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Original Article

Antineoplastic activity of metformin on the A549 human lung carcinoma cell line in vitro

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Abstract: Background: To investigate the effect of metformin on proliferation of the A549 human lung carcinoma cell line and explore the possible mechanisms involved. Methods: A549 cells were exposed to metformin alone or in combination with cisplatin. Cell proliferation was measured by the MTT assay. Cell apoptosis and cell cycle stages were detected through flow cytometry. The Protein expression levels of Bcl-2 were evaluated through Western Blot analysis. Results: Metformin time- and dose-dependently inhibited the growth of A549 cells. Metformin potentiated the effect of cisplatin in vitro. Flow cytometric analysis showed that metformin can induce apoptosis in A549 cells, and arrest cell cycle at the G0/G1 phase. Western blot analysis demonstrated that metformin reduced the protein expression level of Bcl-2. Conclusion: Metformin significantly suppresses the growth of A549 cells and enhances cisplatin-induced cytotoxicity. Additional preclinical studies are being conducted to determine the applicability of metformin in the treatment of lung cancer.

Keywords: Metformin, cisplatin, lung carcinoma, proliferation, apoptosis

Introduction

Lung cancer is a common malignant tumor worldwide, and its morbidity and mortality increased annually. However, the therapeutic effects of surgery, chemotherapy, and radiotherapy are unsatisfactory.

Metformin (1,1-dimethylbiguanide hydrochloride) is commonly used to treat type 2 diabetes because of its hypoglycemic efficacy and safety. Aside from its hypoglycemic effect, metformin has been recently found capable of reducing the risk of cancer by inhibiting the growth of various tumor cells, including those of breast cancer, ovarian cancer, pancreatic cancer and other malignancies [1-4]. Research in the area of lung cancer cells has been relatively less common, and the anti-lung cancer mechanism of the drug remains unclear; thus, further research is necessary.

In this study, we explored the anti-neoplastic effect and underlying mechanism of metformin on the A549 lung carcinoma cell line in vitro. We also evaluated the effect of metformin in combination with cisplatin on A549 cells.

Materials and methods

Reagents and antibodies

Metformin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), MTT, propidium iodide, and RNA kinase were purchased from Sigma (USA). Cisplatin was obtained from the Limited Company of Howson (Jiangsu, China). Dimethyl sulfoxide (DMSO) was procured from Amresco (USA). Anti-Bcl-2 antibody, goat anti-mouse secondary antibody and goat anti-rabbit secondary antibody were purchased from Jinqiao Biotechnology Company (Beijing, China). All other reagents for Western blot analysis were procured from Volson Biotechnology Company (Xi'an, China).

Cell culture

A549 human lung cancer cells were purchased from the Experimental Animal Center of the Fourth Military Medical University. A549 cells were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS) in a humidified 37°C incubator supplemented with 5% CO_2.

