statement
The authors declare that there are no conflicts of interest.

Acknowledgments

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Author Contributions:

Amber Yasmeen, Marie-Claude Beauchamp, Ettie Piura, **Eric Segal**, Michael Pollak, Walter H. Gotlieb. Induction of apoptosis by metformin in epithelial ovarian cancer: Involvement of the Bcl-2 family proteins. Gynecologic Oncology. 2011 Jun 1;121(3):492-8. Epub 2011 Mar 8.

Eric David Segal: I carried out some experiments including tissue culture, colorimetric protein assay, western blotting, caspase 3/7 analysis, cell cycle and apoptosis analysis through FACS, isobolometry and statistical analysis.

Eric D. Segal, Amber Yasmeen, Marie-Claude Beauchamp, Joshua Rosenblatt, Michael Pollak and Walter H. Gotlieb. Modulating role of the OCT1 transporter in the cytotoxic effect of biguanides.

Eric David Segal: I carried out all experiments including tissue culture, colorimetric protein assay, western blotting, alamar assay, siRNA transfection and statistical analysis.

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