RESEARCH ARTICLE

Evaluation of Effects of Metformin in Primary Ovarian Cancer Cells

Seema Patel[1], Neeta Singh[1]*, Lalit Kumar[2]

Abstract

Background: Ovarian cancer is the third most common cause of cancer in Indian women. Despite an initial 70-80% response rate, most patients relapse within 1-2 years and develop chemoresistance. Hence, identification or repositioning of drugs to resensitise ovarian cancer cells to existing chemotherapy is needed. Traditionally immortalized cell lines have been used in research, but these may contain genetic aberrations and chromosomal abnormalities serving as poor indicators of normal cell phenotype and progression of early-stage disease. The use of primary cells, maintained for only short periods of time *in vitro*, may serve as the best representative for studying *in vivo* conditions of the tissues from which they are derived. In this study we have attempted to evaluate the effect of metformin (an antidiabetic drug) in primary ovarian cancer cells because of its promising effect in other solid tumours. Materials and Methods: Primary cultures of epithelial ovarian cancer cells established from ascitic fluid of untreated ovarian cancer patients were used. The cells were treated with metformin at doses standardized by MTT assay and its ability to induce apoptosis was studied. The cells were analysed for apoptosis and apoptosis related proteins by flow cytometry and western blotting respectively. Results: Metformin induced apoptosis in ovarian cancer cells, provoking cell cycle arrest in the G0/G1 and S phase. It induced apoptosis in ovarian cancer cells by, down-regulating Bcl-2 and up-regulating Bax expression. Conclusions: Metformin was able to induce apoptosis in primary ovarian cancer cells by modulating the expression of Bcl-2 family proteins. These data are relevant to ongoing translational research efforts exploring the chemotherapeutic potential of metformin.

Keywords: Metformin - ovarian cancer - apoptosis - Bcl 2 - Bax

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Introduction

Epithelial ovarian cancer (EOC) accounts for 80-90% in all ovarian cancers and is one of the most lethal gynecologic malignancies (Arikan et al., 2014; MhawechFauceglia et al., 2014). An estimated 1 out of 70 women is under life-threatening risk of EOC worldwide (Siegel et al., 2013). EOC is considered to be a silent killer because of its insidious onset and nonspecific early symptoms, thus it is always undiagnosable until advanced stages (Russell et al., 2014; Gasparri et al., 2015). Primary cytoreductive surgery aims at removing as much primary and metastatic tumor as possible in order to facilitate response to subsequent chemotherapy and improve survival (Gadducci et al., 2013; Bian et al., 2014). Currently, cisplatin and paclitaxel based chemotherapy combined with cytoreduction is the main clinical strategy for EOC, but is limited by high recurrence with a rate of approximately 70% and chemoresistance in platinum and paclitaxel resistant tumors (Rai et al., 2014, Zhang et al., 2015). Thus identifying novel prognostic and targeted therapeutics is needed for improving patient's survival (Sakarya et al., 2015) Hence, researchers want to unherald the potential of commonly used decades old antidiabetic drug metformin in cancer.

There is evidence that metformin results in (a) initiation of an LKB1-mediated AMPK-dependent energy stress response (b) inhibition of phosphoinositide 3-kinase/Akt/mammalian target of rapamycin signaling, leading to reduced proliferation of cancer cell lines, which has provided a molecular basis for a direct, insulin-independent antitumor effect and strengthened the rationale to evaluate metformin in cancer clinical trials (Aletti et al., 2007; Zakikhani et al., 2010).

Metformin is a biguanide, widely used for treating type 2 diabetes mellitus. It increases insulin sensitivity and improves glycemic control (Nathan et al., 2009; Nevadunsky et al., 2014). Accumulating evidence suggests that metformin possesses anticarcinogenic properties, and its use is associated with favorable outcomes in several cancers. Metformin association has shown lower prostate cancer recurrence in patients of type 2 diabetes mellitus (Hwang et al., 2015). Further it can reduce the risks of endometrial carcinoma and inhibit endometrial carcinoma

