Herbal product silibinin-induced programmed cell death is enhanced by metformin in cervical cancer cells at the dose without influence on nonmalignant cells

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Abstract

Silibinin is known to display high efficacy against cancer cells and for hepatic protection. Metformin, a well-known antidiabetic agent, has recently been reported to inhibit cancer. In the present study, we investigated the effect of metformin on silibinin-induced programmed cell death in cervical cancer cells (C-33A). MTT assay and Western blot assays were performed to quantify cell viability and the expression of signaling proteins, respectively. Combined treatment with metformin and silibinin decreased cell survival in synergistic manner in C-33A cells at a dose that did not affect nonmalignant cells (HUVECs). Silibinin and metformin increased PTEN and AMPK expression in C-33A cells, respectively. Combined treatment caused a greater increase in the expression of activated caspase-3 or AIF, indicating apoptosis. Combined treatment with silibinin and metformin may induce programmed cell death of human cervical cancer cells at a dose that does not affect HUVECs. This finding reveals a potential therapeutic strategy of cervical cancer

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Introduction

Cervical carcinoma, the popular female cancer around the world, is the seventh leading cause of cancer death in women (Siegel et al., 2014). Nowadays, cervical cytology screening is useful to reduce the mortality of this cancer. Various strategies for reducing cervical cancer have been reported, including immune therapy of cytokines, polyamine synthesis inhibitors, individual micronutrient supplementation and pharmaceutical agents, and all have indicated a limited success (Follen et al., 2003; Bell and Alvarez, 2005). Moreover, human papillomavirus (HPV) vaccine strategies have also been applied to reduce cervical cancer risk. However, the actual impact of cervical cancer initiation remains controversial and managing lesions remains necessary to be investigated.

Milk thistle (Silybum marianum) has widely been utilized in liver diseases as a popular dietary supplement in the United States and Europe (Kroll et al., 2007). Silibinin, a polyphenolic flavonoid, is the major active compound in milk thistle (Singh and Agarwal, 2004; Gazak et al., 2007). Milk thistle is known to be safely and tolerably protecting the liver against chemical or alcohol-related injury (Ball and Kowdley, 2005; Hackett et al., 2013). The inhibitory effect of silibinin has been demonstrated in multiple cancer cell lines, including lung (Chu et al., 2004), liver (Lah et al., 2007; Cui et al., 2009), skin (Mallikarjuna et al., 2004; Mohan et al., 2004), colon (Yang et al., 2003) and prostate cancers (Singh et al., 2002; Tyagi et al., 2002).

Metformin is an anti-diabetic agent widely used to treat diabetic patients. Its action is mainly mediated by the activation of AMP-activated protein kinase (AMPK), which inhibits hepatic gluconeogenesis and enhances glucose uptake in skeletal muscle and adipose tissue (Zhou et al., 2001). In addition, metformin has been reported to reduce cell proliferation in several human tumors including breast cancer (Zhuang and Miskimins, 2011; Lee et al., 2014), pancreatic cancer (Bao et al., 2012) and gastric cancer (Kato et al., 2012). Metformin also inhibited tumor growth in xenograft mouse models of breast cancer (Anisimov et al., 2005), prostate cancer (Ben Sahra et al., 2008), ovarian cancer (Rattan et al., 2011) and melanoma (Janjetovic et al., 2011). The administration of metformin to diabetic patients was associated with lower risks of cancer incidence and mortality (Noto et al., 2012). Colorectal cancer patients with diabetes who were treated with metformin as part of their diabetic therapy appeared to exhibit a superior overall survival rate (Luo et al., 2012; Smiechowski et al., 2013).

Both silibinin and metformin showed anticancer activities. Thus, we administered metformin with silibinin in combination to investigate the anticancer efficacy in C-33A cells. In the present study, an effective dose of this combination of drugs that did not affect nonmalignant cells was found.

