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

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A B S T R A C T

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.

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

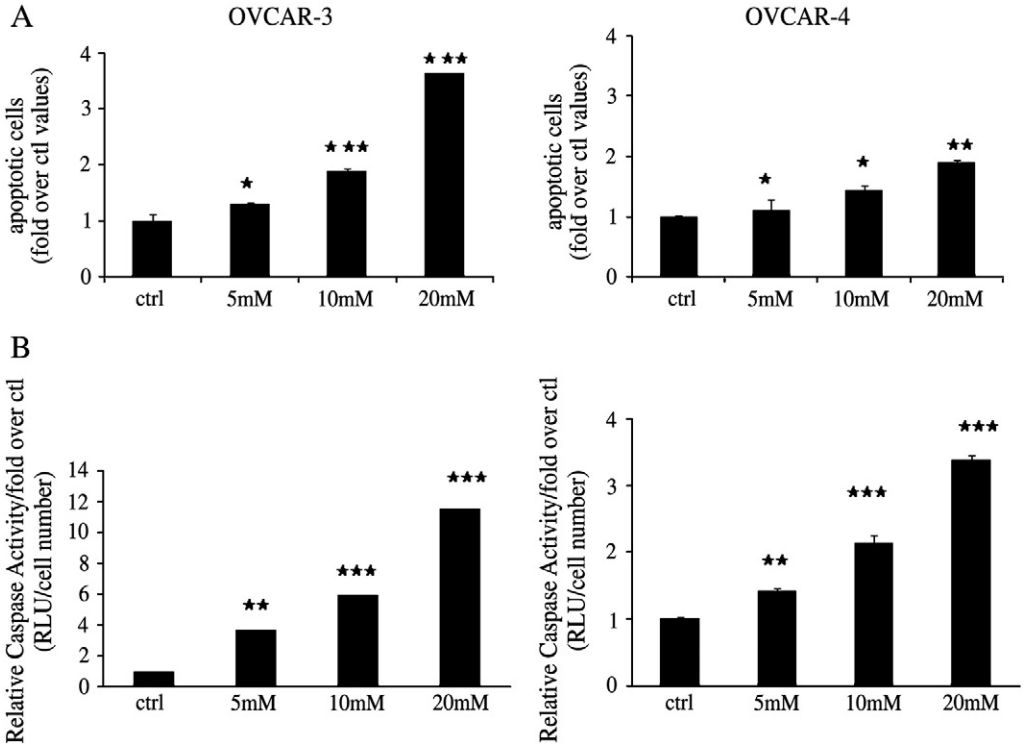

Fig. 1. Dose-dependent effect of metformin on (A) cellular apoptosis and (B) caspase-3/7 activity. OVCAR-3 and OVCAR-4 cells were incubated with 5, 10 and 20 mM metformin for 72 h in 1% FBS and (A) apoptosis levels were determined by flow cytometry. Results represent the mean of three independent experiments. (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

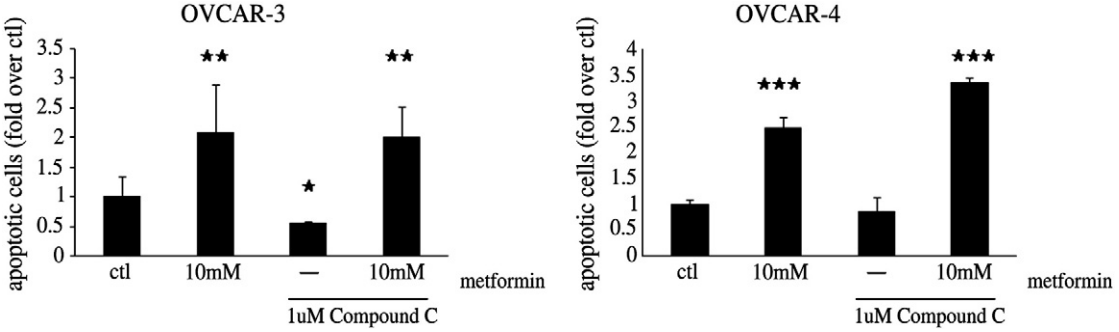

Fig. 2. Metformin induces apoptosis in an AMPK-independent manner in OVCAR-3 and OVCAR-4 cell lines. Cells were incubated for 72 h in 1% FBS in the presence of 10 mM metformin and/or 1 μM compound C and apoptosis levels assessed by flow cytometry. Results represent the mean of three independent experiments.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