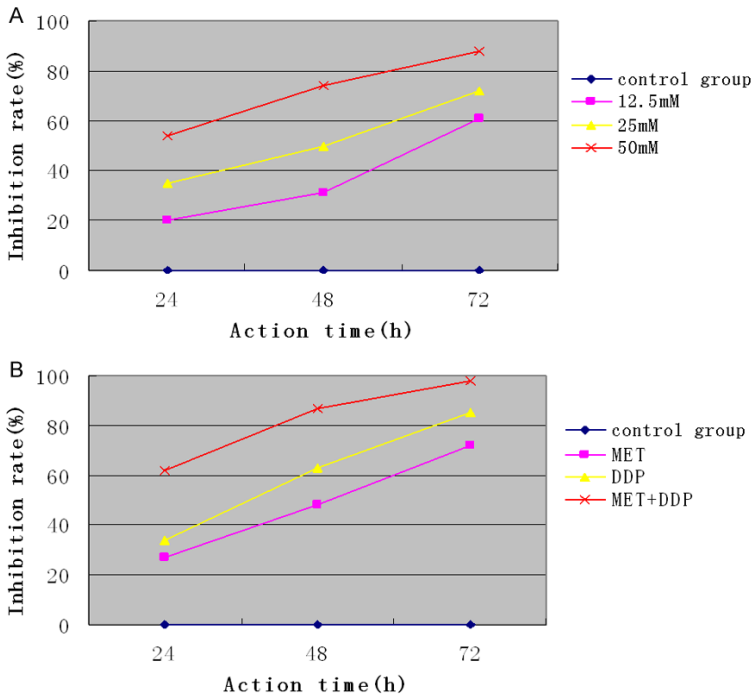

Figure 1. Metfromin inhibits A549 cell proliferation in vitro and potentiates cisplatin. A. A549 cells treated with different doses of metformin (0, 12.5, 25, and 50 mmol/L) for 24, 48, and 72 h. Metformin inhibits cell proliferation in a dose- and time-dependent manner ($P < 0.05$ vs control). B. A549 cells treated with metformin (24.5 mmol/L) and cisplatin (3.5 mg/ml) in combination or separately for 24, 48, and 72 h. Combined metformin and cisplatin treatment effectively inhibits cell proliferation ($P < 0.05$ vs monotherapy).

Proliferation assay

A549 cells at the logarithmic phase were harvested and resuspended at 4 × 10^4 cells/mL. The Cell suspensions were seeded into 96-well plates at 200 μL per well and then incubated overnight. Then, the cells were treated with metformin (0, 12.5, 25, and 50 mmol/L) or cisplatin (0, 2, 4, and 8 mg/mL) for 24, 48, and 72 h. After removing the medium, each well was added with 20 μL of fresh MTT solution and then incubated for another 4 h. Subsequently, each well was added with 150 μL of DMSO and the thoroughly shaken for 5 min to dissolve the crystals. The absorbance at 492 nm was determined using a DG3022-based Micro ELISA plate reader. A549 cells were treated with the IC50 of metformin alone or in combination with cisplatin for 24, 48, and 72 h, after which cell proliferation was evaluated. The cells in the control group were only supplemented with RPMI-1640 complete medium. The experiment was performed three times. The cell inhibition rate was calculated using the following formula: cell inhibition rate (%) = (OD of control cells-OD of treated cells)/(OD of control cells) × 100.

Analysis of cell cycle and apoptosis

Tumor cells (2 × 10^5 cells/2 mL) were seeded into six-well plates and cultured in RPMI-1640 without 10% FBS for 24 h. The medium was removed and replaced by a fresh culture medium containing 0 or 24.5 mmol/L metformin, and then incubated for another 48 h. After washing with PBS, the cells were fixed with ice-cold 70% ethanol at 4°C overnight. The cells were stained for total DNA content with 150 uL of PI/RNA kinase staining buffer for 30 min in the dark at room temperature. Cell cycle distribution was analyzed using a flow cytometer (Becton Dickinson, San Jose, CA, USA) and ModFit software. Apoptosis and necrotic cell death were analyzed by double staining with FITC-conjugated Annexin V and PI in accordance with the manufacturer's instructions. Apoptosis was analyzed through flow cytometry, and data were processed using Flow Jo software.

Western blot analysis

A549 cells were seeded into six-well plates at a density of 2 × 10^5 cells per well and the treated with 24.5 mmol/L metformin (IC50) for 24, 48, and 72 h. The cells were collected and then lysed in RIPA buffer (150 mM NaCl, 10 mM Tris-HCl, pH8.0, 1% NonidetP-40), 0.5% deoxycholic acid, 0.1% SDS, 5 mM EDTA) containing 0.7% phenylmethylsulfonyl fluoride, 0.2% aprotinin, 0.2% leupeptin, and sodium metavanadate. Samples (50 μg protein) were incubated at 100°C for 5 min, separated by 10% (w/v) SDS-PAGE gel electrophoresis, and then transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with a solution containing 5% nonfat milk powder in TBS/Tween20 (TBS/T) for 2 h at room tempera-