Materials and methods

Materials

Antibodies against activated caspase-3 and apoptosis induce factor (AIF) were purchased from Millipore (Millipore, Bedford, MA, USA). Antibodies against the phosphatase and tensin homolog (PTEN), signal transducer and phosphorylated (p-) protein kinase B (Akt), phosphorylated 5’-adenosine monophosphate (AMP)-activated protein kinase (p-AMPK) and β-actin (actin) were the products of Abcam (Cambridge, MA, USA). Metformin and silibinin were purchased from Sigma-Aldrich (St Louis, MO, USA).

Cell culture

Human cervical cancer cells (C-33A) and human umbilical vein endothelial cells (HUVECs) were purchased from the Culture Collection and Research Center of the Food Industry Institute (Hsin-Chiu City, Taiwan). C-33A cells were cultured in α-MEM (HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and gentamicin sulfate (10 μg/ml) (GIBCO). HUVECs were nonmalignant epithelial cells which are widely used as normal cells in cancer studies (Kamat et al., 2011; Dil and Banerjee, 2012; Hoang et al., 2012). HUVECs were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO2 in air. After approximately 60% confluence, the medium was replaced with serum-free cell medium, to prevent the cell growth in the period of treatment, containing various concentrations of silibinin (0, 50, 100, 150 or 200 μM) (Lah et al., 2007; Cui et al., 2009) and metformin (0, 5, 10, 15 or 20 mM) (Janjetovic et al., 2011; Luo et al., 2012) and the cells were cultured for 24 h as performed in previous studies (Yu et al., 2012; Su et al., 2013a,b). The cells were harvested via treatment with 0.25% trypsin and 0.2 g/l EDTA for further studies.

Cytotoxicity assay

Cell viability and survival was determined via the 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Scudiero et al., 1988). Briefly, 104 cells were seeded on 96-well plates in triplicate and treated with different combinations of concentrations of metformin and silibinin for 24 h. After treatment, the medium was replaced with 100 μl/well of fresh medium and 10 μl of MTT (final concentration of 0.5 mg/ml) was added to each well. The plates were incubated at 37 °C for 4 h, allowing the viable cells to reduce the yellow tetrazolium salt into dark blue formazan crystals. The formazan crystals were dissolved using a solution of 0.01 M HCl/10% SDS. Finally, the absorbance of each individual well was determined at 595 nm using a Synergy HT Multi-Mode Microplate Reader (BioTek, U.S.). The results of the assays were expressed as the means ± SEM. The data were collected from at least three independent experiments.

Analysis of synergy by combination index (CI)

The Loewe additivity model was used as a second method of analyzing the interaction between silibinin and metformin (Lee et al., 2007). The interaction between the compounds is reported as the combination index in the following equation:

\[ CI = \frac{d_1}{D_{50,1}} + \frac{d_2}{D_{50,2}} \]
In the equation, $d_1$ and $d_2$ represent the concentrations of the compounds in combination required to achieve a $x$ effect. $D_{x,1}$ and $D_{x,2}$ represent the concentrations of the same compounds individually that would quantitatively achieve the same $x$ effect. A CI $< 1.0$ indicates that the combination is synergistic, and a CI $> 1.0$ indicates an antagonistic interaction. The combination indexes for this study were determined by using concentrations corresponding to combine of silybinin (0.01–0.1 mM) and metformin (1–10 mM).

**ApoTox-Glo Triplex Assay**

Apoptotic response was measured by the detection of DNA histone complexes released from the nucleus to the cytosol of cells using ApoTox-Glo Triplex Assay (Promega, WI, USA) (Liu et al., 2013). It was performed to assess viability, cytotoxicity and caspase-3/7 activation within a single assay well. Briefly, $10^4$ cells were seeded on 96-well plates in triplicate and treated with different combinations of concentrations of metformin and silybinin for 24 h. Each well contained a final volume of 200 ml of culture medium. Each group was mixed with the viability/cytotoxicity reagent using orbital shaking (500 r.p.m. for 30 s) and incubated for 1 h at 37 °C. The fluorescence was measured to assess viability and cytotoxicity. Otherwise, after the addition of 100 μl caspase-Glo 3/7 to all wells, samples were briefly mixed by orbital shaking (500 r.p.m. for 30 s). After incubating for 30 min at room temperature, luminescence was measured by using a microplate reader to assess apoptosis. Fluorescence was measured at 380 nm (excitation)/510 nm (emission) for viability, 485 nm (excitation)/520 nm (emission) for cytotoxicity, and luminescence for apoptosis.