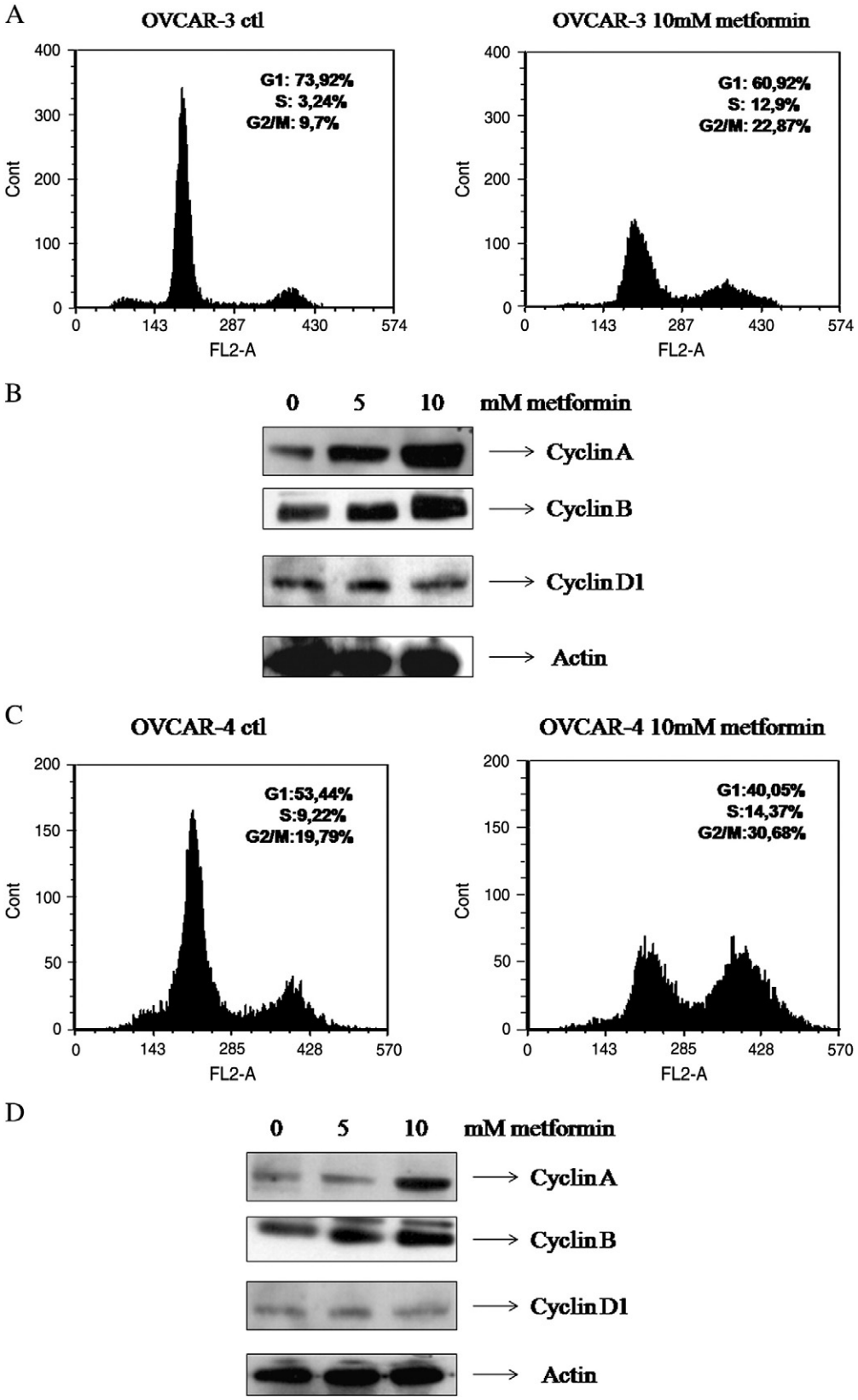

Fig. 3. Metformin blocks cell cycle progression in S phase. (A) OVCAR-3 and (C) OVCAR-4 cell lines were incubated with or without 10 mM metformin for 72 h in 1% FBS and cell cycle distributions were analyzed by flow cytometry. OVCAR-3 (B) and OVCAR-4 (D) cell lysates were subjected to Western blot for 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