*[1]Department of Biochemistry, [2]Institute of Rotary and Cancer Hospital (IRCH), AIIMS, New Delhi, India *For correspondence: singh_neeta26@rediffmail.com, singh_neeta@hotmail.com*

cells growth involving PI3K/Akt pathway (Goodwin et al., 2008; Pollak., 2010; Xie et al., 2011; Emami et al., 2013; Zhang et al 2015). Moreover, researchers think that metformin prevents lung cancer caused by smoking by reducing IGF-1 and insulin levels by suppressing mTOR which is a protein that promotes the growth of lung cancer cells (Leone et al., 2014). Further detailed analyses and clinical trials will be necessary to identify the specific molecular mechanisms, and to determine whether metformin would have similar effects *in vivo*. We have tried to explore this mechanism in ascitic fluid of ovarian carcinoma patients in this study. Metformin causes cell cycle arrest on MCF-7 Breast Cancer Cells *in vitro* (Topcul et al., 2015), improves the prognosis of patients with Pancreatic Cancer (Zhang et al., 2015).

Similarly metformin has shown promising effect in ovarian cancer. Metformin intake is associated with better survival in ovarian cancer (Kumar et al., 2013), reduces ovarian cancer risk in Taiwanese women with type 2 diabetes mellitus (Tseng et al., 2015), targets ovarian cancer stem cells *in vitro* and *in vivo* (Shanka et al., 2012), limits the adipocyte tumor promoting effect on ovarian cancer (Tebbe et al., 2014), prevents aggressive ovarian cancer growth driven by high energy diet (Wahab et al., 2015). Moreover, metformin inhibits ovarian cancer growth and increases sensitivity to paclitaxel in mouse models (Lengyel et al., 2015).

Basic and translational (or preclinical) ovarian cancer research has traditionally been conducted with a repertoire of immortalized cell lines, which have homogeneous phenotypes and have adapted to long-term tissue culture (Niranjan et al., 2013). However, most have acquired significant genetic aberrations from their cells of origin and hence poorly represent the diversity, heterogeneity and drug-resistant tumors occurring in patients and multidrug resistant cell lines (Wang et al., 2013). The derivation and short-term culture of primary cells from solid tumors have thus gained significant importance in personalized cancer therapy. These well characterized primary tumor cells redefine cancer therapies with high translational relevance (Mitra et al., 2013). Primary cell culture provides a model system that allows a broader spectrum of cell types from a greater number of patients to be studied in the absence of artificially induced genetic mutations. Nevertheless, it can be technically challenging even for laboratories experienced in immortalized cell culture (Niranjan et al., 2013).

The effects of an anticancer drug on tumor cells are dependent on not only its inhibition of tumor cell proliferation, but also the induction of apoptosis. Apoptotic cell death is often associated with distinctive characteristics, such as nuclear fragmentation, cytoplasmic blebbing, and nucleosomal fragmentation of DNA (Gupta et al., 2013; Wang et al., 2015). Emerging evidence suggests that deregulated programmed cell death or apoptosis is a major contributor to tumor initiation, progression and development of acquired resistance to anticancer therapies. Therefore, therapeutic manipulation of the apoptotic pathways may be an attractive avenue to improve the clinical response of ovarian cancer patients (Hanahan et al., 2010).

We therefore decided to evaluate whether metformin could stimulate apoptosis in primary cultures established from ascitic fluid. We also sought to identify the pathways involved in this effect.

Materials and Methods

All ovarian cancer ascitic samples for establishing primary ovarian cell cultures were obtained after signed informed consent and Institutes ethical committee approval from IRCH, AIIMS. Patient ascites was obtained from epithelial Ovarian cancer patients at the time of diagnostic laparotomy. All ascites samples were obtained from chemotherapeutically naive patients with Stage III epithelial ovarian carcinoma.

Ovarian cancer cell isolation and culture

Primary human OC cells were isolated from patient ascitic fluid. Establishment of culture was attempted only when grape like clusters of cells, characteristic of ovarian cancer cells culture, was seen under the microscope. Ascitic fluid usually contains a large number of red blood cells; however, they do not interfere with cell plating. MCDB 105 medium and Medium 199 (Sigma Aldrich, USA) were taken in the ratio 1:1 containing 10% FCS and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin) to culture the ovarian cancer cells. The ascitic fluid (200ml) was centrifuged at 8000rpm for 10 minutes at 4^0C. Then the pellet was suspended in 15ml of ascitic fluid and 15 ml of MCDB 105 medium and medium 199 media and incubated in 75 mm^2 flasks in a humidified atmosphere of 5% CO_2 at 37^OC. After 4 days the media was removed and the cells washed with PBS. The clusters of ovarian cancer cells attach to the flask surface. The cells were maintained in the above mentioned media till they grew to 80% confluency. The cells were then trypsinized and sub-cultured (Dunfield et al., 2002).