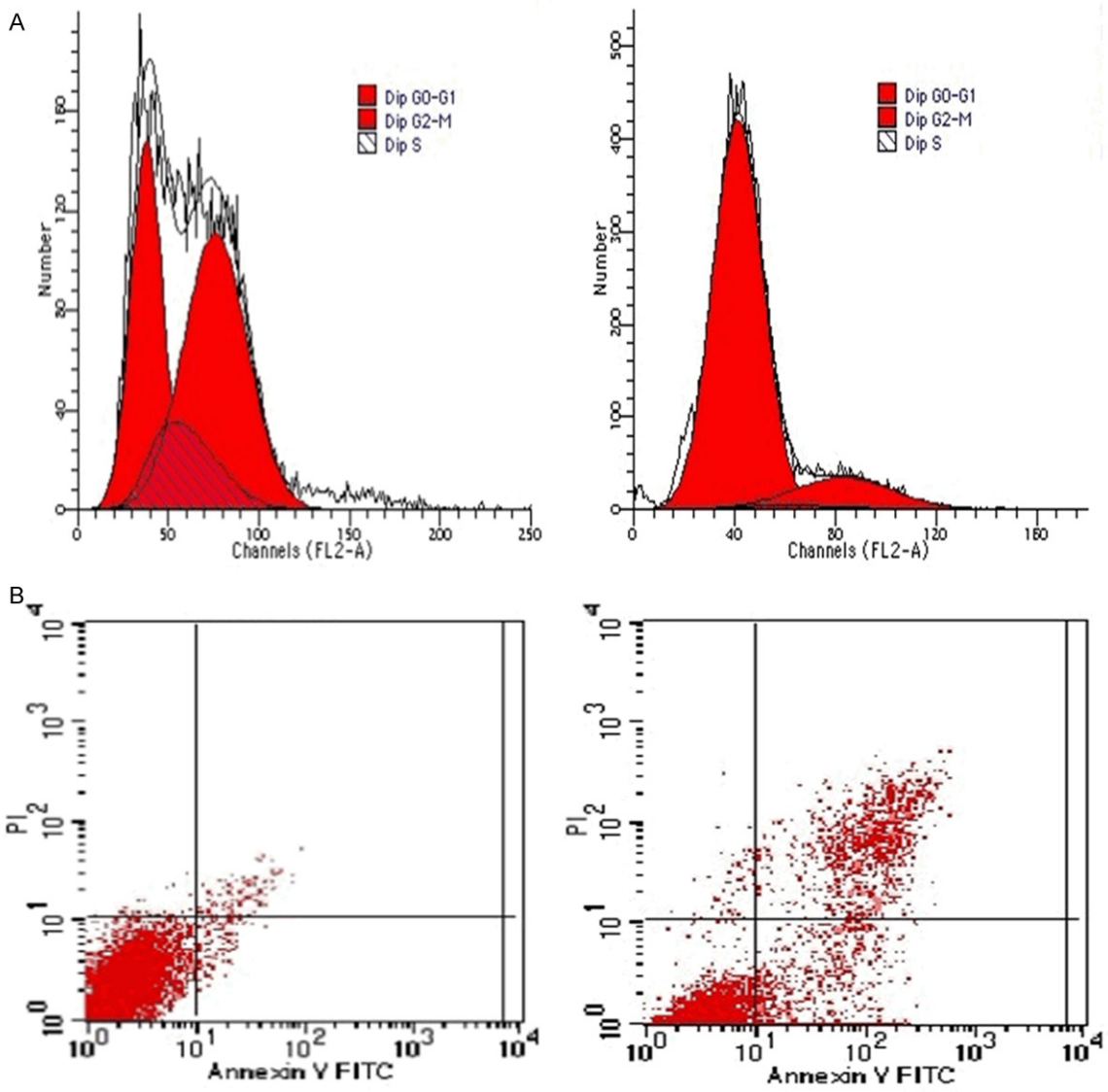

Figure 2. Effect of metformin on cell cycle profile and apoptosis was analyzed through flow cytometry. A. Metformin induced cell cycle arrest at the G1-phase and decreased the percentage of cells at the S, and G2/M phases (right). B. Metformin promoted cell apoptosis and increased the percentage of apoptotic cells (right).