**Western blotting analysis**

Protein was extracted from cell lysates using ice-cold radio-immuno-precipitation assay (RIPA) buffer supplemented with phosphatase and protease inhibitors (50 mM sodium vanadate, 0.5 mM phenylmethylsulphonyl fluoride, 2 mg/ml aprotinin, and 0.5 mg/ml leupeptin). The protein concentrations were determined via the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Total protein samples (30 μg) were separated via SDS/polyacrylamide gel electrophoresis (10% acrylamide gel) using the Bio-Rad Mini-Protein II system. The proteins were transferred to polyvinylidene difluoride membranes (PerkinElmer, Waltham, MA, USA) using a Bio-Rad Trans-Blot system. After transfer, the membrane was blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and incubated for 2 h. The membrane was then washed in TBS-T and hybridized with primary antibodies, which were diluted to a suitable concentration in TBS-T for 16 h. Specific antibodies for activated caspase-3, AIF, PTEN, p-Akt (Ser473) and p-AMPK (Thr 172) (1:1000 dilution) were used. Additionally, the membranes were incubated with goat polyclonal antibody to bind the β-actin (Actin) (1:10,000 dilutions) serving as the internal control. Incubation with secondary antibodies and detection of the antigen–antibody complex were performed using an ECL kit (Amersham Biosciences, UK). After comparing with the marker for specificity, the immunoblots of β-actin (43 kDa), activated caspase-3 (17 kDa), AIF (57 kDa), PTEN (42 kDa), p-Akt (60 kDa) and p-AMPK (62 kDa) were quantified with a laser densitometer (Avegene Life Science, Taipei, Taiwan).

**Statistical analysis**

Data are expressed as the mean ± standard error (SE). Statistical analysis was performed in the Microsoft EXCEL. Data were evaluated using the Student’s t-test at the significance level at $2a = 0.05$.

**Results**

**Silibinin and metformin inhibit cell viability synergistically**

Fig. 1A shows the survival rate of C-33A cells and human umbilical vein endothelial cells (HUVEC) treated with the combination of silibinin and metformin at various concentrations for 24 h. Respectively, silibinin and metformin at their highest dose, in combination or individually, exerted an inhibitory effect on the survival of C-33A cells. However, individual treatment with silibinin or metformin at their highest respective dose exerted a toxic effect on nonmalignant cells (HUVECs), and the cell death rate was even greater following combined treatment. Furthermore, combined treatment with 100 μM silibinin and 10 mM metformin appeared to be more effective than treatment with 50 μM silibinin and 5 mM metformin without altering the survival of HUVECs. Fig. 1B shows the survival curves of C-33A cells treated with silibinin (100 μM) and metformin (10 mM) in combination or individually at different exposure times. More, Fig. 1C shows the survival curves of C-33A cells treated with silibinin (0–100 μM) and metformin (0–10 mM) in combination or individually. The survival curves shifted after a longer exposure of combined treatment or in dose dependent manner respectively while individual treatment did not affect the survival of C-33A. The synergistic effect of silibinin + metformin on C-33A cells was further confirmed using an alternative approach of calculating the combination index. The combination of silibinin + metformin at concentrations below 100 μM silibinin with 10 mM metformin had a combination index $< 1$, indicating synergism between two compounds (Fig. 1D). Thus, we applied the combination of 100 μM silibinin with 10 mM metformin to compare the effects of this treatment with individual treatment on C-33A cells after 24-h incubation in the following experiments.

**Silibinin and metformin induced cytotoxicity and apoptosis in C-33A cells**

To further evaluate the effect of combined treatment in vitro, we measured C-33A cell viability/cytotoxicity and apoptosis using an Apo-Tox Glo assay. As shown in Fig. 2, combined treatment with 100 μM silibinin and 10 mM metformin appeared to induce cytotoxicity and apoptosis in C-33A cells while it is failed to do so individually.
Combined metformin and silibinin (M + S) treatment inhibits Akt phosphorylation by enhancing PTEN expression

The effects of M + S treatment on the expression of PTEN were examined via Western blot analysis. As shown in Fig. 3, the expression level of phosphorylated protein kinase B (AKT) (Ser473) was decreased and that of PTEN was increased in C-33A cells after silibinin or M + S treatment for 24 h.