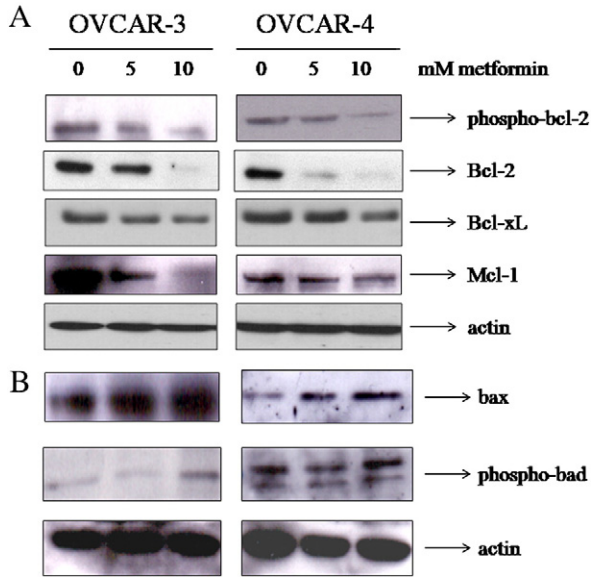

Fig. 4. Metformin inhibits anti-apoptotic (A) and induces pro-apoptotic (B) proteins from the bcl-2 family in a dose-dependent manner. OVCAR-3 and OVCAR-4 cells were treated with 10 mM metformin for 72 h in 1% FBS. Cell lysates were subjected to Western blot for phospho-AMPK, AMPK, phosphor-bcl-2, bcl-2, 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].

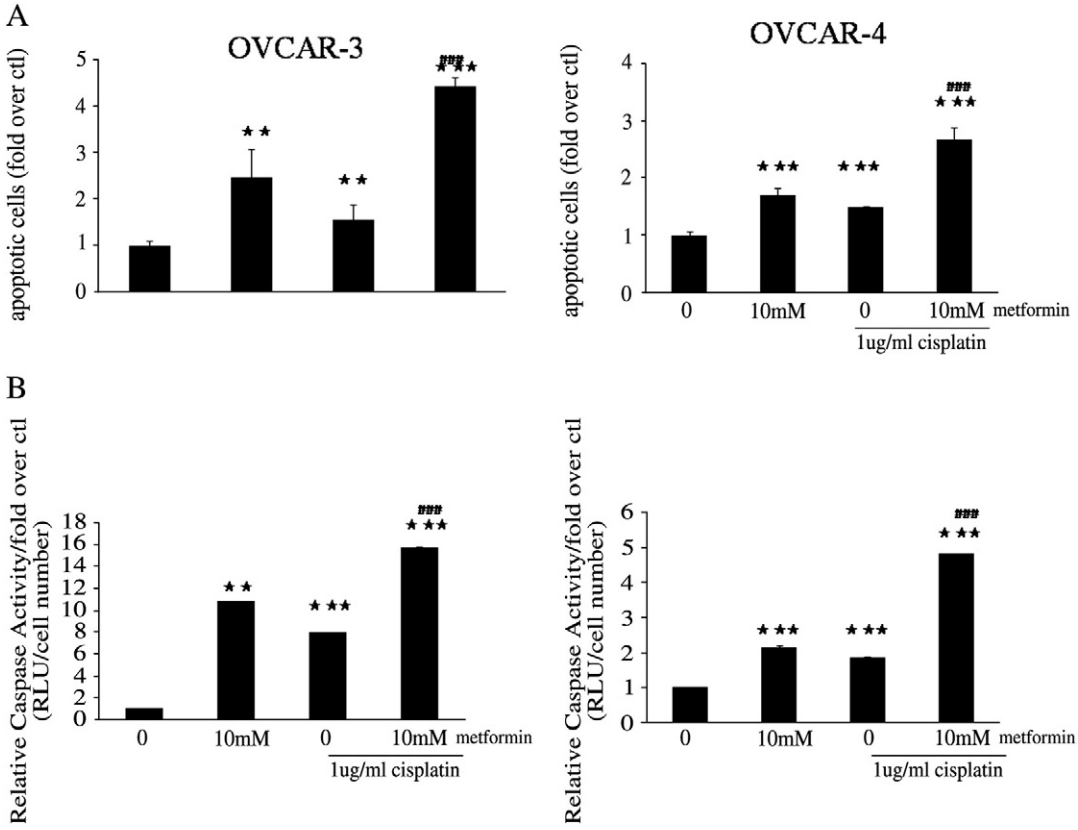

Fig. 5. Combination effect of metformin and cisplatin on (A) cellular apoptosis and (B) caspase-3 activity. OVCAR-3 and OVCAR-4 cells were incubated in the presence of 10 mM metformin and/or 1 μg/ml cisplatin for 48 h in 1% FBS and (A) apoptosis levels were determined by flow cytometry. Results represent 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.