We were able to establish 15 primary cultures out of 30 samples. Ovarian cancer cells have a limited growth potential (6-8 passages) and will eventually exhibit the flattened appearance of senescent cells that fail to divide. Therefore most experiments were conducted when the cells were at a low passage number (4-8). The experiments were conducted in triplicate.

MTT assay for cell viability/proliferation

The doses of Metformin were determined using MTT assay. Briefly, $5x10^4$cells/well were plated in 96-well culture plates. After overnight incubation, the cells were treated with varying concentrations of metformin for 24, 48, 72 hours. The cells were incubated with 100 μl of 5 mg/ml MTT for 4hrs at 37^0C. The formazan crystals thus formed were dissolved in DMSO and the absorbance was measured at 570nm using an ELISA reader and 620nm as the reference wavelength. The doses of 3mM metformin for 48 hrs duration were used for treatment of after standardization.

Flow cytometry

Ascitic ovarian cancer cells were treated with 3mM Metformin for 48 hrs, and then harvested. 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 and cell cycle analysis, after collection and washing, the 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 (BD Facs, USA) using Win Mdi 2.9 software.

Protein extraction and Western blot analysis

Cells were lysed in RIPA buffer (25 mMTris–HCl pH 7.6, 150 mMNaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail tablets (G biosciences, USA). Determination of total protein content was done according to the Bradford method. Briefly, clarified protein lysates (60-100µg) were resolved electrophoretically on 10%-15% denaturing SDS polyacrylamide gels and transferred to nitrocellulose membranes. After blocking in 5% milk, membranes were probed with the primary antibodies specific to Bcl-2, Bax and β-actin. Immunobloted proteins were visualized using Alkaline Phosphatase conjugated secondary antibodies. Final detection was performed with BCIP/NBT substrate (Promega, USA). Appropriate positive and negative controls were run simultaneously. The bands were analyzed and quantitated using Alphaimager scanning densitometer (Alpha Innotech, USA). The protein expression is expressed in Relative Units (RU).

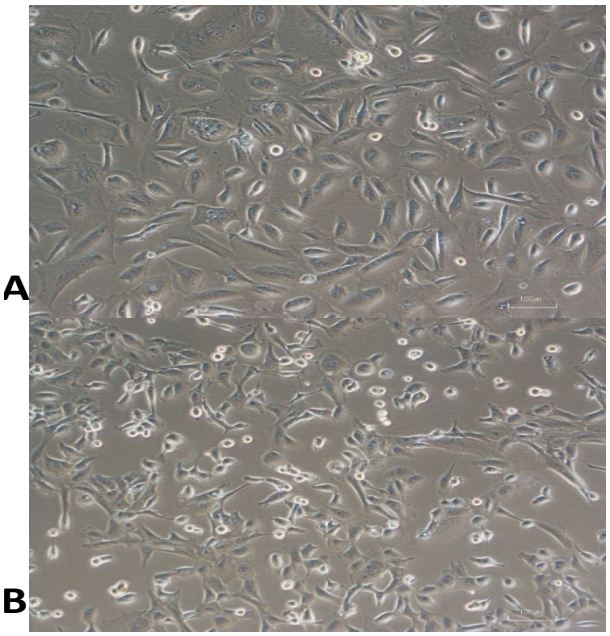

Figure 1. (A): Establishment of Primary Culture from Ascitic Fluid of Ovarian Cancer Patients (Representative Experiment is shown, 10 X magnification). (B): Metformin Treatment of Ovarian Cancer Cells

