The mechanism of treatment of multiple myeloma with metformin by way of metabolism

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Abstract

Introduction: Multiple myeloma (MM) is a malignant hematologic tumor. Although many new drugs are currently found to significantly improve the median survival, MM is still not curable due partly to drug resistance recurrence. Epidemiological studies have shown that patients with type 2 diabetes have a high risk of malignancy, and patients' treatment with metformin could reduce the risk of cancer as well as associated mortality.
Material and methods: We used chemotherapeutics – melphalan combined with metformin or the single drug – to treat RPMI8226 cells and used a series of tests to detect the drug sensitivity, apoptotic rate, DNA damage and the concentration of ATP. SPSS 17.0 was used to analyze the data.
Results: The inhibitory effect of melphalan on RPMI8226 cells was significantly increased after metformin was added ($p < 0.05$), and the inhibitory effect was enhanced with the increasing concentration of melphalan. The comet assay showed that metformin increased melphalan-induced DNA damage and increased the apoptotic rate from 12.7 ±2.8% to 18.8 ±1.5% ($p < 0.05$). In the ATP concentration test, the concentration of ATP in the tumor cells was significantly decreased from 0.42 ±0.01 μmol/l to 0.08 ±0.02 μmol/l ($p < 0.05$).
Conclusions: Metformin can promote DNA damage induced by melphalan and decrease the concentration of ATP in the process of repairing DNA damage to hinder the anti-apoptotic process of tumor cells, which showed the pesticide effect of the enhanced sensitivity of multiple myeloma cells to melphalan.

Key words: metformin, multiple myeloma, metabolism, synergy.

Introduction

Multiple myeloma (MM) is a proliferative disorder of malignant hematological tumor, accounting for 1% of malignancies in the USA [1]. Indications for treatment are based on end-organ damage including hypercalcemia, renal impairment, anemia, bone damage and other markers of active disease [2, 3]. Although with the application of novel agents (bortezomib, thalidomide, thalidomide, etc.) consistent improvements in progression-free survival (PFS) and overall survival (OS) have been made in past years, multiple myeloma is still a malignant disease that cannot be cured at present. Patients generally suffer from the complications and chemotherapy side effects, as well as the resistance of chemotherapy drugs and frequent relapse. Therefore it is necessary to explore new

treatments or novel approaches to reduce or alleviate the adverse effects of the treatment process.

Metformin is a well-known hypoglycemic agent and is widely used as a front-line drug in patients with type 2 diabetes after dietary care and motor control failure. Epidemiological studies and meta-analysis showed that patients with type 2 diabetes had a higher risk of developing malignancies (including MM) [4, 5]. But compared with patients treated with sulfonylureas and insulin drugs, patients with metformin had a lower risk of cancer and related mortality, and metformin exhibits potent and persistent antiproliferative effects in a variety of tumors, such as breast cancer [6], hepatocellular carcinoma [7], colorectal cancer [8], ovarian cancer [9], head and neck carcinoma [10], and prostate cancer [11]. Metformin has a synergic anti-cancer effect in combination with a variety of drugs, effectively inhibiting AKT and Mtorc1 phosphorylation and biotin MCL-1 expression [12] and GRP78 expression, as well as interference with autophagy [13]. Metformin affects the cell metabolism and energy supply. As the result of that, the main mechanism for the treatment of diabetes with metformin is to inhibit gluconeogenesis and to inhibit the uptake of glucose in the intestinal wall cells, thereby reducing the concentration of glucose in blood. Thereby metformin will thwart the growth or proliferation of tumor cells, finally leading to apoptosis. Researchers believe that biguanide drugs inhibit the expression of IGF-IR, and hinder the PI3K/AKT/MTOR pathway, altering the energy condition of cancer and eradicating the MM cells with dexamethasone in a synergistic effect [14]. Meanwhile it has been confirmed that metformin reduces the oxygen consumption of mitochondria by inhibiting respiratory complex I NADH, and sharply reduces the proportion of ATP/ADP, whose function will last even when metformin is cleared [15].