ture and then hybridized with antibodies against β-actin or Bcl-2 in TBS/T containing 5% BSA at 4°C overnight. Subsequently, the membranes were probed with IRDye 800 CW goat anti-mouse secondary antibody or goat anti-rabbit secondary antibody at a dilution of 1:10 000 for 2 h at room temperature. The antibody-antigen complexes were detected with the Odyssey W Infrared Imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

Statistical analyses were performed using SPSS13.0. All data were presented as means ± SD ($\bar{x} \pm S$). Statistical differences among groups were assessed with one-way ANOVA followed by a post-hoc test. Comparisons between two groups were assessed using unpaired *t*-tests. Statistical significance was considered at $P < 0.05$.

Results

Metfromin inhibits A549 cell proliferation in vitro and potentiates cisplatin toxicity

Treatment with various concentrations of metformin (0, 12.5, 25, and 50 mmol/L) for 24, 48,

Table 1. Metformin causes cell cycle arrest at the G1-phase and induces the apoptosis of A549 cells

A. Treatment of metformin (24.5 mmol/L) for 48 h induced cell cycle arrested at the G1 phase

Group	G1 (%)	S (%)	G2/M (%)
Control	36.53±2.67	14.84±0.72	48.63±2.53
Metformin	82.75±1.44*	1.60±0.36*	15.65±1.62*

The percentages of cells in the metformin group were 82.75%, 1.60% and 15.65% at the G1, S and G2/M phases, respectively (*P < 0.01 *vs* control).

B. Cells treated with metformin (24.5 mmol/L) for 48 h enhanced apoptosis in A549 cells

Group	Apoptotic rate (%)
Control	4.13±1.06
Metformin	30.44±9.08*

The rate of cell apoptosis was 30.44% in the metformin group (*P < 0.01 *vs* control).

and 72 h inhibited the growth of A549 cells in a dose- and time- dependent manner. The differences between the control (0 mmol/L of metformin) and all experimental groups were significant (P < 0.05). The proliferation inhibition rate increased from 20% to 31% and 61% at 12.5 mmol/L, from 35% to 50% and 72% at 25 mmol/L, and from 54% to 74% and 88% at 50 mmol/L when the treatment time was prolonged from 24 h to 48 h and 72 h, respectively (**Figure 1A**).

We studied the inhibitory effect of combination treatment with metformin and cisplatin (24.5 mmol/L and 3.5 mg/ml, respectively) on A549 cells. The proliferation inhibition rate increased from 62% to 87% and 98% when the treatment time was prolonged from 24 h to 48 h and 72 h, respectively (**Figure 1B**). The combination of metformin and cisplatin exhibited significantly stronger inhibitory effects on tumor cell proliferation than metformin or cisplatin alone (P < 0.05) or than the control treatment (P < 0.05). No significant differences were noted between the cisplatin and metformin groups at the various incubation times (P > 0.05). These data demonstrate that metformin can increase the cytotoxicity of cisplatin to A549 cells.

Metformin induces G1 cell cycle arrest and apoptosis in A549 cells

The possible effects of metformin on cell cycle progression in A549 cells were examined by flow cytometry. Treatment with 24.5 mmol/L metformin for 48 h delayed the entry into the S phase of the cells and arrested them at the G1 phase (**Figure 2A**). compared with control (0 mmol/L of metformin), this treatment increased the percentage of cells at the G1 phase (82.75% VS 36.53%, *P < 0.01), but reduced cell percentage at the S phase (1.60% VS 14.84%, *P < 0.01) and G2/M phases (15.65% VS 48.63%, *P < 0.01) (**Table 1A**). To determine whether or not metformin induces apoptosis, cells were treated with 24.5 mmol/L metformin for 48 h. The percentage of apoptotic cells was determined through flow cytometry (**Figure 2B**). Results showed that metformin dramatically increased the percentage of apoptosis cells (30.44% vs 4.13%, *P < 0.01) (**Table 1B**).