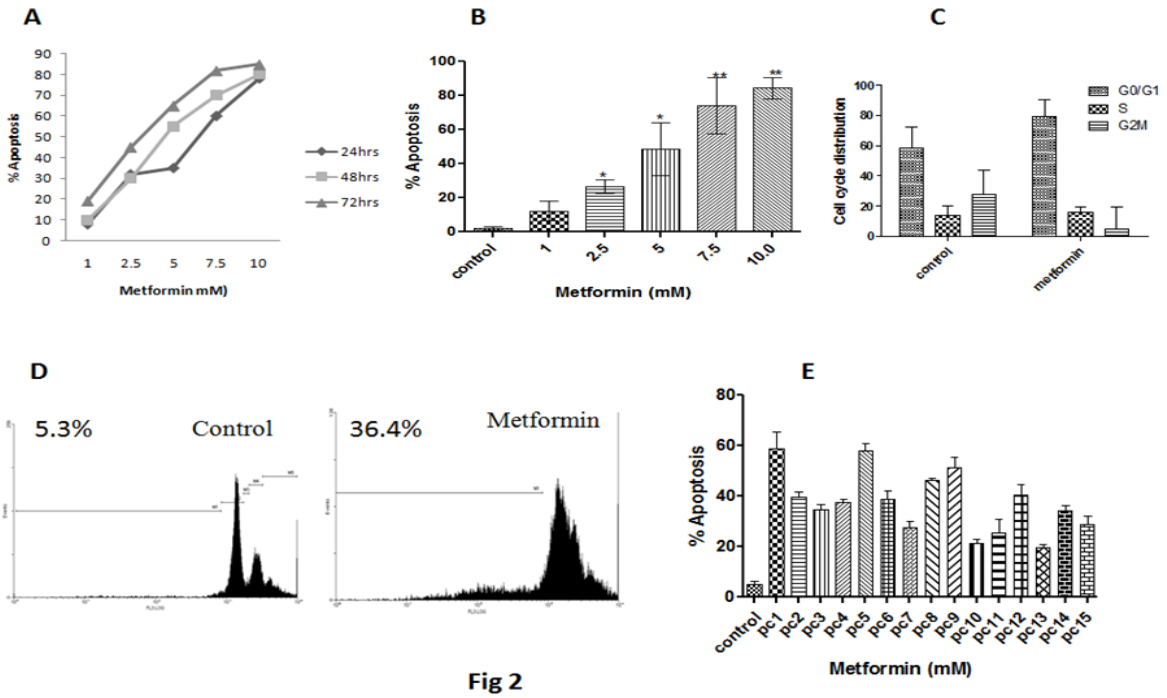

Figure 2. (A): Representative Experiment Showing dose Response Curve with Various doses of Metformin for 24, 48 and 72 hrs. (B): Dose-dependent effect of metformin on cellular apoptosis. Results represent the mean % apoptosis of three independent experiments of one primary culture. The doses were standardized for metformin 3 mM for 48hrs for further experiments. (C): Primary ovarian cancer cells were incubated with 3 mM metformin for 48 hrs and cell cycle distributions were analyzed by flow cytometry. One representative experiment out of 15 primary cultures is shown. (D): Representative experiment showing flowcytometric analysis for percentage apoptosis in control and treated primary ovarian cancer cells. The horizontal margin drawn from Y axis to peak represents % of apoptotic cells. (E): Mean percentage apoptosis induced in control and treated primary ovarian cancer cells as measured by flowcytometry. The diagram represents mean ±SD of 15 primary cultures. *P<0.05 versus control, **P<0.01 versus control, ***P<0.001versus control

The density of the control was taken as 1 and the results of treatments were expressed in relation to the control.

Statistical analysis

All values are expressed as mean±SD (standard deviation). For multiple comparisons, data were analyzed by one-way ANOVA test followed by the Post hoc Bonferroni test. P<0.05 was considered significant. All statistical analyses were carried out using Graph Pad Prism v.5 software.

Results

Establishment of primary culture from ascitic fluid of ovarian cancer patients

Small clusters of ovarian cancer cells freshly isolated from an ascitic fluid were seen. A colony of ovarian cancer cells attached and started to spread on the culture flask after four days followed by outgrowth of ovarian cancer cells forming a cluster. The contaminating fibroblasts were removed after each passage. Confluent monolayer of epithelial ovarian cancer cells of typical epithelial cobblestone morphology were seen after 3-4 weeks (Figure 1A).

Metformin treatment inhibits cell viability

To examine the effects of metformin on cancer cell growth, we treated primary ovarian cancer cells with metformin. Cell viability was determined with increasing doses of metformin for 24, 48 and 72 hours and measured by MTT assay. As shown in Figure 2(A, B) metformin, inhibited cell viability, as related to control in a dose dependent manner. The dose of 3 mM metformin 48 hrs duration was used for treatment of ascitic ovarian cancer cells after standardization.