When we treat multiple myeloma with an alkylating agent, the chemical compound will result in DNA damage, the formation of cross-linking strands, and termination of cell replication. The impaired cells need to restore the DNA damage for survival with base repair, homologous recombination, cross-injury repair, nucleotide excision repair as well as other appropriate mechanisms. The research results of Petermann showed that it is necessary for ATP to participate in the course of DNA repair, indicating ATP reduction in the desired repairing cells [16]. The increase in cell apoptosis resulted from oligomycin hindering ATP synthesis [17]. Lyamzaev *et al.* also came to the conclusion ATP deficiency is one of the main factors leading to apoptosis and necrosis [18].

However, metformin affects the metabolism of tumor cells and breaks DNA repair by reducing the amount of ATP following apoptosis. An alkylating agent is a common reason to generate much DNA damage in the treatment course of MM patients. Then the hypothesis arises that metformin would increase synergistically the apoptosis of myeloma cells treated with melphalan by affecting ATP concentration, which would provide a more theoretical basis for metformin applied to the clinical treatment of multiple myeloma.

Material and methods

Material

The human multiple myeloma cell line RPMI8226 was kindly offered by Professor Jianfeng Zhou (Department of Hematology, Tongji Hospital of Huazhong University of Science and Technology), and the original cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI 1640 medium (Gibco, USA) containing 10% fetal bovine serum(Gibco, USA) at 37°C in a humidified atmosphere containing 5% CO_2. The medium was replaced every 2 days. Melphalan, DMSO, metformin and rotenone (Sigma, USA) were used to treat the cells with different concentrations. A CCK8 kit (Tongren, Japan) was used to detect the inhibitory effect of drugs on cell growth. A flow cytometry anti-kit (Beyotime, China) was applied to detect the apoptosis rete. An ATP bioluminescence test kit (Beyotime, China) was used to detected the concentration of ATP in cells. A comet experimental kit (Trevigen, USA) was applied to assess the extent of DNA damage in cells.

CCK-8 assay

The inhibitory rate of drugs was detected by CCK-8 assay. RPMI 8226 cells were seeded into 96-well culture plates (5×10^5 cells/well, 100 µl/well) and incubated for 24 h at 37°C and atmospheric conditions of 5% CO_2. The cells were treated with various concentrations of metformin (0 mmol/l, 1.25 mmol/l, 2.5 mmol/l, 5 mmol/l, 10 mmol/l, 20 mmol/l, 40 mmol/l) for 48 h and melphalan (0 µmol/l, 0.08 µmol/l, 0.4 µmol/l, 2 µmol/l, 10 µmol/l, 50 µmol/l, 250 µmol/l) for 24 h respectively. Finally we found that when the RPMI8226 cells were treated with metformin (10 mmol/l) and melphalan (2 µmol/l to 250 µmol/l), the growth inhibition of RPMI8226 cells was significantly enhanced, and the difference between concentrations was moderate. The cells were also incubated in the presence of metformin and melphalan together. At the end of the culture, the CCK-8 reagent (10 µl) was added to each well. After 4 h of incubation at 37°C , the OD value was read at 450 nm wavelength using a microplate reader (DTX880, Beckman, Germany). All experiments were repeated in triplicate.