M + S treatment enhances AMPK phosphorylation

The effects of M + S treatment on the phosphorylation of AMPK (Thr 172) were examined via Western blot analysis. As shown in Fig. 4, the expression level of phosphorylated AMPK (Thr 172) increased in C-33A cells after treatment with metformin or M + S treatment for 24 h.

M + S treatment induces C-33A cell apoptosis

The levels of activated caspase-3 and apoptosis induce factor (AIF) were estimated by Western blotting analysis to assess programmed cell death as described previously (Corbiere et al., 2004; Ferraud et al., 2012; Yu et al., 2012; Leon et al., 2013; Su et al., 2013b). M + S treatment increased the levels of activated caspase-3 and AIF in C-33A cells, whereas individual treatment did not display these effects (Fig. 5). Thus, M + S-induced inhibition is primarily mediated by the induction of programmed cell death in cancer cells.

Discussion

In the present study, we found that co-treatment with of silibinin (100 μM) and metformin (10 mM) direct inhibits the survival of human cervical cancer cell line (C-33A cells). In addition, the combination of silibinin (100 μM) and metformin (10 mM) at the dose that synergistically inhibited C-33A cell survival did not affect normal HUVECs. Furthermore, Apo-Tox Glo assay indicated that combined treatment with 100 μM silibinin and 10 mM metformin appeared to induce cytotoxicity and programmed cell death in C-33A cells while it is failed to do so individually. The specific inhibition of cancer cells using
this combinatory therapy was considerable. Moreover, silibinin alone or in combination with metformin increased the PTEN expression level resulting in a significant reduction in phosphorylated Akt (at Ser473) in C-33A cells. Moreover, metformin alone or in combination with silibinin increased the phosphorylation of AMPK in C-33A cells. Finally, the combination of silibinin and metformin increased caspase-3 activation or AIF expression, which were widely used as indicators of programmed cell death (Corbiere et al., 2004; Perraud et al., 2012; Yu et al., 2012; Leon et al., 2013; Su et al., 2013b). Therefore, we suggest that the combination of silibinin and metformin more effectively induced programmed cell death in C-33A cells than each individual treatment at a dose that displayed no effect on normal cells.

The important roles of PTEN/Akt signaling in carcinogenesis and cancer progression have been indicated (Wan et al., 2003; Sansal and Sellers, 2004). Phosphorylation of Akt (p-Akt), results in its activation, promotes cell cycle progression by phosphorylating several other key proteins (Vivanco and Sawyers, 2002; Gershtein et al., 2007). The PTEN/PI3K/Akt pathway is associated with silibinin-induced inhibition of the growth in human hepatocellular carcinoma cells (Lah et al., 2007). Many events are involved in silibinin-induced programmed cell death. Silibinin could cause the loss of mitochondrial membrane potential and increase cytochrome C release from mitochondria and Bax, as well as the decrease in expression of Mcl-1 proteins, indicating that silibinin-induced programmed cell death is mediated through caspase-dependent and -independent mechanisms (Singh et al., 2005). At the non-cytotoxic dose, silibinin could obviously inhibit MMP-2 enzymatic activity and ERK 1/2 phosphorylation in HepG-2 cells (Momeny et al., 2008). Additionally, our previous reports show that silibinin-induced increase in PTEN expression and/or decrease in p-Akt expression was associated with the decreased survival rate and enhanced programmed cell death in FaDu oral cancer cells (Su et al., 2013b) and C-33A cervical cancer cells (Yu et al., 2012). In this study, we found that silibinin alone or in combination with metformin increased the PTEN expression, resulting in decreased p-Akt in C-33A cells, consistent with our previous reports.