Effect of metformin on cell cycle

To further evaluate the mechanism of growth inhibition by metformin the cell cycle profile was analyzed by flowcytometry after treatment with metformin (Figure 2C). Majority of cells presented in the G1-phase of the cell cycle, and small part in the G2/M-phase and the rest of the cells were found to be in the S-phase in control cells. However metformin treatment resulted in an increase in number of cells in G0/G1 and S phase compared to untreated control cells. Thus it was seen that at the cellular level, metformin interferes with cell cycle leading to G0/G1 or S phase arrest.

Response of primary cultures of ovarian cancer cells to metformin

Out of the thirty ascitic fluid samples collected only fifteen primary cultures of ovarian cancer cells could be established. The cells were treated with metformin (3mM). This dose was established by doing a dose response curve and MTT assay. Morphological changes characterized by membrane blebbing and formation of apoptotic bodies were also observed. The control and treated cells were then analysed by flowcytometry (Figure 2D). As evident from bar diagrams (Figure 2E), there was a wide variation in response of individual primary cultures to metformin. On treatment with metformin in primary cultures % apoptosis was ranging from 19.3% - 58.8% compared to control.

Effect of metformin on pro-survival and anti-survival proteins of the Bcl-2 in primary ovarian cancer cells with metformin

We further, attempted to elucidate the pathway

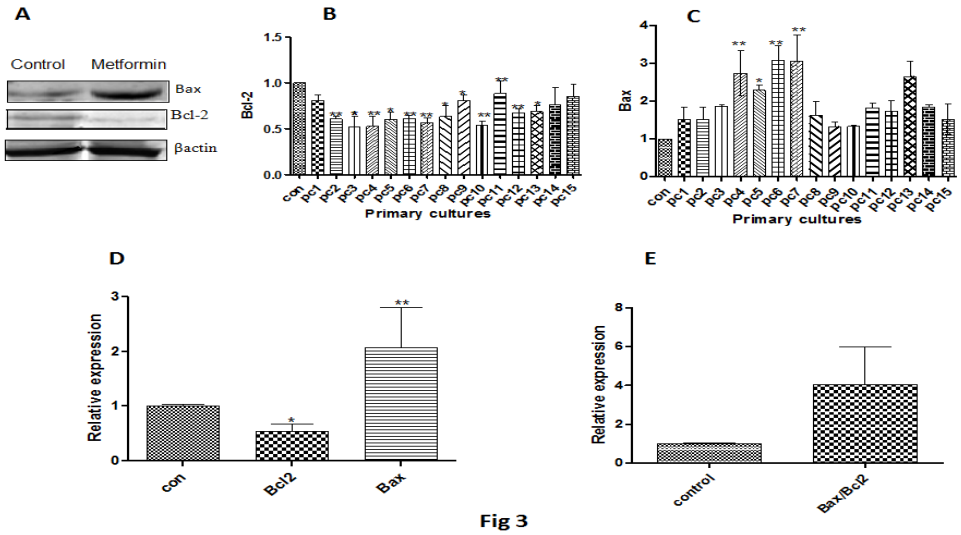

Figure 3. (A) Effect of Metformin on the Expression of Anti-apoptotic and Pro-apoptotic Proteins (Bcl-2(26 KDa), Bax (23KDa) and β Actin (42KDa) in Ovarian Cancer Cells Treated with 3 mM Metformin. Cell lysates were subjected to Western blot, one representative blot out of 15 is shown. Densitometric analysis of protein expression of (B): Bcl-2 (C) Bax in control and treated primary ovarian cancer cells culture (pc) as measured by western blot analysis. The bar diagram represents the mean of three independent experiments (D): Mean protein expression of Bcl2 and Bcl-xL in control and treated primary ovarian cancer cells as measured by western blot analysis. The bar diagram represents the mean of 15 primary cultures (pc). (E): Bax/Bcl-2 ratio in primary ovarian cancer cells. The bar diagram represents the mean of 15 primary cultures (pc).*P<0.05 versus control, **P<0.01 versus control, ***P<0.001versus control

involved in induction of apoptosis by metformin. To do so, we measured protein expression of the Bcl-2 and Bax (Figure 3A) in the presence of metformin (3mM). We observed decrease in protein expression of Bcl-2 and increasing expression of Bax in primary ovarian cancer cells (pc) (Figure 3B & 3C). The protein expression of Bcl-2 decreased by 1.8 fold and Bax increased by 2.1fold respectively (Figure 3D) compared to control resulting in increase in Bax/Bcl-2 ratio (Figure 3E).