Metformin induces the apoptosis of A549 cells by downregulating the protein expression of Bcl-2

To investigate the mechanism by which metformin induces apotosis in A549 cells, the total protein from tumor cells treated with saline solution (control group), cisplatin (3.5 mg/ml), metformin (24.5 mmol/L), or combined cisplatin and metformin (3.5 mg/ml and 24.5 mmol/L, respectively) for 48 h and the protein levels of apoptosis-related Bcl-2 were measured by Western blot analysis. The protein expression of Bcl-2 time-dependently decreased in the metformin, cisplatin, and combination treatment groups, among which the combination group clearly had the weakest expression. This result indicates that metformin can enhance the inhibitory effect of cisplatin (**Figure 3A**). The differences between the control and metformin groups were significant (P < 0.05) (**Figure 3B**).

Discussion

Metformin has been widely used as an antidiabetic agent for more than 50 years [5]. Metformin functions as an insulin sensitizer, mainly by activating the adenosine monophosphate-activated protein kinase (AMPK) signaling pathway to inhibit glycogen generation and output, increases muscle and adipose tissue glucose utilization, and reduces blood sugar levels [6-8]. Increased AMP levels activate AMPK, and intracellular energy sensor actuates ATP-requiring processes. A Previous study

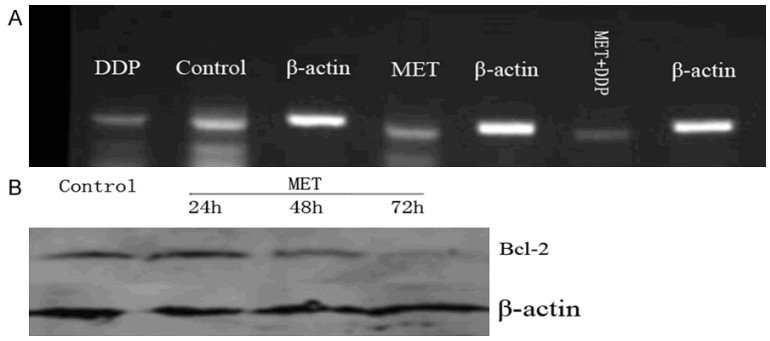

Figure 3. Effects of metformin on Bcl-2 protein expression in A549 cells. Total cell lysates were analyzed through Western blot analysis with antibodies against the Bcl-2 protein. A. Bcl-2 protein expression in different treatment groups. Treatment of A549 cells with saline solution (control group), metformin (24.5 mmol/L), cisplatin (3.5 mg/ml), or combined cisplatin and metformin (3.5 mg/ml and 24.5 mmol/L) for 48 h decreased Bcl-2 protein expression. B. Downregulation of Bcl-2 protein expression after metformin treatment. The effects became prominent with time ($P < 0.05$ vs control). β-actin was used as the loading control.

demonstrated that AMPK is involved in tumor cell proliferation, apoptosis, p53-p21 pathway activation, and cell cycle arrest at the G1 phase. These results indicate that metformin may have antitumor effects [9]. Several clinical studies have shown that metformin treatment of patients with type 2 diabetes can reduce the incidence of breast, ovarian, pancreatic cancers, among others [10, 11]. Recent studies have shown that metformin can inhibit the growth of various tumor cells in vitro, including breast, prostate, lung, and colon cancer cells [1-3]. Rattan et al. demonstrated that metformin treatment blocks the proliferation, protein synthesis, angiogenesis, and metastasis of ovarian cancer in vivo [12]. Hirsch et al. reported that Metformin selectively kills cancer stem cells [13]. Accumulative evidences have also suggested that metformin can enhance the cytotoxicity of chemotherapy drugs, such as cisplatin, etoposide and doxorubicin [14-16]. The present study demonstrated that metformin can inhibit the proliferation of A549 cells in a time- and dose-dependent manner, and that its combination with cisplatin can exert a synergistic anticancer effect. The results of previous studies coincided with the present findings. Another study showed that metformin combined with gefitinib or erlotinib produces a synergistic inhibitory effect on the proliferation, migration and invasion of cell lines resistant to TKIs in vitro and in vivo [17].