Silibinin causes cell-cycle arrest at the G1 phase in HT-29 human colon carcinoma cells and inhibits the cell proliferation rate at concentrations of 75–100 μg/ml (approximately 150–200 μM) (Agarwal et al., 2003). In addition, suppression of growth in human colorectal carcinoma (SW480) cells by silibinin at a concentration of 200 μM has been detected (Kaur et al., 2010). Prostate cancer cells treated with silibinin (200 μM) displayed inhibited invasion, motility and migration via the

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**Fig. 2 – ApoTox-Glo Triples Assay of C-33A cells treated with silibinin and metformin in combination or individually.** ApoTox-Glo Triples Assay showing the viability (A), cytotoxicity (B) and apoptosis (C) are performed by C-33A cells treated with silibinin (100 μM) and metformin (10 mM) in combination (M + S) or individually. The data are expressed as the means ± SE (n = 6 for each group). *Statistically significant as compared with control C-33A cells.
suppression of vimentin and MMP-2 expression (Wu et al., 2009). In summary, silibinin exerts anticancer effects at the dosage of 200 μM. In this study, we are the first to reveal that co-treatment of silibinin (100 μM) with metformin (10 mM) more effectively induced programmed cell death than treatment with silibinin 100 μM alone. Moreover, this combined treatment did not affect normal HUVECs. Thus, the specific inhibition of cancer cells using this combinatory therapy was considerable.

Metformin has widely been used to treat type 2 diabetic patients (Zhou et al., 2001; Hettihewa et al., 2008; Han et al., 2011; Bacha et al., 2012; Smiechowski et al., 2013). Interestingly, the concept that this compound may be a promising anticancer agent was first developed in the early 1970s (Dilman, 1971). Later on, two population-based studies provided preliminary evidence that metformin may reduce cancer risk and improve prognosis in type 2 diabetic patients (Bowker et al., 2006; Berstein et al., 2012). Inhibition of cancer cells by metformin is to induce the activation of AMPK and the suppression of mammalian target of rapamycin (mTOR) (Fruman and Edinger, 2008; Hadad et al., 2008; Yasmeen et al., 2011). Metformin treatment also led to a significant decrease of cyclin D1 protein level and retinoblastoma protein (pRb) phosphorylation. Cyclin-dependent kinase (CDK) 4 and CDK6 were markedly reduced by metformim (Sikka et al., 2012; Kobayashi et al., 2013). Moreover, a significant down-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-xl and the up-regulation of pro-apoptotic protein Bax were observed in malignant cells following metformin treatment (Luo et al., 2012; Lin et al., 2013; Ma et al., 2014). In this study, we found that metformin alone or in combination with silibinin increased the expression of phosphorylated-AMPK (Thr 172), consistent with previous reports (Fruman and Edinger, 2008; Hadad et al., 2008; Kusmic et al., 2010).

Metformin at 20 mM has previously been found to be effective against breast cancer (Zakikhani et al., 2006), melanoma (Janjetovic et al., 2011) and gastric cancer (Kato et al., 2012). In fact, the concentration of metformin administered to type 2 diabetic patients is approximately 30–60 μM (Martin-Castillo et al., 2010). Thus, the doses of metformin that were effective against cancer cells appear to be approximately 300-fold greater than that which is administered for diabetic disorders. In this study, we applied a low dose of metformin (10 mM) in combination with silibinin (100 μM) more effectively induce programmed cell death than application of
enhance programmed cell death in cancer cells. Thus the effect of this combinatorial therapy on other cancer cell lines is still unknown and it could be generated in the future.

**Conclusion**

Combined treatment of silibinin with metformin synergistically enhanced the inhibition of C-33A cell survival via increased PTEN expression and AMPK phosphorylation, resulting in the induction of caspase-3 and AIF. The obtained findings suggest a novel therapeutic strategy for human cervical cancer that induces few side effects.

**Conflict of interest**

None.

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**References**


Ben Sahra, I., Laurent, K., Loubat, A., Giorgetti-Peraldi, S., Colosetti, P., Auburger, P., Tanti, J.F., Le Marchand-Brustel, Y., Bost, F., 2008. The antidiabetic drug metformin exerts an...
antimutual effect in vitro and in vivo through a decrease of cyclin D1 level. Oncogene 27, 3576–3586.