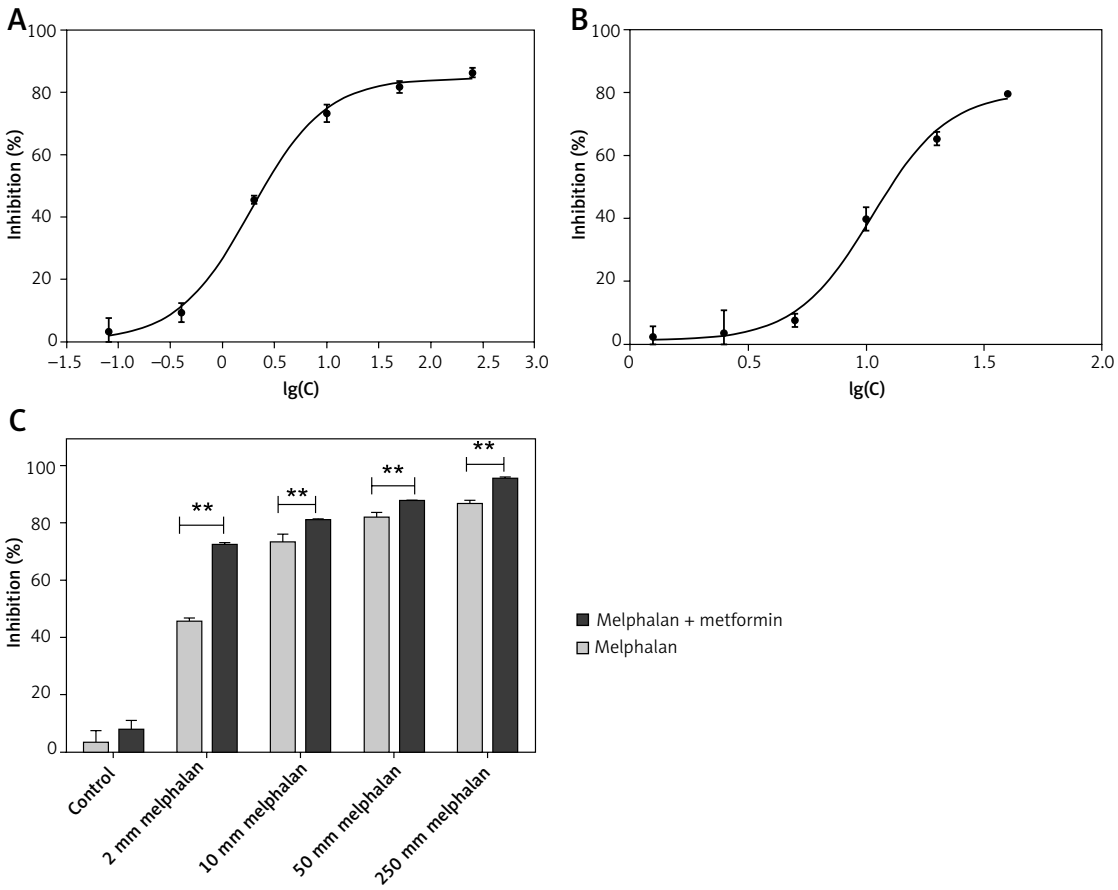

Figure 1. Metformin can inhibit synergistically RPMI 8226 cell proliferation with each concentration of melphalan. **A** – The anti-proliferative effects of melphalan on RPMI 8226 cell line treated with various concentrations (0.08 µmol/l to 250 µmol/l) of active substances. **B** – The anti-proliferative effects of metformin on RPMI 8226 cell line treated with various concentrations (1.25 mmol/l to 40 mmol/l) of active substances. **C** – RPMI 8226 cells were exposed to concomitant metformin (10 mmol/l) and various concentrations of melphalan (2 µmol/l to 250 µmol/l), or separate melphalan with various concentrations (2 µmol/l to 250 µmol/l). The cell viability was measured by CCK-8 assay. Lg (c) is the logarithm of the concentration of metformin to ten. The results are presented as mean ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test (**$p < 0.01$)

Apoptosis assay

RPMI 8226 cells were seeded in 6-well plates at 1×10^6 cells/well and cultured overnight. The cells were incubated with single melphalan (10 µmol/l), metformin (10 mmol/l) or rotenone (1 mmol/l) for 24 h, the first two particular concentrations of which were obtained through a CCK-8 experiment, showing about 50% of the growth inhibition effect in the presence of both, as well as the combination of melphalan with one of the two other drugs. At the end of the treatment, cells were harvested and washed with PBS once. Flow cytometry analysis of apoptotic cells was carried out using an Annexin V-FITC/PI staining kit. The cells were re-suspended in binding buffer (100 mmol HEPES, pH 7.4, 100 mmol NaCl, and 25 mmol $CaCl_2$) followed by staining with Annexin V-FITC/PI at room temperature in darkness for 15 min. Apoptotic cells were then evaluated by gating PI and Annexin V-positive cells in fluorescence activated cell-sorting flow cytometry [19].