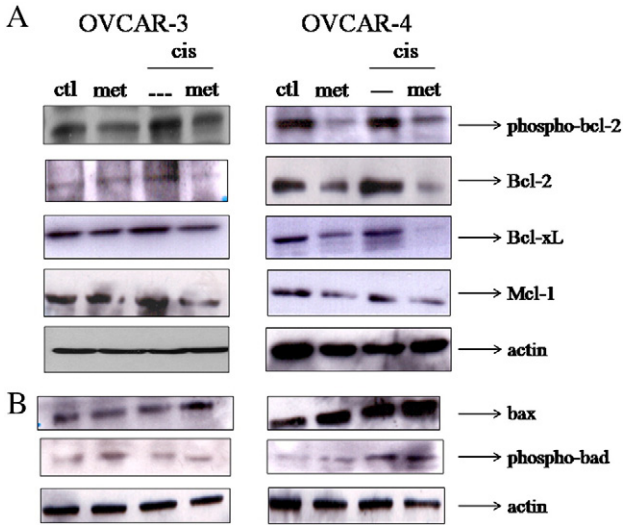

Fig. 6. Combination effect of metformin and cisplatin on the expression of anti-apoptotic (A) and pro-apoptotic (B) proteins from the bcl-2 family. OVCAR-3 and OVCAR-4 cells were treated with 10 mM metformin and/or 1 μg/ml cisplatin for 72 h in 1% FBS. Cell lysates were subjected to Western blot for 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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References