Discussion

Despite the significant advances in our understanding of various aspects of cancer initiation, progression and metastasis we have achieved limited clinical success. Consequently, there is a great need to develop improved upfront and salvage therapies for ovarian cancer. Preclinical studies with cancer cell lines have played an important role in our understanding of tumor biology and high throughput screening for drug development. However, genetically altered cancer cell lines under *in vitro* condition do not truly represent clinical scenarios (Kirk et al., 2012). Thus it may be difficult to comprehend the genetic and epigenetic diversities of millions of patients from small number of cancer cell lines (Lima et al., 2010). These disparities in clinical responses and patient dependent tumor variability are the driving force behind personalized medicine and provide the impetus to develop methods of culturing primary tumor cells from patients that will enable effective bench to bed side translation (Mitsiades et al., 2011; Trusheim et al., 2011). More than one third of ovarian cancer patients present with ascites at diagnosis, and almost all have ascites at recurrence. The presence of ascites correlates with the peritoneal spread of ovarian cancer and is associated with poor disease prognosis. The ability to culture epithelial ovarian cancer cells from patients provides an important experimental system that has the potential to resemble the patient situation more accurately (Ahmed et al., 2013).

Metformin is one of the most commonly used medications for diabetes worldwide. It has been previously demonstrated that metformin has anticancer effects in ovarian cancer both *in vitro* and vivo (Gotlieb et al., 2008). We have seen in this study the apoptotic potential of metformin in primary ovarian cancer cells corroborating its anticancer role.

We have evaluated the effects of metformin on cell cycle distribution and progression. Concurrently, ovarian cancer cells were blocked in G0/G1 and S phases on exposure to metformin. Again, differences exist between studies regarding the effect of metformin on cell cycle distribution. 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) (Takane et al., 2008). The role of OCT1 in metformin uptake by ovarian cancer cells is unknown at the moment and is under investigation.

Data concerning the effect of metformin on apoptosis in cancer cells are limited and somewhat inconsistent. It has been seen that metformin blocked the cell cycle in the G0/G1 phase in prostate cancer cells and did not induce apoptosis (Sahra et al., 2010). Similarly, breast cancer cells did not undergo apoptosis in response to metformin (Alimova et al., 2009) whereas it stimulated apoptosis in pancreatic cancer cells. 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

Many antiapoptotic and proapoptotic genes are involved in apoptosis. When the ratio of pro-apoptotic Bcl-2 family members (Bax) to anti-apoptotic Bcl-2 family (Bcl-2, Bcl-xL) members increases, pores form in the outer mitochondrial membrane, liberating apoptogenic mitochondrial proteins to activate caspases and induce apoptosis. Consequently, we wanted 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 Bcl-2 anti-apoptotic proteins in cells treated with metformin (Figure 3A & 3B). Concomitantly, we have observed that the pro-apoptotic proteins, Bax are induced in the cells exposed to metformin (Figure 3A & 3C).

Most of the *in vitro* and preclinical studies have used doses ranging from 1-100mM (Zakikhani et al., 2010; Rattan et al., 2011). While this seems initially unattainable *in vivo*, metformin is not metabolized and has been found to be highly concentrated with the cells, especially within mitochondria. Thus, in order to achieve the concentrated cellular metformin levels and study the potential changes that occur *in vivo* over longer periods of treatment, high doses over short time courses must be used *in vitro*. However further studies with lower dose of metformin and longer treatment would be more relevant to mimic the doses in patients since primary culture does not proliferate like conventional ovarian cancer cell line and the effect might be better with longer time period.

Thus a detailed understanding of apoptotic mechanisms and factors that can compromise them is critical to design of more potent, specific and effective personalized cancer therapies (Chumworathayi et al., 2013). Futhermore it emanates the necessity to study metformin's chemoadjuvant potential.

In conclusion, in this study we have tried to offer a basis for further studies in primary ovarian cancer cells cultured from ascitic fluid to study the apoptotic potential of metformin. Primary cell cultures more closely mimic the physiological state of cells *in vivo* and generate more relevant data representing living systems. Hence, primary tumor cell lines as personalized strategies are essential requisite for assessing the effect of so-called tailor made adjuvant therapy.

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