Comet assay

The comet assay, a gel electrophoresis-based method that can be used to measure DNA double-strand breaks, was performed as previously described [20]. It is versatile, relatively simple to perform and sensitive. The cells were pretreated with melphalan (10 µmol/l) and melphalan combined with metformin (10 mmol/l) to induce DNA damage for 24 h, and then harvested and diluted into a 1×10^5 cells/ml cell suspension with ice frozen to stop the life activity. According to the protocol we mixed typically cell fluid with low melting agarose, then the compound was spread on a special slide and frozen slightly in the refrigerator for 10 min. Then the solidification was subjected to cracking, which was performed with a fresh preparation of precooling cracking liquid (pyrolysis liquid : DMSO – 9 : 1) to promote DNA double strand cleavage at the temperature of 4°C for 1 h, and alkaline electrophoresis for 30 min with the supply of 300 mA

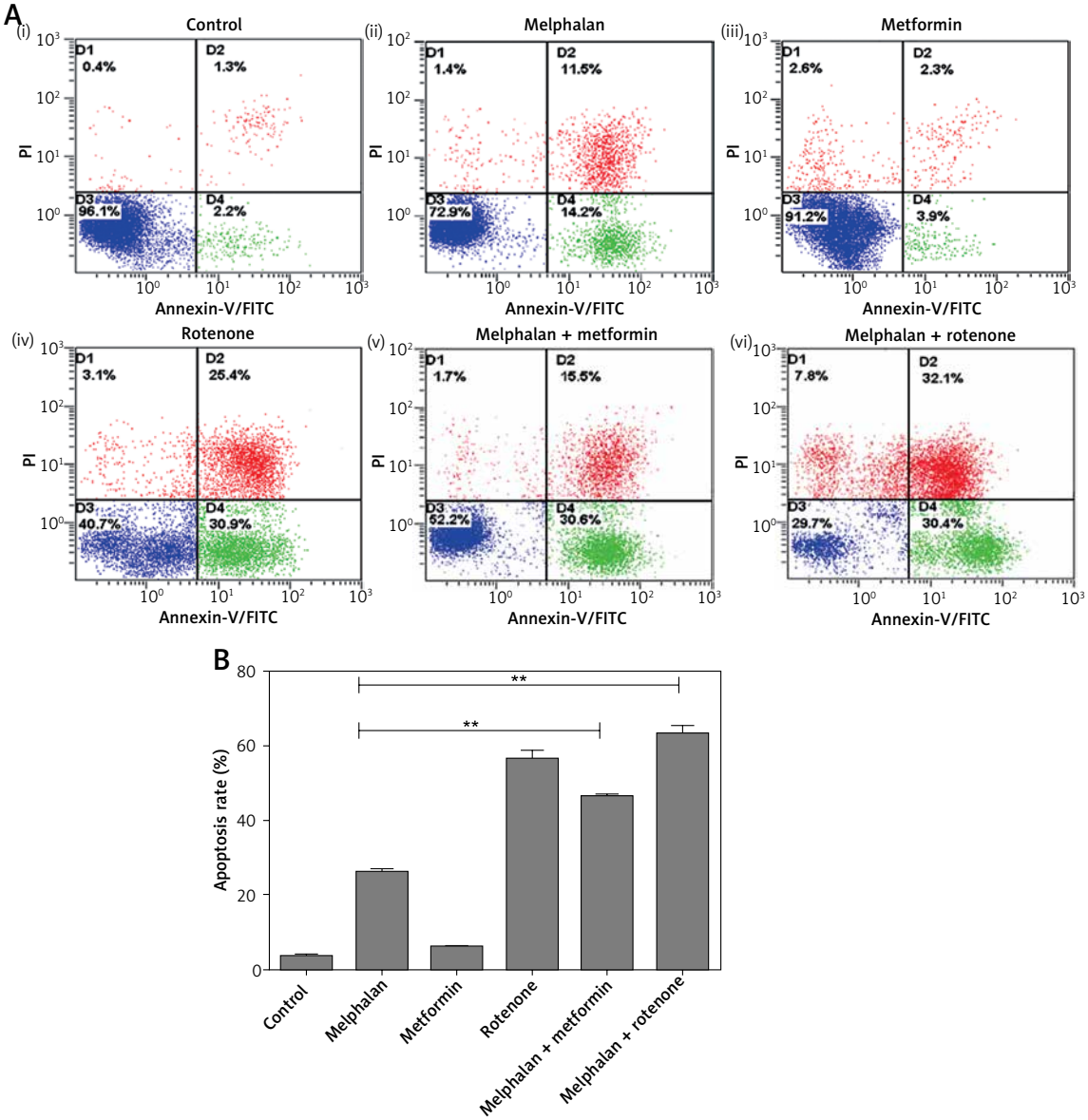

Figure 2. Metformin induced apoptosis combined with melphalan. **A** – i. Control; ii. Melphalan; iii. Metformin; iv. Rotenone; v. Melphalan + metformin; vi. Melphalan + rotenone. **B** – Comparison of apoptosis rates in each group. Cell apoptosis was analyzed by Annexin V-FITC/PI assay. The results are presented as mean ± standard deviation (SD). Statistical analysis was performed using Student *t*-test (**$p < 0.01$)

constant current power. After that the compound slide was washed three times with 0.4 mmol/l Tris-HCl, and placed into gradient alcohol for dehydration. The final specimen was stained with PI (20 µl) for 10 min in darkness. Subsequent to thorough washing in running water for 20 min, the slide was photographed under a fluorescence microscope and analyzed by CASP software.

ATP bioluminescence assay

ATP bioluminescence assay was performed as previously described [21]. First the ATP standard curve was made. The standard solution of ATP was diluted to six concentration gradients (10^{-12} to 10^{-17} mol/µl). Then we made the ATP standard curve and drew the fitting curve with GraphPad Prism 5.0. We elicited the similar fitting function. Before being placed in 96-well plates, RPMI8226 cells were pretreated with melphalan (2 µmol/l, 10 µmol/l, 50 µmol/l, 250 µmol/l) or anti-cancer compounds of every concentration of melphalan plus metformin (10 mmol/l) for 24 h. The LUM value of lysed cells fluid with the presence of assay solution was read. The corresponding ATP concentration was calculated by fitting the function in the presence of LUM.

Statistical analysis

SPSS version 17.0 (Chicago, IL, USA) was used for the t-test between the comparison groups for