[1] Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71–96.
[2] Chien JR, Aletti G, Bell DA, Keeney GL, Shridhar V, Hartmann LC. Molecular pathogenesis and therapeutic targets in epithelial ovarian cancer. J Cell Biochem 2007;102:117–29.
[3] Aletti GD, Gallenberg MM, Cliby WA, Jatoi A, Hartmann LC. Current management strategies for ovarian cancer. Mayo Clin Proc 2007;82:751–70.
[4] Bowker SL, Majumdar SR, Veugelers P, Johnson JA. Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin. Diab Care 2006;29:254–8.
[5] Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. BMJ 2005;330:1304–5.
[6] Libby G, Donnelly LA, Donnan PT, Alessi DR, Morris AD, Evans JM. New users of metformin are at low risk of incident cancer: a cohort study among people with type 2 diabetes. Diab Care 2009;32:1620–5.
[7] Jiralerspong S, Palla SL, Giordano SH, Meric-Bernstam F, Liedtke C, Barnett CM, et al. Metformin and pathologic complete responses to neoadjuvant chemotherapy in diabetic patients with breast cancer. J Clin Oncol 2009;27:3297–302.
[8] Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, et al. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science 2005;310:1642–6.
[9] Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 2001;108:1167–74.
[10] Fruman DA, Edinger AL. Cancer therapy: staying current with AMPK. Biochem J 2008;412:e3–5.
[11] Hadad SM, Fleming S, Thompson AM. Targeting AMPK: a new therapeutic opportunity in breast cancer. Crit Rev Oncol Hematol 2008;67:1–7.
[12] Wang W, Guan KL. AMP-activated protein kinase and cancer. Acta Physiol (Oxf) 2009;196:55–63.
[13] Gotlieb WH, Saumet J, Beauchamp MC, Gu J, Lau S, Pollak MN, et al. In vitro metformin anti-neoplastic activity in epithelial ovarian cancer. Gynecol Oncol 2008;110:246–50.
[14] Frenzel A, Grespi F, Chmelewskij W, Villunger A. Bcl2 family proteins in carcinogenesis and the treatment of cancer. Apoptosis 2009;14:584–96.
[15] Yip KW, Reed JC. Bcl-2 family proteins and cancer. Oncogene 2008;27:6398–406.
[16] Marone M, Scambia G, Mozzetti S, Ferrandina G, Iacovella S, De Pasqua A, et al. bcl-2, bax, bcl-XL, and bcl-XS expression in normal and neoplastic ovarian tissues. Clin Cancer Res 1998;4:517–24.
[17] Skirnisdottir I, Seidal T, Gerdin E, Sorbe B. The prognostic importance of p53, bcl-2, and bax in early stage epithelial ovarian carcinoma treated with adjuvant chemotherapy. Int J Gynecol Cancer 2002;12:265–76.
[18] Kassim SK, Ali HS, Sallam MM, Fayed ST, Seada LS, abd-Elkawy E, et al. Increased bcl-2 expression is associated with primary resistance to chemotherapy in human epithelial ovarian cancer. Clin Biochem 1999;32:333–8.
[19] Anderson NS, Bermudez Y, Badgwell D, Chen R, Nicosia SV, Bast Jr RC, et al. Urinary levels of Bcl-2 are elevated in ovarian cancer patients. Gynecol Oncol 2009;112:60–7.
[20] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[21] Berenbaum MC. Criteria for analyzing interactions between biologically active agents. Adv Cancer Res 1981;35:269–335.
[22] Silber JH, Rosenbaum PR, Polsky D, Ross RN, Even-Shoshan O, Schwartz JS, et al. Does ovarian cancer treatment and survival differ by the specialty providing chemotherapy? J Clin Oncol 2007;25:1169–75.
[23] Herzog TJ, Pothuri B. Ovarian cancer: a focus on management of recurrent disease. Nat Clin Pract Oncol 2006;3:604–11.
[24] Dilman VM. Age-associated elevation of hypothalamic, threshold to feedback control, and its role in development, ageine, and disease. Lancet 1971;1:1211–9.
[25] Rattan R, Giri S, Hartmann L, Shridhar V. Metformin attenuates ovarian cancer cell growth in an AMP- kinase dispensable manner. J Cell Mol Med 2009.
[26] Ben Sahra I, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auberger P, et al. The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. Oncogene 2008;27:3576–86.
[27] Alimova IN, Liu B, Fan Z, Edgerton SM, Dillon T, Lind SE, et al. Metformin inhibits breast cancer cell growth, colony formation and induces cell cycle arrest in vitro. Cell Cycle 2009;8:909–15.
[28] Wang LW, Li ZS, Zou DW, Jin ZD, Gao J, Xu GM. Metformin induces apoptosis of pancreatic cancer cells. World J Gastroenterol 2008;14:7192–8.
[29] Liu B, Fan Z, Edgerton SM, Deng XS, Alimova IN, Lind SE, et al. Metformin induces unique biological and molecular responses in triple negative breast cancer cells. Cell Cycle 2009;8:2031–40.
[30] Ben Sahra I, Laurent K, Giuliano S, Larbret F, Ponzio G, Gounon P, et al. Targeting cancer cell metabolism: the combination of metformin and 2-deoxyglucose induces p53-dependent apoptosis in prostate cancer cells. Cancer Res 2010;70:2465–75.
[31] Isakovic A, Harhaji L, Stevanovic D, Markovic Z, Sumarac-Dumanovic M, Starcevic V, et al. Dual antiglioma action of metformin: cell cycle arrest and mitochondria-dependent apoptosis. Cell Mol Life Sci 2007;64:1290–302.
[32] Cantrell LA, Zhou C, Mendivil A, Malloy KM, Gehrig PA, Bae-Jump VL. Metformin is a potent inhibitor of endometrial cancer cell proliferation—implications for a novel treatment strategy. Gynecol Oncol 2010;116:92–8.
[33] Takane H, Shikata E, Otsubo K, Higuchi S, Ieiri I. Polymorphism in human organic cation transporters and metformin action. Pharmacogenomics 2008;9:415–22.
[34] Kang MH, Reynolds CP. Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. Clin Cancer Res 2009;15:1126–32.
[35] Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science 1998;281:1322–6.
[36] Martin-Castillo B, Vazquez-Martin A, Oliveras-Ferraros C, Menendez JA. Metformin and cancer: doses, mechanisms and the dandelion and hormetic phenomena. Cell Cycle 2010;9:1057–64.
[37] Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. Biochem J 2000;348(Pt 3):607–14.