Cell cycle is an important process in the proliferation of malignant cells. A previous study reported that metformin can inhibit the proliferation of prostate cancer cells by decreasing CyclinD1 expression and inhibiting cell cycle progression [18]. In the present study, the results of flow cytometry showed that treatment with or without metformin after 48 h significantly increased the proportion of G1 phase cells and significantly decreased that of S and G2/M phase cells. The regulatory effect of metformin on A549 cell cycle mainly occurs to prevent the entry of these cells into S phase and cause cell cycle arrest at the G1 phase, thereby inhibiting the DNA synthesis of lung cancer cells. These results agree with the findings of Xiong et al. [19].

Thus far, the Bcl-2 gene is the most studied apoptosis suppressor gene, which encodes a membrane-integrated protein [20]. According to their function and structure, members of the Bcl-2 gene family, are divided into two categories: Anti-apoptotic proteins include Bcl-2, Bcl-xl, Bcl-w, and Mcl-1; pro-apoptotic proteins include Bax, Bak, Bad, Bid, and Bim. Under normal circumstances, the organization of genes does not promote or limit expression. A shift in expression is caused by the high expression of the Bcl-2 gene in lung tissue, thereby inhibiting apoptosis and ultimately leading to tumor formation. In the present study, A549 cells were treated with or without metformin for 48 h. Flow cytometry results showed that the apoptotic rate of the metformin-treated cells was significantly higher than that of the control cells. Xiong et al. reported that treatment with metformin decreases the expression ofBcl-2 and Bcl-xl but increases that of Bax in Hep-G2 cells [19]. In the present study, the results of Western blot analysis showed that metformin decreased Bcl-2 protein expression in A549 cells after 24, 48, and 72 h of treatment. Thus, the rate of cell apoptosis increased, which is consistent with the results of flow cytometry.

Recent evidence has suggested that metformin induces cytotoxicity by decreasing the expres-

sion levels of thymidine phosphorylase and excision repair cross-Complementation1 in NSCLC cells; the involved molecular mechanisms may partially be linked to the attenuation of MEK1/2-ERK1/2 signaling by metformin [21]. Previous studies on breast and lung cancer revealed that the IGF-I pathway plays a critical role in the promotion of tumor cell growth and survival [22, 23]. Salani et al. demonstrated that Caveolin-1 is essential for the inhibitory effect of metformin on IGF-1-induced events in NSCLC cells [24]. Zhang et al. have found that metformin downregulates the secretion of IGF-1 and the expression of IGF-1R to suppress endometrial carcinoma cell growth involving the PI-3K/Akt pathway [25]. Furthermore, metformin induces apoptosis in a dose-dependent manner in human colorectal cancer cells by increasing the expression of p-AMPK and decreasing that of p-mTOR [26].

In summary, metformin significantly inhibits the growth of human lung carcinoma A549 cells and potentiates cisplatin activity. Metformin possibly exerts this inhibitory effect by inducing cell cycle arrest at the G0/G1 phase, downregulating Bcl-2 protein expression, and promoting cell apoptosis. Furthermore, metformin combinated with cisplatin exhibits a synergistic antiproliferative effect. This study provides novel insights into the treatment of lung cancer. However, the molecular mechanisms underlying the anticancer effects of metformin warrant further elucidation.

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Disclosure of conflict of interest

None.

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