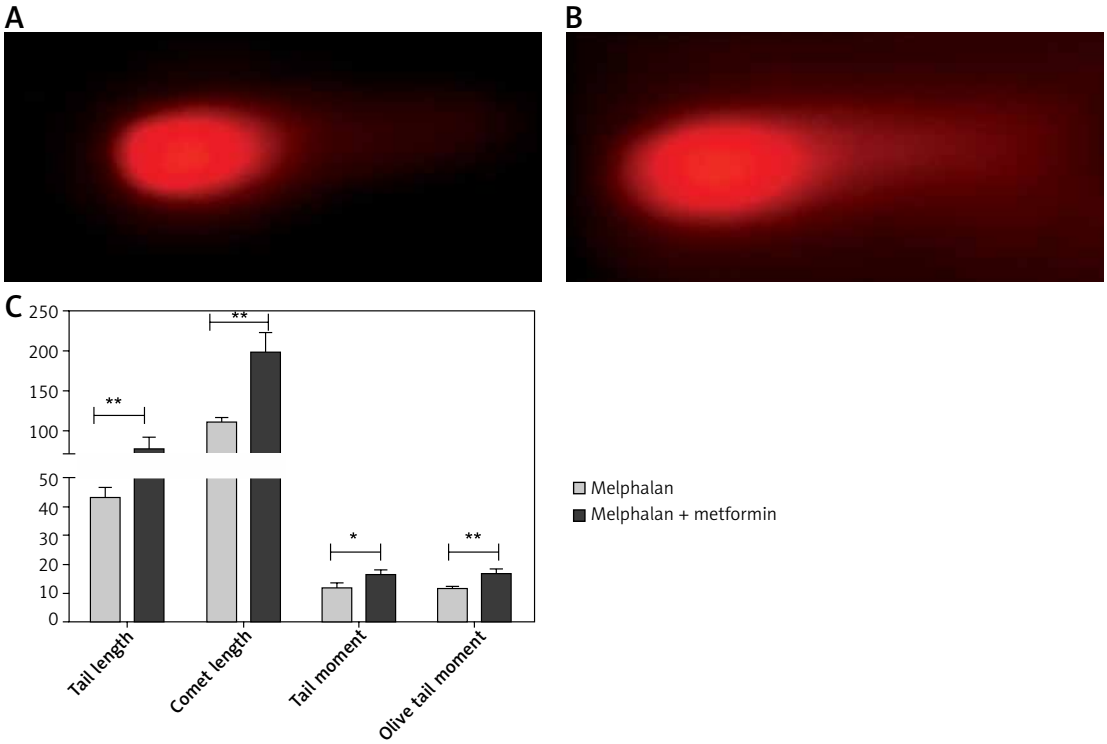

Figure 3. Determination of DNA base damage by alkaline comet assay. Comet assay was performed with melphalan (**A**) and melphalan combined with metformin (**B**) treatment. The results show that the comet length, tail length at the concomitant applied melphalan and metformin are significantly increased (**C**). The results are presented as mean ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test (*$p < 0.05$, **$p < 0.01$)

all the data which are expressed as mean ± standard deviation. A *p*-value of less than 0.05 was considered statistically significant.

Results

Metformin can inhibit synergistically RPMI 8226 cell proliferation with each concentration of melphalan

As shown in Figures 1 A and B, melphalan as well as metformin (10 mmol/l) inhibits the growth of RPMI8226 cells, and the inhibitory effect is enhanced with the increase of drug concentration. When RPMI8226 cells were treated with metformin and melphalan together (Figure 1 C), the CI (combination index, CI less than 1 means synergism, CI greater than 1 means antagonism, CI equal to 1 indicates increased effect) [22] was calculated by CompuSyn version 1.0. The results show (CI < 1) that metformin has a synergistic effect on the growth inhibition of the cells.

Synergistic effects of various drugs combined with melphalan on cell apoptosis

Consistent with the results of the CCK-8 assay, flow cytometric analysis of melphalan, metformin, rotenone single and in combination treated RPMI 8225 cells indicated an increased effect of cell apoptosis (Figures 2 A and B). The difference between the effect of single melphalan and concomitant melphalan and metformin on the apoptosis rate is statistically significant. Similarly, rotenone, which is a standard inhibitor of NADH in the respiratory chain, combined with melphalan can also significantly increase the apoptosis rate of tumor cells.

Metformin increases DNA damage induced by melphalan

The alkaline version of the comet assay was used to determine the DNA-damaging potential after melphalan and melphalan combined with metformin treatment of RPMI 8226 cells. The resulting data are shown as the comet length, tail length, Olive moments and tail moments of RPMI8226 cells (Figure 3). The results showed observable DNA damage after 24 h with melphalan and metformin. There was a significant increase of comet length and tail length with the concomitantly applied melphalan and metformin compared to single melphalan. The data suggest a rather stronger effect of metformin on DNA damage induced by melphalan.

Melphalan reduces the concentration of ATP in RPMIN 8226 cells with the application of metformin

The ATP standard curve was made according to the standard ATP concentration, and the fit-

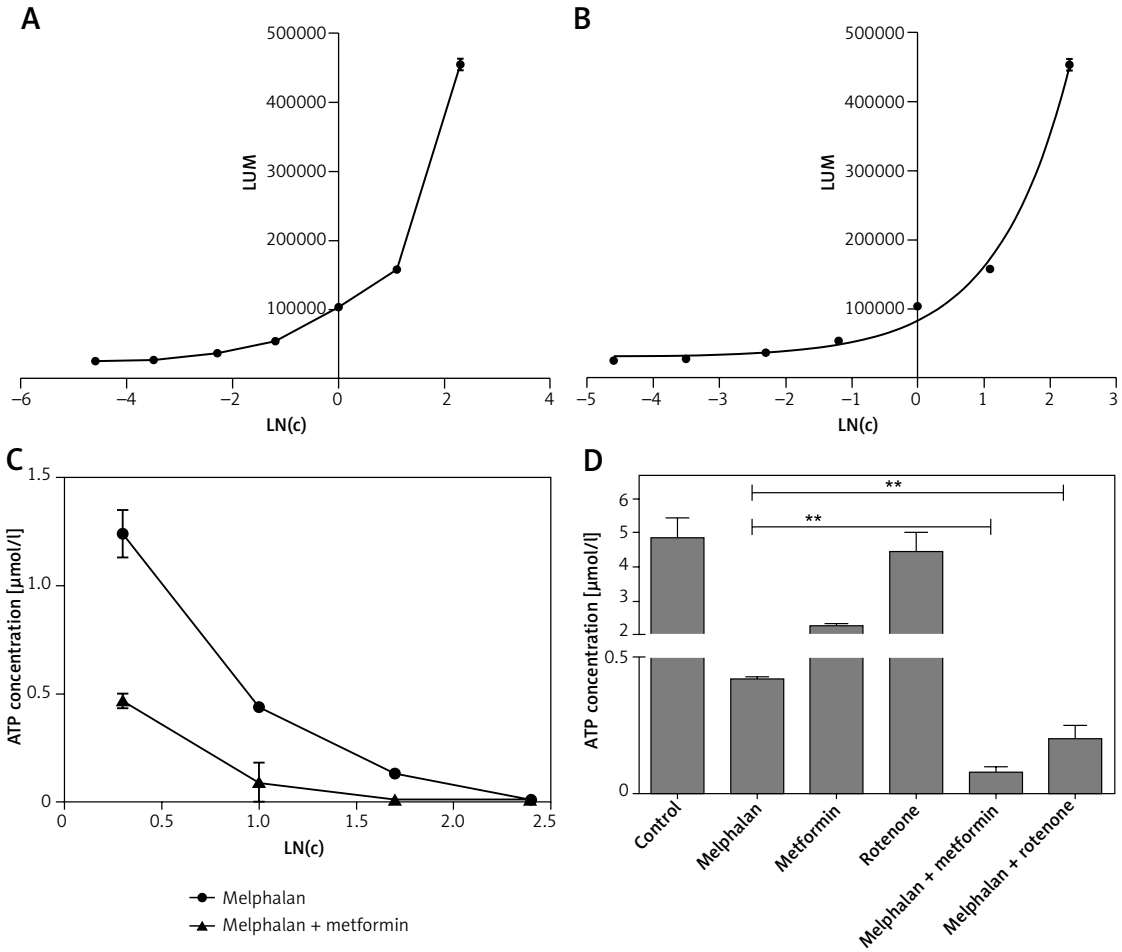

Figure 4. Melphalan reduces the concentration of ATP in RPMIN 8226 cells with the application of metformin. **A** – ATP standard curve. **B** – ATP fitting curve. **C** – ATP concentrations drop significantly in RPMI 8226 cells with the treatment of melphalan (2 µmol/l to 250 µmol/l) and metformin (10 mmol/l). Lg(c) is the logarithm of the concentration of melphalan to ten. **D** – When combined with rotenone (1 mmol/l), ATP concentrations also drop significantly. The results are presented as mean ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test (**$p < 0.01$)

ting curve was drawn using the graphic software GraphPad Prism 5.0 (Figure 4 B). We finally worked out the similar fitting function:

$$Y = 30686 + (1.51e + 09-30686)/(1 + 10 \wedge ((11.37-X) * 0.3916))$$

when X is LN (c), representing the natural logarithm of ATP concentration. The effect of anti-cancer drugs on ATP concentration was calculated. It was found that ATP concentration in RPMI8226 cells decreased with the increasing concentration of melphalan (Figure 4 C). When metformin was combined with various concentrations of melphalan, ATP concentrations in tumor cells significantly decreased. Rotenone can also significantly reduce the concentration of ATP in tumor cells in coordination with melphalan (Figure 4 D).

Discussion

Tumor cells are highly metabolic; therefore they need to synthesize more ATP than normal cells by the glycolysis pathway. The transformation to the aerobic glycolysis pathway is a feature of cancer cells which may be used as a general clinical examination for neovascularization. If we hinder this metabolic shift, a new treatment for cancer may arise. In addition, to meet the basic life activities, cancer cells need an additional amount of ATP compared with normal cells for unrestricted cell proliferation, metastasis, and response to chemotherapy-induced damage to self-repair. Alkylating agents as melphalan can cause inter-chain cross-linking by the treatment of multiple myeloma cells, resulting in a large amount of DNA damage. Only to repair this damage can tumor cells resist the process of apoptosis. ATP is an indispensable energy supplier in the restoration process [17]. If we reduce the concentration of ATP in tumor cells at this time, we can not only affect the life activities of undamaged tumor cells, but also hinder the damaged DNA repair process and increase the efficacy of chemotherapy drugs. Metformin, which is an inhibitor of NADH, effectively

inhibits the transmission of electrons in the respiratory chain and reduces the production of energy, thereby affecting the metabolic process of cancer cells to exert an anticancer effect [15, 23]. Rotenone, an inhibitor of complex I NADH, can also mimic this process. Metformin is a targeted drug that can reduce inflammatory factor precursors IL-1β induced LPS in macrophages, but with no effect on TNF-α [24]. Therefore, metformin combined with the alkylating agent melphalan can increase the sensitivity of chemotherapy and promote tumor cell apoptosis with few side effects.

It was found that metformin can increase the anti-tumor effect in combination with melphalan. CCK-8 showed that the anti-tumor effect was enhanced with the increasing concentration of melphalan. According to the experiments of flow cytometry, comet assay and ATP concentration measurement, the conclusions are that the combination of melphalan and metformin will make the apoptosis rate increased, DNA damage serious, and ATP concentration reduced significantly. Rotenone with the same mechanism of action also caused the same effect. In order to rule out the compound factors that many viable cells caused by the enhanced cytotoxic effect of melphalan combined with metformin from death to a lower level of ATP, another group of melphalan with the same apoptosis rate as the combination of melphalan (10 μmol/l) and metformin (10 mmol/l) was examined. As we expected, the reduction of ATP concentration between the two groups was different. The conclusion was demonstrated for a step further that metformin can promote melphalan on reducing the ATP level in tumor cells.

In conclusion, metformin can promote DNA damage induced by melphalan and decrease the concentration of ATP in the process of repairing DNA damage, hindering the anti-apoptotic process of tumor cells, and increase the sensitivity of multiple myeloma cells to melphalan.

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Conflict of interest

The authors declare no conflict of interest.

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